Innate killers at work

Strategies to exploit neutrophils for cancer treatment

Martínez Sanz, P.

Publication date
2023

Document Version
Final published version

INNATE KILLERS AT WORK:

Strategies to exploit neutrophils for cancer treatment

Paula Martínez Sanz

INNATE KILLERS AT WORK

Strategies to exploit neutrophils for cancer treatment

Paula Martínez Sanz

Innate killers at work: Strategies to exploit neutrophils for cancer treatment
Paula Martínez Sanz
ISBN: 978-94-6483-353-9

Provided by thesis specialist Ridderprint, ridderprint.nl
Printing: Ridderprint
Layout and design: Rowen Aker, persoonlijkproefschrift.nl
Cover Design: Alex Martínez Sanz & DALL·E - OpenAI

Innate killers at work
Strategies to exploit neutrophils for cancer treatment

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. P.P.C.C. Verbeek
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op donderdag 19 oktober 2023, te 13.00 uur

door Paula Martínez Sanz
geboren te St. Cugat del Vallès Barcelona

Promotiecommissie

Promotor:	prof. dr. T.W. Kuijpers	AMC-UvA
Copromotores:	dr. H.L. Matlung	Sanquin Research
	dr. G.A.M. Tytgat	Prinses Máxima Centrum
Overige leden:	prof. dr. T.D. de Gruijl	Vrije Universiteit Amsterdam
	prof. dr. M.D. Hazenberg	AMC-UvA
	prof. dr. E.F. Eldering	AMC-UvA
	dr. M.C. Wolkers	Sanquin Research
	dr. J.J. Molenaar	Universiteit Utrecht
	prof. dr. G. de Haan	AMC-UvA

Faculteit der Geneeskunde

TABLE OF CONTENTS

Chapter 1	General Introduction	7
Chapter 2	Plasticity in pro- and anti-tumor activity of neutrophils: Shifting the balance	33
Chapter 3	G-CSF as a suitable alternative to GM-CSF to boost dinutuximab-mediated neutrophil cytotoxicity in neuroblastoma treatment	71
Chapter 4	CD47-SIRPα checkpoint inhibition enhances neutrophil-mediated killing of dinutuximab-opsonized neuroblastoma cells	107
Chapter 5	FcαR-bearing CAR NB4 neutrophils display built-in antigen-specific cytotoxic activity towards solid tumor targets	135
Chapter 6	Humanized MISTRG as a preclinical *in vivo* model to study human neutrophil-mediated immune processes	159
Chapter 7	General Discussion	195
Annexes	English summary	217
	Nederlandse samenvatting	225
	Contributing authors	233
	List of publications	237
	PhD portfolio	238
	About the author	240
	Acknowledgements	241

CHAPTER 1

GENERAL INTRODUCTION

THE GLOBAL FIGHT AGAINST CANCER

Cancer is nowadays a dominant disease, sadly present in every day's and everyone's life in one way or another. As the world population is growing and aging, the global number of cancer deaths is increasing, placing it as the second leading cause of death worldwide, second only to cardiovascular diseases[1–3].

Cancer is defined by the National Cancer Institute as a disease in which cells uncontrollably divide and spread to nearby tissues[4]. But actually, cancer is not just one disease but a collection of diseases. From the medical point of view, one would even dare to say that every cancer is a different disease. For patients and families, instead, cancer is sometimes considered a "total disease", an illness that takes over patients not only physically, but also socially and emotionally. These days, *fight* or *battle* metaphors are often used to refer to cancer: defeating cancer, fighting cancer, beating cancer. We seem to be in a constant battle with cancer, although this does not feel as a regular fight, but as a rather pressing one. The reality is that patients with cancer who are going to succumb to the disease this year cannot wait[5]; and neither can the physicians and nurses who treat these patients, nor the scientists who devote their lives trying to crack the code to "cure" cancer. Just as seen for infectious diseases that once were fatal, for which the morbidity and mortality rates plummeted since the discovery and development of their cure with antibiotics[6], professionals in the cancer field also seem to be racing against the progression of the patient's disease and the rate of global cancer research. But in fact, it is now believed that the goal should not necessarily be to defeat cancer today; instead, we should seek to survive long enough to benefit from the upcoming medical advancements that are about to arrive. This thesis is aimed at putting in perspective some of the newest preclinical advances in the field of cancer immunotherapy engaging a subtype of innate immune cell: the neutrophil.

CONVENTIONAL CANCER THERAPIES: CUT, BURN, POISON

Up until 2011, the terms "cut, burn and poison" summarized the three conventional treatments for cancer, referring to surgery, radiotherapy and chemotherapy[7–10]. In the early days (around 3000 years ago), the excision of cancerous tissue from visible and palpable tumors was the only available method to eliminate cancer[7]. This was a rather dangerous approach due to the absence of antibiotics and antiseptic measures, which also came with high chances of leaving cancerous cells behind. Those cells left behind that were invisible to the human eye and that went unnoticed to surgeons would be the main culprits of early relapses back in those days. It was not until the end of the 19th century that another method to treat cancer was discovered[8]. The delivery of ionizing radiation which caused DNA damage and subsequently induced tumor cell death was an approach used after surgery to further control tumor growth locally. Yet, such radiation was soon found to also induce cancer, and despite recent improvements aiming to reduce off-target effects to

surrounding healthy tissue, complete cancer elimination was found to be rather challenging. Since dissemination of cancer cells was found to be a common phenomenon, the focus for the treatment of cancer started to gradually shift to treating cancer systemically with the goal to also reach those disseminated malignant cells in the body. This was achieved with the development of chemotherapy, which is the chemical way to stop the proliferation of fast-growing cells with cytostatic drugs discovered 50-60 years later[9]. Chemotherapeutic drugs have come a long way and are reckoned as effective weapons against cancer. Even so that they were once considered the "penicillin for cancer", as oncologist Dusty Rhoads liked to describe it[5]. Nevertheless, they also cause the body significant damage. They weaken healthy cells such as those of the hematopoietic system or the digestive track, among others, and thereby this anti-cancer therapy often comes with serious side-effects.

Today, combinations of these "cut, burn and poison" techniques are often used to tackle the tumor both locally and systemically, leading to more effective cures. But although it is now anticipated that these therapies can cure cancer in roughly half of the people who develop it (which is in itself already an outstanding medical achievement), the other half still succumbs to it[11]. This is not particularly unexpected since a cancerous cell is a normal cell that has mutated and changed, and it will continue to change. And unfortunately, a cancer drug, such as a chemotherapeutic drug, has not (*yet*) been designed to continuously adapt to an endlessly mutating cancer cell[11]. The traditional anti-cancer therapies may temporarily harm or poison the cancer, but the remaining cancer cells will continue to change and grow abnormally. Therefore, it appears unlikely that the conventional anti-cancer therapies will ever truly cure cancer. As the author Charles Graeber from The Breakthrough likes to put it: "The drug dances with the cancer, but the cancer dances away"[11].

THE FOURTH PILLAR OF CANCER TREATMENT: IMMUNOTHERAPY

Fortunately, we have a living immune system, an exceptionally developed and specialized system that is capable of adapting to any disease, including cancer. This system has been extensively studied in the field of infectious diseases. Briefly, the innate arm of the immune system is able to recognize and kill the "usual suspects" of disease, and provides an effective response strong enough to get rid of most invading threats in just a couple of days. Normally the innate response is sufficient to contain them, but sometimes they may need reinforcements, or other times the invaders may be unfamiliar, and here is when the adaptive arm comes into play. The adaptive arm is capable of facing new challenges and it does so by adapting, fighting and remembering the invaders that the body has never encountered before in case there is a future encounter with those. This is especially needed because diseases evolve and adapt, and so our living army should also be able to adapt to them[12].

Our immune system, however, did not seem to attack cancer and scientists did not understand why. We know that immune cells distinguish cells that belong in the body (self-cells) from those that do not belong (non-self-cells)[12]. But cancer is different, cancer is not an invader. It is, in fact, a sick body cell that is not infected but mutated, hence, it doesn't manifest itself like any other infectious disease. Cancer cells were thought to be too similar to a normal self-cell for the immune system to recognize as foreign and mount an immune response against it. This initially led to the idea that making use of our immune system to fight cancer (what is now called *immunotherapy*) would never work and it was a futile pursuit. But not everyone was willing to stick to the idea that making use of the immune system to fight cancer was flawed.

Among the first scientific attempts to modulate the patients' immune system to cure cancer can be attributed to the American surgeon William B. Coley, known today as the "Father of immunotherapy", who already in 1891 found a number of his cancer patients go into spontaneous remissions after he had (deliberately) injected their tumors with *streptococcus pyogenes*[13]. Nevertheless, the therapeutic confirmation of Coley's principle was only demonstrated roughly 80 years later with the use of Bacille Calmette-Guerin (BCG) tuberculosis vaccine when its use showed cures in the treatment of bladder cancer[14]. The next milestone in the cancer immunotherapy field came with the theory of "cancer immunosurveillance" proposed by Thomas and Burnet in the 1950s, who suggested that our immune cells acted as watchmen to identify and eliminate mutated cells in our body[15–17]. At the same time, other scientists such as the renowned immunologist Steven A. Rosenberg, also seemed to be strongly convinced that there was already a mechanism present in the body that helped the immune system recognize cancer cells, as he started noticing that patients with compromised immune system developed cancer at greater rates than those with normal immune system[11]. The theory of cancer immunosurveillance was corroborated at the end of the 20th century by the teams of Robert D. Schreiber, Gavin P. Dunn and Lloyd J. Old, who for the first time experimentally showed the ability of our immune system to provide anti-tumor surveillance and to induce anti-tumor responses[18].

These constituted the first glimmers in the darkness of cancer immunotherapy, which together with the development of immunology research propelled the development of a multitude of strategies aimed at getting the immune cells in our body to kill the cancer cells as quickly, powerful and selectively as possible. Such that in 2011 the new "cancer's penicillin moment" came when the first new-generation immunotherapeutic cancer drug, consisting of a checkpoint blockade drug, Ipilimumab, used to unleash T lymphocytes (described later in this chapter), received FDA approval for the treatment of skin cancer, which was considered the breakthrough of the year a couple of years later[19]. From that point onwards we count with immunotherapy as the fourth pillar of cancer treatment, which

instead of treating the disease site (such as with surgery and radiotherapy), it treats the specific tumor biologic characteristics and its interaction with the immune system. And just like that, immunotherapy started to be considered as the best available tool to fight cancer.

The term immunotherapy is actually an umbrella term that encompasses a wide variety of concepts and methods for the treatment of cancer, and the menu of these methods is rapidly evolving. The main existing types of immunotherapy that are currently being thoroughly investigated are described in **Figure 1**. Most immunotherapies are focused on engaging cells of the adaptive immune system (mainly T lymphocytes), but more and more are being applied to the innate arm. In this thesis, a selection of those that engage neutrophils, a subtype of innate immune cell, will be described.

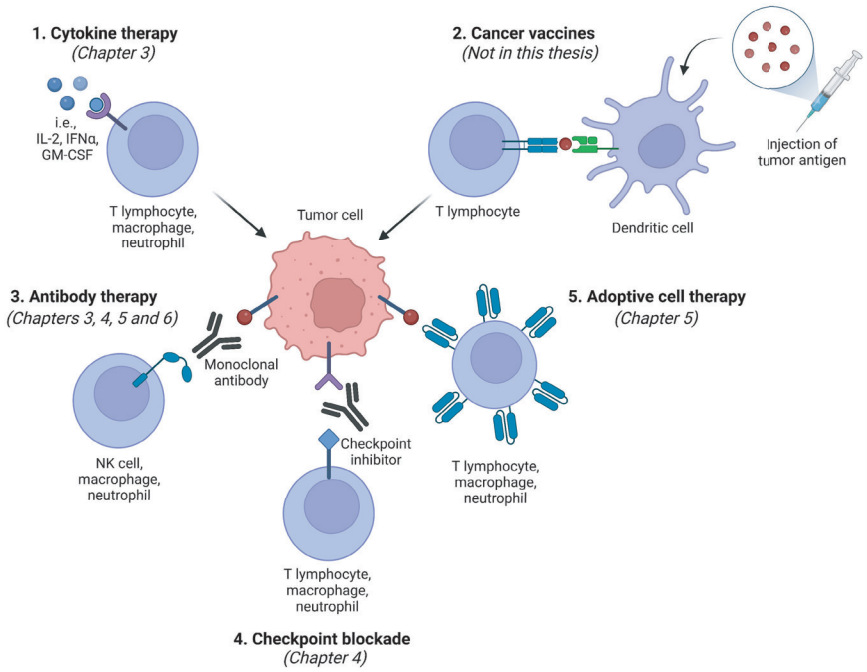


Figure 1. *The five main types of immunotherapy can be classified in 1) cytokine (or growth factor) therapy with hormone-like proteins that stimulate proliferation and/or activation of specific immune cells to fight cancer (described in chapter 3), 2) cancer vaccines that stimulate adaptive immune responses through the injection of a tumor antigen (not described in this thesis), 3) antibody therapy with opsonizing monoclonal antibodies that specifically target tumor antigens directly on tumor cells (described in chapters 3, 4, 5 and 6), 4) checkpoint blockade with blocking antibodies specifically interfering with immune checkpoints between effector immune cells tumor cells (described in chapter 4), and 5) adoptive cell therapy (ACT) with genetically engineered immune cells that are in vitro modified and expanded to better recognize and kill tumor cells when reinfused back in the patient (described in chapter 5). Created with BioRender.com.*

NEUTROPHILS: FROM HOST DEFENSE TO EFFECTORS IN CANCER

Immune cells originate in the bone marrow from hematopoietic stem cells (HSCs). HSCs are self-renewing cells with the unique ability to differentiate into all the different blood cell lineages, a process known as hematopoiesis. Among the different blood cell lineages, the myeloid lineage is the one giving rise to cells of the innate phagocytic system, including monocytes, macrophages and granulocytes. Neutrophils, belonging to the myeloid compartment, together with the less abundant eosinophils and basophils, are known as granulocytes, mainly due to the presence of granules in their cytoplasm. Despite comprising the largest white blood cell population circulating in our bloodstream (50-70% of the cells in human blood are neutrophils), neutrophils are short-lived cells with a half-life of less than 24 h in circulation. Hence, they are renewed in the bone marrow at a daily rate of 10^{11} via a process called granulopoiesis[20]. In fact, the neutrophil reserve in the bone marrow is 5 times larger than the pool of circulating neutrophils to make sure they are available in case of a severe infection[21]. Neutrophil differentiation comprises a number of developmental stages in which the neutrophil's nucleus undergoes several morphological changes until it becomes a non-dividing end-stage polymorphonuclear cell[22,23]. The promyelocyte is the most immature neutrophil progenitor. This is still an actively proliferating cell characterized by a round nucleus. The metamyelocyte is next in the maturation sequence. At this stage cell division terminates and the nucleus turns into a kidney-shaped nucleus. The metamyelocyte matures to a band cell, with a band-shaped nucleus, and this to a segmented cell which is the end-stage neutrophil characterized by a multilobulated and hypersegmented nucleus[20].

As part of our innate army of soldiers, neutrophils are equipped with broadly effective anti-microbial mechanisms divided in four major strategies: degranulation, the release of reactive oxygen species (ROS), phagocytosis, and the release of neutrophil extracellular traps (NETs)[24]. The neutrophil's granules are filled with anti-microbial enzymes that are released upon an encounter with invading pathogens contributing to their killing[23,25]. Next to degranulation, neutrophils are also able to produce ROS such as hydrogen peroxide (H_2O_2) or superoxide (O_2^-), a process called the respiratory burst[26,27]. These ROS can be released extra- or intracellularly as a tool to fight microbes. The reason to perform the respiratory burst intracellularly is because neutrophils are also exceptional phagocytes, and after engulfment of microbial particles, the release of intracellular ROS within the phagolysosome will result in their destruction[28]. When these particles are too large to engulf, neutrophils – while dying – can actively release large, extracellular, web-like structures composed of DNA-histone complexes and antimicrobial proteins known as NETs as a last attempt of attack or containment[24,29]. The release of theses NETs has been suggested to result in the entrapment of these pathogens contributing to their neutralization.

More recently neutrophils have been discovered to be more than just a first-line of defense against infections. They have been found to play an active role in tumor immunity as well. Although they are more often known for their myeloid-derived suppressor cell (MDSC) activity contributing to the proliferation of the tumor (pro-tumor effect), they also have a role in tumor elimination (anti-tumor effect)[30,31]. More on the functional plasticity of neutrophils in the tumor microenvironment can be found in **chapter 2**. In contrast to their rather well-established role in host defense, we are only beginning to understand the precise role of neutrophils as effector cells against cancer. Despite initially thought differently, it has now been demonstrated that none of the above-mentioned major anti-microbial mechanisms of neutrophils participate in cancer cell destruction[32,33]. In fact, neutrophils alone do not seem to be capable of recognizing nor interact with cancer cells. Instead, the presence of neutrophils in tumors is often associated with poor prognosis, especially in solid tumors[34,35]. Nonetheless, neutrophils are endowed with Fc receptors, a family of cell surface receptors that are expressed on a wide variety of leukocytes, including NK cells, macrophages, monocytes, and granulocytes[36]. Neutrophils specifically express FcγRI, FcγRIIa, FcγRIIIb and FcαR which can recognize and bind tumor targeting antibodies and mediate tumor cell destruction via a mechanism that was given the name of antibody-dependent cellular cytotoxicity or ADCC[37–39]. A more detailed explanation of how ADCC works in the context of antibody therapy in cancer is described later in this chapter.

Additionally, although only lately being more carefully studied, neutrophils have been shown to also initiate potent adaptive immune responses (*i.e.* they can act as danger sensors or as antigen-presenting cells; read more on this on **chapter 2**), which for cancer therapy it is believed to be a crucial step to achieve successful long-term anti-tumor immunity[40,41]. All in all, in the last decades neutrophils have been put in the spotlight as effector cells to be exploited in different immunotherapeutic strategies to combat cancer.

CYTOKINE (OR GROWTH FACTOR) THERAPY

The first main type of cancer immunotherapy is cytokine (or growth factor) therapy. Cytokines or growth factors are small hormone-like proteins that are naturally produced and secreted by a number of cells in the body and are considered “immune system modulators”. These signaling molecules come in many different flavours (meaning that each cytokine activates or stimulates a specific set of immune cells) and usually have limited range and longevity to prevent overreaction[42]. The man-made versions of these proteins can also be generated for its use in cancer treatment to stimulate the proliferation and activation of certain effector immune cells which will keep cancer cells from growing or will contribute to their killing. The most well-known example of a manufactured cytokine is interleukin-2 (IL-2), specifically stimulating T lymphocytes in the tumor microenvironment, which was

first approved for the treatment of metastatic kidney cancer and metastatic melanoma[43]. Other cytokines or growth factors used to specifically stimulate and activate cells of the myeloid compartment such as macrophages, monocytes or neutrophils, are granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). These growth factors stimulate granulopoiesis and therefore are commonly used to treat chemotherapy-induced neutropenia, among others, in cancer patients so as to boost neutrophil production in the bone marrow[44,45]. However, cytokine therapy has shown limited efficacy when given as monotherapy, and this is the reason why cytokine-based approaches are usually combined with other kinds of immunotherapy (*i.e.* antibody therapy).

ANTIBODY THERAPY: FC-MEDIATED ADCC AND TROGOCYTOSIS

The discovery of antibodies dates back to 1890 and have since become well-established forms of treatment in a wide spectrum of diseases, including cancer[15,17,46]. Antibodies, also known as immunoglobulins (Ig), are sticky Y-shaped molecules produced by plasma cells in our body that hold on to foreign or non-self-cells, and mark them for death by either the complement system or by specific immune cells[46]. This immune process of using opsonins to "tag" pathogens or cells is called "*opsonization*". Given that they were assumed to be the proteins in the bloodstream that neutralized toxins, they were originally called "antitoxins". The structure of an antibody consists of four chains, subdivided into two heavy and two light chains (**Figure 2**). Moreover, the Y-shaped structure allows the antibody to carry out dual functions: the arms of the immunoglobulin constitute the variable domain or antibody binding region (also known as F(ab') region) that will recognize the antigen to which it has been targeted; instead, the stem of the molecule is the constant domain or the region that will actively participate in the antibody-mediated effector functions (also known as Fc tail) by interacting with cell surface receptors of specific immune cells. There are five immunoglobulin classes of antibody molecules found in humans: IgA, IgD, IgE, IgG and IgM. Each isotype has its own characteristics regarding the type of heavy-chain they contain, their half-life, the antigens they target or the immune cells they engage.

When it comes to antibody therapy for cancer treatment, scientists are able to manufacture synthetic antibodies in the lab. These are the so-called monoclonal antibodies. Since the IgG class is the most abundant isotype in human serum with an average half-life of 25.8 days[47], currently all approved ADCC-inducing therapeutic monoclonal antibodies for clinical use are IgG-based, and more specifically of the (human) IgG1 subclass. Research on antibody-based therapies bloomed in the last decades and resulted in the development of different forms of man-made monoclonal antibodies. These can come in different forms: i) naked (or unconjugated) antibodies, which work alone and are the most commonly used, ii) conjugated (or antibody-drug conjugates), which are coupled to chemotherapeutic drugs

or radioactive compounds to directly kill the targeted cells, or iii) bispecific antibodies, which combine two different monoclonal antibodies with the aim to target two different proteins or cells simultaneously (**Figure 2**)[48]. And in fact, many more innovative combinations of engineered antibodies for their application in many clinical diseases are being generated and studied nowadays[49].

As the most extensively studied and used, the first FDA-approved monoclonal antibody against cancer was the IgG version of rituximab in 1997[50]. Rituximab binds to CD20 protein present on the surface of immature B cells for the treatment of non-Hodgkin lymphoma. Upon binding of the therapeutic antibody to its target, these immunoglobulins exert their biological effects through several mechanisms. On the one hand, the opsonizing antibody can exert direct effects through the F(ab') domain by interfering with the intrinsic functions of the cell (*i.e.* preventing tumor cell survival signals from happening). However, the most important effector functions of these anti-cancer therapeutic antibodies are mediated through the engagement of the complement system inducing complement-dependent cytotoxicity (CDC), or via the crosslink of the Fc tail to Fc receptors expressed on diverse immune cells. The latter results in the indirect Fc-mediated killing of the targeted cells.

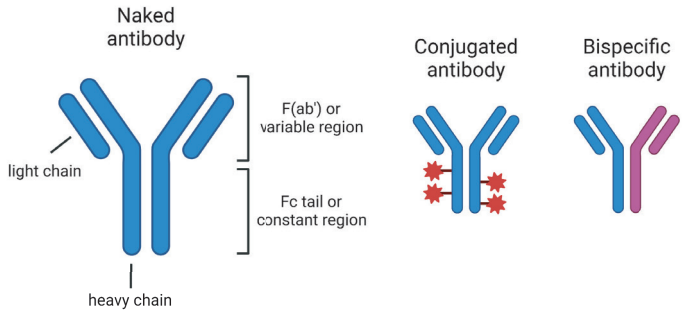


Figure 2. *Immunoglobulins (Ig) consist of Y-shaped molecules that are divided in two heavy and two light chains. The binding domain of an Ig is composed of the F(ab') or variable region that will recognize the target of interest and is unique for every antibody. The stem of the Ig is the Fc tail or constant region which will be recognized by the Fc receptors on Fc-expressing immune cells and thereby is involved in mediating the effector functions of the antibody. Manufactured monoclonal antibodies come in different forms: naked (or unconjugated) antibodies, conjugated to chemotherapeutic or radiation agents (also called antibody-drug conjugates or ADCs), and bispecific antibodies that bind two different antigens (blue part of the antibody will bind one antigen and magenta part will bind a different one). Created with BioRender.com.*

The engagement of one Fc-expressing cell type or another may invoke a different aftermath towards the antibody-targeted tumor cell, although they will all result in its destruction[38]: while NK cells induce apoptosis of the target cell by the release of cytotoxic granules via ADCC, macrophages make use of their phagocytic capacities to engulf the antibody-targeted cell resulting in antibody-dependent cellular phagocytosis or ADCP. For neutrophils, instead, the cytotoxic mechanism by which they induce tumor cell destruction in the context of antibody therapy has for long remained largely unclear. In the last years, however, convincing evidence demonstrated the ability of neutrophils to trogocytose antibody-opsonized tumor cells, a process involving the active transfer of plasma membrane from a donor cell to an acceptor cell during intercellular contact[51]. In fact, in 2018, a direct association between neutrophil-mediated trogocytosis and tumor cell death of antibody-opsonized solid cancer cells was described. Here, the neutrophil started ripping off pieces of the antibody-opsonized tumor cell membrane until its lysis[33]. This necrotic type of cell death was termed "trogoptosis", and as an antibody-mediated mechanism, Fc receptor downstream signaling is intrinsically involved in the process. A more detailed explanation on the specific contribution of each Fc receptor expressed on neutrophils – each having different affinities to the different immunoglobulins and thereby conveying stronger or weaker effector functions – on the killing of antibody-opsonized cells is described in **chapter 2**. If an activating Fc receptor is engaged, the downstream signaling will result, among others, in the activation of CD11b/CD18 or Mac-1 integrin within the neutrophil.

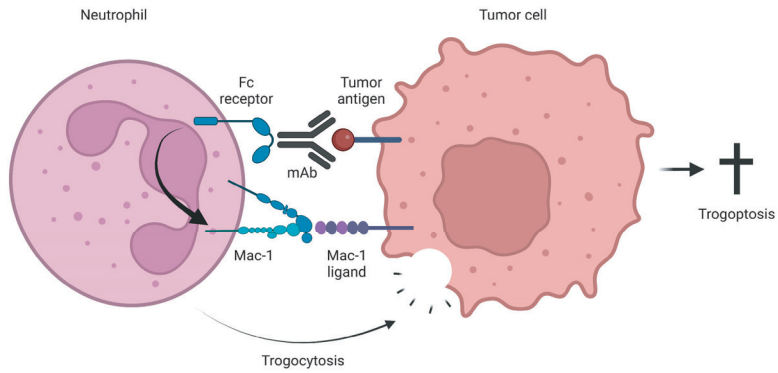


Figure 3. *The neutrophil-mediated killing of tumor cells requires the binding of the Fc tail of the therapeutic monoclonal antibody (mAb) opsonizing the targeted tumor cells to the Fc receptor on neutrophils. The engagement of an activating Fc receptor induces downstream signaling pathways within the neutrophil that activate CD11b/CD18 or Mac-1 integrin. The activated integrin forms a cytotoxic synapse between the tumor cell and the attacking neutrophil which starts taking "bites" of the tumor cell membrane by trogocytosis until its disintegration, resulting in the tumor cell lysis via the necrotic type of cell death: trogoptosis. Created with BioRender.com.*

This allows an intimate cell-cell contact between the neutrophil and the tumor cell, from which the neutrophil can start taking "bites" of the tumor cell membrane contributing to its destruction (**Figure 3**). In fact, CD11b/CD18 activation is indispensable for neutrophil-mediated ADCC to take place[33,52,53]. Nonetheless, trogocytosis has also been described as a mechanism by which tumor cells evade tumor immunity, as it may involve the shaving of the targeted antigen from the plasma membrane by the effector immune cells. More details on the consequences of antibody-dependent trogocytosis depending on the circumstances are described on **chapter 2**.

CHECKPOINT BLOCKADE

Unfortunately, antibody therapy alone is most of the times not curative and often needs to be used in combination with other forms of treatment, such as chemotherapy, radiation therapy or other immunotherapies. The highly immunosuppressive tumor microenvironment giving rise to immune resistance mechanisms of all kinds contributes to this. In addition, our immune system functions under highly strict regulatory mechanisms and the reason for that is simple: an allergic reaction can occur when the immune system overreacts, and an autoimmune disease may take place if the immune system misidentifies normal self-cells and attacks them. Hence, there must be some sort of physiological safety mechanism in place to prevent such unwanted reactions from happening. These protective controls are the so-called "checkpoints" that act as a system of breaks telling the immune system "don't attack". It is a way of asking the immune system if it is completely sure about what it is about to do.

As far as cancer is concerned, it is now widely known that cancer cells use several strategies, including these checkpoints, to their benefit to stop the immune system from attacking them. The expression of these immune checkpoints make cancer cells look like normal body cells, thereby tricking our defender cells. In fact, the ability of cancer cells to evade immune destruction has been proposed as one of the ten hallmarks of cancer[54]. Consequently, a major area of interest in current cancer immunotherapy research has arisen from the discovery of these evasive mechanisms prompting the development of a new type of drugs aimed at inhibiting these checkpoints which received the name of "checkpoint inhibitors"[17]. These checkpoint inhibitors are basically monoclonal man-made antibodies that block the cancer's "secret handshake"[11] with immune cells unleashing the immune's system capacity to recognize and kill the tumor cells. Very rapidly, these new drugs were tested as possible cancer immunotherapies, until the first checkpoint inhibitor drug, Ipilimumab, was approved by the FDA in 2011[55]. Ipilimumab specifically targets the inhibitory receptor CTLA-4 on activated T lymphocytes contributing to the potentiation of the T lymphocyte's capacity to kill cancer cells.

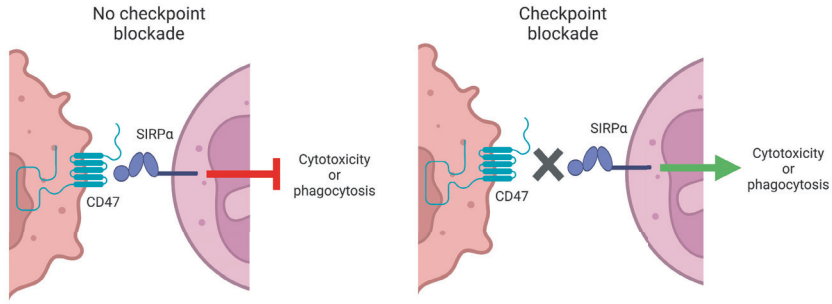


Figure 4. *CD47/SIRPα axis constitutes the first innate inhibitory checkpoint to be investigated in the context of cancer therapy. CD47, often overexpressed by tumor cells, acts as a "don't eat me signal" which upon binding to its ligand SIRPα, expressed on innate cells (neutrophils and macrophages), results in the inhibition of the respective effector functions (i.e. cytotoxicity or phagocytosis). By inhibiting this axis in the presence of a blocking antibody (checkpoint inhibitor), directed to either of the molecules, the inhibitory signals are halted within the effector cell. The "break" is now released and the effector cell becomes fully capable of performing its effector functions towards tumor cells. Created with BioRender.com.*

Despite most efforts are focused on the targeting of immune checkpoints especially expressed on T lymphocytes (*i.e.* PD1/PDL-1, CTLA-4/CD80-86, LAG-3/MHC-II, TIGIT/CD155)[56], innate cells also express some of them, which have become of recent interest to block. Neutrophils and macrophages have a variety of these inhibitory checkpoints in place so as to suppress their activity when necessary, which turns them into potential therapeutic targets for checkpoint blockade therapy too[57]. Signal regulatory protein alpha (SIRPα) is an important and well-investigated one which binds to CD47. CD47 is ubiquitously expressed on healthy cells (*i.e.* on red blood cells) and acts as a "don't eat me signal" when bound to SIRPα, resulting in the inhibition of macrophage and neutrophil effector functions, which apart from phagocytosis also include additional cytotoxic mechanisms[58,59]. This checkpoint is especially needed to prevent events such as healthy red blood cell phagocytosis which would continuously happen if these safety mechanism would not exist. Cancer cells, however, tend to overexpress CD47 as a way to hide from the immune system to avoid being recognized and eliminated by innate cells (**Figure 4**). Preclinically, CD47/SIRPα interactions have been shown to negatively regulate neutrophil- and macrophage-mediated cytotoxicity both *in vitro* and *in vivo* for a number of cancers when used in combination with opsonizing therapeutic antibodies[33,60,61]. Interfering with this axis reverts this picture and further enhances their cytotoxic capabilities, leading to high levels of tumor cell killing[53,62,63]. As a result, many efforts have been put into developing blocking agents to inhibit CD47/SIRPα axis for their use in cancer therapy[64], which have entered the clinical stage for multiple cancer indications[65]. In particular, the CD47 inhibitor Hu5F9-G4 (Magrolimab)

showed high efficacy with minimal adverse effects when combined with rituximab for the treatment of Non-Hodgkin's lymphoma[66], and its use is now being tested for solid tumors too[67]. Importantly, some new preclinical evidence described the activation of T cell responses resulting from the CD47/SIRPα blockade approaches[68], bridging once again innate and adaptive responses, a necessary step to accomplish lasting anti-tumor immunity.

ADOPTIVE CELL THERAPY: CHIMERIC ANTIGEN RECEPTORS

The name for this type of cancer immunotherapy is easier to understand when split in two parts. A "cellular therapy" is any treatment that employs a whole living cell as the drug, instead of a laboratory-produced engineered antibody, for instance. On the other hand, the term "adoptive" means to bring something as one's own. When these terms are coupled together, it receives the name of Adoptive Cell Therapy or Adoptive Cell Transfer (ACT), referring to the acquisition of a cellular therapy in one's body. This group of treatments consists of removing a subtype of the patient's effector immune cells via leukapheresis to expand their numbers or to genetically tweak them in a lab to help them better recognize and kill the patient's cancer once reinfused back in the patient (**Figure 5**). Given the fact that T lymphocytes are the ultimate cytotoxic cells in cancer therapy, they are excellent cells to harness for ACT. In addition, they can persist in the body for a very long time. There are several ways of modifying cells but one of the most successful nowadays is the generation of Chimeric Antigen Receptor (CAR) T cells. CAR T cells were first described in the 1990s[69] and they consist of the generation of tumor-specific T lymphocytes that have been genetically engineered to express a special receptor directed to a tumor antigen of interest. Such receptor is a chimeric molecule composed of an ectodomain, generally a portion of an antibody that recognizes an antigen (scFv or Fab fragments are the most commonly used), that is coupled to an endodomain or intracellular part consisting of at least one signaling domain (**Figure 5**). Commonly used signaling domains for CAR T cells are the CD3ζ chain and a costimulatory receptor, such as CD28 or 4-1BB[70]. The binding of the CAR T cell to the tumor antigen results in the activation of the T cell cytotoxic activity towards the targeted cell contributing to the cancer cell's destruction.

The first FDA-approved CAR T cell drug, Kymriah™, came in 2017 for the treatment of children with relapsed B-cell acute lymphoblastic leukemia[71], which was followed by approval of other CAR T cell drugs for other types of lymphomas the same year[72]. However, in spite of the remarkable success of these tumor-specific lymphocytes for their application in hematological malignancies, much less success has been obtained with ACT for the treatment of solid tumors[73]. While there likely are numerous reasons for this, the limited T cell trafficking and the immune resistance mechanisms arising from the highly suppressive tumor microenvironment characteristic of solid tumors are thought to be two of the main

hurdles for CAR T cell therapy[74,75]. Likewise, CAR T cell therapy has to be administered and monitored with caution as patients may develop cytokine storm syndrome, a potentially lethal adverse effect[76].

In an attempt to optimize CAR therapy in terms of efficacy and safety to broaden its application to other malignancies, efforts were made to use other immune cell types, such as natural killer (NK) cells and myeloid cells, as a basis for CAR therapy[77]. NK cells display rapid and potent immunity, especially in solid tumors, and therefore received the second most attention in CAR therapy research, leading to several clinical trials aimed at treating both hematological and non-hematological malignancies[73]. CAR-myeloid cells with neutrophils, monocytes and macrophages, have been described preclinically, but no clinical trials have been initiated thus far. Several advantages come with the harness of myeloid cells for CAR therapy over CAR T cells. In contrast to T lymphocytes, myeloid cells infiltrate solid tumors well. At the same time, a first attack by these (*i.e.* macrophage-induced ADCP or neutrophil-mediated ADCC) contributes to the release of tumor antigens in the tumor microenvironment that will be caught by dendritic cells and subsequently presented to cytotoxic T lymphocytes. The subsequent activation of T cell cytotoxic responses towards the tumor will result in better therapeutic outcomes leading to long-lasting tumor control. Last, although the use of innate CARs may provide an obstacle for maintaining durable responses, resulting from their rapid turnover, this may actually add to the restriction of serious side effects as seen for CAR T cells.

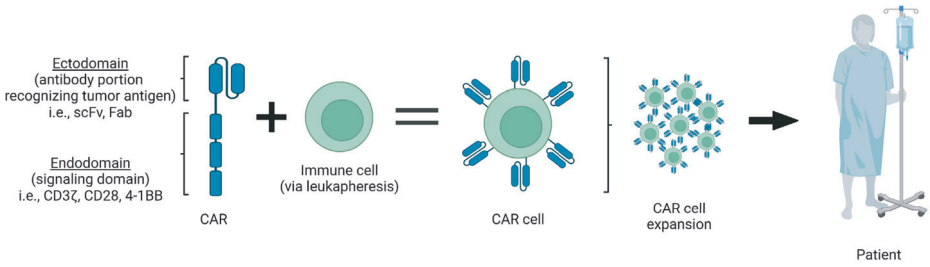


Figure 5. *Chimeric Antigen Receptor (CAR) therapy is a subtype of Adoptive Cell Therapy (ACT) consisting on the generation of cells (previously removed from the patient via leukapheresis) that have been genetically engineered to express a chimeric receptor composed of two parts: the ectodomain is the antibody portion that will recognize the tumor antigen of interest, and the endodomain which encompasses the signaling domains that will activate the effector cell's functions towards the tumor cell upon activation. The genetically engineered CAR cells are then expanded in vitro and reinfused back in the patient where they will exert their effects. Created with BioRender.com.*

Thanks to the availability of continuously expanding myeloid-like cell lines (*i.e.* THP-1, U937, NB4 and HL-60 cell lines), the possibilities to generate CAR-myeloid cells start with using these as test or proof of concept models, as they provide an unlimited source of effector cells that can be exploited for genetic engineering. Klichinsky et al., generated several versions of CAR-macrophages from the THP-1 macrophage-like model cell line, which successfully induced antigen-specific phagocytosis *in vitro*[78]. Furthermore, they also transduced primary human macrophages with an anti-HER2 CAR, another possibility for the generation of CAR-myeloid cells, which in their hands efficiently reduced tumor burden in an ovarian cancer xenograft model that also promoted anti-tumor T cell activity[78]. Yet, to obtain therapeutically relevant CAR-myeloid cells and the right numbers of cells that can be infused into the patient, the most appropriate approach appears to be the gene transduction of human hematopoietic stem cells (HSCs) followed by expansion and selective differentiation to cells of the different lineages to generate CAR-expressing granulocytes, monocytes, or macrophages. As a proof of concept, De Oliveira et al., retrovirally transduced cord blood-derived human $CD34^+$ HSCs with an anti-CD19 CAR that were differentiated into cells of the myeloid lineage generating CAR-bearing myeloid cells[79]. As far as neutrophils are concerned, in 1998, Roberts et al., already described transduction of human HSCs with an anti-CD4-CD3ζ CAR from which CAR-expressing neutrophils were isolated and tested against Raji cells *in vitro*[80]. More recently, CAR-neutrophils were generated from genetically engineered human pluripotent stem cells which displayed enhanced anti-tumor activities and induced improved survival in a glioblastoma xenograft model compared to the untransduced versions[81]. Naturally, the signaling domains that drive T lymphocyte anti-tumor activities differ from those driving anti-tumor effector functions by neutrophils or macrophages. Hence, other non-classical intracellular domains involved in ADCC or ADCP may also be exploited as signaling domains for CAR-myeloid cells[70]. Altogether, the advancements in the field of ACT are paving the way to myeloid cell-based therapeutic strategies that could complement and boost current cancer immunotherapeutic approaches.

IN VIVO MODELS FOR THE PRECLINICAL STUDY OF NEUTROPHILS

Studies of human immune cells are primarily constrained to *in vitro* or *ex vivo* assays, whereas studies in living organisms (*in vivo*) allow manipulation and monitoring of the immune system in an organismal setting. The use of animals in *in vivo* studies addresses many of the shortcomings of *in vitro* studies, where the safety, toxicity and efficacy of a specific therapeutic can be evaluated in a more complex level. Mice are considered valuable tools for the study of *in vivo* immune responses as they can model human disease states, and in fact, many of the main fundamental concepts of immunology are derived from these laboratory animals. However, notwithstanding that mice and humans are biologically of use

to compare and relatively similar on many levels of the immune system, there are important human-to-mouse differences that have to be taken in consideration. Strong evolutionary pressures over the years have led to significant changes in the overall organization of the murine and human immune systems[82–84]. While signatures of lineage-specific gene expression as well as other aspects in cell identity are conserved between species, notable differences are described in relation to the effector and regulatory mechanisms of some human and mouse immune cells[85]. One such cell type are neutrophils, for which major cross-species alterations affecting their intrinsic biology have been described. These include differences key neutrophil molecules (*i.e.* selectins, Fc receptors[86–88]), the expression of cytokines (*i.e.* IL-10, IL-17[89,90]), the activation pathways to induce the respiratory burst[91,92] and NO generation by NO synthases[93], as well as the cytotoxic or immunosuppressive mechanisms against infections or cancer (*i.e.* defensins, serine proteases, arginase-1[94–98]). Altogether, this emphasizes the need for models that support the *in vivo* study of human neutrophils, specifically, as murine findings mostly fail to translate to humans. For instance, murine tumor models are being generated from the transplantation of cell lines derived from advanced tumors that have already been subjected to immune selection and therefore grow rapidly[99]. As a consequence, these models appear to completely bypass the initial phases of tumor evolution that are expected to occur during the slow evolution of human tumors, and thus do not accurately mirror the gradual stages of tumor development[100]. This may already by itself – also in a humanized setting though – have a profound impact on tumor behavior as well as on the function of tumor-infiltrating cells, including that of neutrophils. Neutrophils are believed to acquire a tumor promoting phenotype in advanced tumor stages leading to potentially more pro-tumoral mechanisms of neutrophils prevailing over the anti-tumorigenic ones[101,102].

Novel mouse models aimed at overcoming some of the obstacles between the different immunological responses of mouse and humans have been generated. These are the so-called "humanized mice" which are highly immunodeficient mice (*i.e.* mutations in *Rag2* or *IL2R*γ genes lead to the development of mice with a dysfunctional murine immune system lacking B, T and NK cells, and allowing better xenografts) that have been reconstituted with a human hemato-lymphoid system by transplantation of human $CD34^+$ HSCs right after birth[103,104]. In this way, mice containing human immunological properties are generated. Examples of these are the *Rag2*$^{-/-}$*IL2R*γ$^{-/-}$ or the *NOD/scid/IL2R*γ$^{-/-}$ (NSG) mouse strains, which allow for the development of a very much complete human immune lymphoid compartment[105,106]. Undoubtedly, these humanized mouse strains have provided a lead forward for studying human immune functions *in vivo*[103,104,107]. Yet, a major limitation of all current humanized mouse models is the inability to establish a reliable human myeloid compartment, thus hindering the study of cells from the innate immune system. This is possibly due to species-related differences of cytokines and growth factors[108], which have

led researchers to develop ways to generate improved models by expressing human-specific cytokines via a knock-in technology within these mice.

The MISTRG mice, transduced with the human versions of *M-CSF*, *IL-3*, *GM-CSF*, *TPO* and *SIRPα*, comprise the first next-generation humanized mouse model allowing successful development of human myeloid cells, including macrophages, NK cells and granulocytes, thus achieving multilineage human immune reconstitution[109]. Despite the achievements gained with these improved humanized mouse model, there are still a number of limitations. Early studies have shown cross-reactivity between some human and mouse growth factors[108,110–112], and although such cross-reactivity does not always equal biologic activity, it may still induce uncontrollable phenotypic changes. Furthermore, although granulocyte progenitors are detected in the bone marrow of the murine host, these appear unable to appropriately circulate in the bloodstream nor reach tissues[109,113]. Last, graft-versus-host disease-related red blood cell destruction may occur, leading to severe anemia, as a result of the high levels of human hematopoietic engraftment in these mice (*i.e.* the phagocytic tolerance toward the mouse host is lost)[104,109].

As a matter of fact, the rapid development of new models will enable researchers to preclinically study specific aspects of the human immune system and their involvement in human pathologies in a much deeper and detailed level. At the end of the day, one has to thoroughly evaluate which model is the most appropriate one relative to the research question that has to be answered.

SCOPE OF THE THESIS

In this thesis we investigated a number of immunotherapeutic strategies in which neutrophils can be harnessed to improve cancer treatment of solid tumors in particular. To start with, in **chapter 2** we have given a general overview of the opposing roles that neutrophils can acquire in cancer where we discussed potential ways to therapeutically silence their tumor promoting activity and to activate or enhance their anti-tumor functions. From this point onwards, each of the following chapters (except for chapter 6) touches upon different types of immunotherapeutic approaches where neutrophils mediate tumor cell cytotoxicity, mainly in the context of antibody therapy.

In **chapter 3** we highlighted the importance of cytokine (or growth factor) therapy by showing how the stimulation of neutrophils with either GM-CSF or G-CSF results in enhanced neutrophil-mediated killing of anti-GD2 opsonized neuroblastoma cells. More specifically, our data showed how G-CSF can be used as a suitable alternative to GM-CSF for the treatment of high-risk neuroblastoma patients, given that the latter is not clinically

available anywhere outside Northern America. In addition, we discarded any unfavorable effects of G-CSF at altering the neuroblastoma cells' phenotype towards a more immune-resistant one, an effect that was previously described in literature despite not being sufficiently investigated.

In line with the above, in **chapter 4** we described another way to improve treatment for neuroblastoma patients with a checkpoint blockade strategy. Here, we first demonstrated how neuroblastoma cells tend to overexpress CD47 as a tumor evasion mechanism to escape an attack from the immune system, highlighting the relevance of the innate immune checkpoint CD47/SIRPα in this cancer type. Next, we investigated the therapeutic potential of combining anti-GD2 antibody with CD47/SIRPα checkpoint inhibition and confirmed an enhancement of the neutrophil's cytotoxic ability to kill the targeted tumor cells, further improving responsiveness to anti-GD2 therapy.

In **chapter 5** we explored the ability of neutrophils as the cell basis for CAR therapy. We used the neutrophil-like NB4 cell line to generate different CAR constructs directed towards three well-established solid tumor antigens (GD2 – neuroblastoma, EGFR – epithelial carcinomas, HER2/neu – breast cancers) which were coupled to the signaling domain of an activating Fc receptor known to induce potent cytotoxicity upon antibody binding by neutrophils. We showed evidence of the capacity of these genetically modified cells to intrinsically recognize and kill tumor cells in the absence of tumor-targeting antibody, offering an alternative therapeutic approach to overcome the challenges faced by CAR T cells for the treatment of solid tumors.

All the immunotherapeutic approaches described in this thesis have been studied *in vitro*, but naturally, findings at this level do not take into account the complexity of a living organism. As mentioned, there is a need to find an *in vivo* model that is more representative to study many of the above-mentioned. In **chapter 6**, we demonstrated that the humanized MISTRG mouse strain serves as a potential *in vivo* model for the study of neutrophil biology and their involvement in complex diseases, such as cancer, thereby conferring a model system for the preclinical evaluation of neutrophil-mediated immunotherapeutic strategies.

In **chapter 7** the key findings of this thesis are summarized, trying to put them in a broader perspective, further evaluating the potential of neutrophils as effector cells supporting antibody therapy for cancer in the near future.

REFERENCES

1. Ritchie H, Spooner F, Roser M. Causes of death - Our World in Data. ourworldindata.org. Accessed November 29, 2022. https://ourworldindata.org/causes-of-death#cancers
2. Cancer. who.int. Accessed November 29, 2022. https://www.who.int/news-room/fact-sheets/detail/cancer
3. Naghavi M, Abajobir AA, Abbafati C, et al. Global, regional, and national age-sex specific mortality for 264 causes of death, 1980–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet*. 2017;390(10100):1151-1210.
4. What Is Cancer? - NCI. cancer.gov. Accessed November 29, 2022. https://www.cancer.gov/about-cancer/understanding/what-is-cancer
5. Mukherjee S. *The Emperor of All Maladies: A Biography of Cancer*. 1st ed. Scribner; 2011.
6. Riley J. Estimates of Regional and Global Life Expectancy, 1800-2001. *Popul Dev Rev*. 2005;31:537-543.
7. History of Cancer Treatments: Surgery. cancer.org. Accessed November 29, 2022. https://www.cancer.org/treatment/understanding-your-diagnosis/history-of-cancer/cancer-treatment-surgery.html
8. History of Cancer Treatments: Radiation Therapy. cancer.org. Accessed November 29, 2022. https://www.cancer.org/treatment/understanding-your-diagnosis/history-of-cancer/cancer-treatment-radiation.html
9. History of Cancer Treatments: Chemotherapy. cancer.org. Accessed November 29, 2022. https://www.cancer.org/treatment/understanding-your-diagnosis/history-of-cancer/cancer-treatment-chemo.html
10. Yaqub F. Cut Poison Burn. *Lancet Oncol*. 2012;13(6):578.
11. Graeber C. *The Breakthrough*. 1st ed. Scribe; 2018.
12. Abbas A, Lichtman A, Pillai S. Cellular and molecular immunology: Properties and overview of immune responses. In: 9th ed. Elsevier; 2016.
13. Coley WB. The Treatment of Malignant Tumors by Repeated Inoculations of Erysipelas: With a Report of Ten Original Cases. *Clin Orthop Relat Res*. 1991;262. https://journals.lww.com/clinorthop/Fulltext/1991/01000/The_Classic__The_Treatment_of_Malignant_Tumors_by.2.aspx
14. Morales A, Eidinger D, Bruce AW. Intracavitary Bacillus Calmette-guerin in the Treatment of Superficial Bladder Tumors. *J Urol*. 1976;116(2):180-182.
15. Dobosz P, Dzieciątkowski T. The Intriguing History of Cancer Immunotherapy. *Front Immunol*. 2019;10:2965.
16. A Brief History of Immunotherapy. Accessed November 30, 2022. https://www.targetedonc.com/view/a-brief-history-of-immunotherapy
17. Oiseth SJ, Aziz MS. Cancer immunotherapy: a brief review of the history, possibilities, and challenges ahead. *J Cancer Metastasis Treat*. 2017;3:250-261.
18. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol*. 2002;3(11):991-998.
19. Couzin-Frankel J. Cancer Immunotherapy. *Science (1979)*. 2013;342(6165):1432-1433.
20. Heifets L. Centennial of Metchnikoff's discovery. *J Reticuloendothel Soc*. 1982;31(5):381-391.
21. Cartwright GE, Athens JW, Wintrobe MM. Analytical Review: The Kinetics of Granulopoiesis in Normal Man. *Blood*. 1964;24(6):780-803.
22. Borregaard N. Neutrophils, from marrow to microbes. *Immunity*. 2010;33(5):657-670.

23. Cowland JB, Borregaard N. Granulopoiesis and granules of human neutrophils. *Immunol Rev*. 2016;273(1):11-28.
24. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol*. 2018;18(2):134-147.
25. Borregaard N, Sørensen OE, Theilgaard-Mönch K. Neutrophil granules: a library of innate immunity proteins. *Trends Immunol*. 2007;28(8):340-345.
26. Dahlgren C, Karlsson A. Respiratory burst in human neutrophils. *J Immunol Methods*. 1999;232(1):3-14.
27. El-Benna J, Hurtado-Nedelec M, Marzaioli V, Marie JC, Gougerot-Pocidalo MA, Dang PMC. Priming of the neutrophil respiratory burst: role in host defense and inflammation. *Immunol Rev*. 2016;273(1):180-193.
28. Lee WL, Harrison RE, Grinstein S. Phagocytosis by neutrophils. *Microbes Infect*. 2003;5(14):1299-1306.
29. Delgado-Rizo V, Martínez-Guzmán M, Iñiguez-Gutierrez L, García-Orozco A, Alvarado-Navarro A, Fafutis-Morris M. Neutrophil extracellular traps and its implications in inflammation: an overview. *Front Immunol*. 2017;8.
30. Treffers LW, Hiemstra IH, Kuijpers TW, van den Berg TK, Matlung HL. Neutrophils in cancer. *Immunol Rev*. 2016;273(1):312-328.
31. Grecian R, Whyte MKB, Walmsley SR. The role of neutrophils in cancer. *Br Med Bull*. 2018;128(1):5-14.
32. Dallegri F, Patrone F, Frumento G, Sacchetti C. Antibody-dependent killing of tumor cells by polymorphonuclear leukocytes. Involvement of oxidative and nonoxidative mechanisms. *J Natl Cancer Inst*. 1984;73(2):331-339.
33. Matlung HL, Babes L, Zhao XW, et al. Neutrophils Kill Antibody-Opsonized Cancer Cells by Trogoptosis. *Cell Rep*. 2018;23(13):3946-3959 e6.
34. Templeton AJ, McNamara MG, Seruga B, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst*. 2014;106(6):dju124.
35. Gentles AJ, Newman AM, Liu CL, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med*. 2015;21(8):938-945.
36. Nimmerjahn F, Ravetch J V. Fc-Receptors as Regulators of Immunity. *Adv Immunol*. 2007;96:179-204.
37. Gale R, Zighelbloim J. Polymorphonuclear leukocytes in antibody-dependent cellular cytotoxicity. *J Immunol*. 1975;114(3):1047-1050.
38. Bakema JE, van Egmond M. Fc receptor-dependent mechanisms of monoclonal antibody therapy of cancer. *Curr Top Microbiol Immunol*. 2014;382:373-392.
39. Heemskerk N, van Egmond M. Monoclonal antibody-mediated killing of tumour cells by neutrophils. *Eur J Clin Invest*. 2018;48 Suppl 2:e12962.
40. Zhu EF, Gai SA, Opel CF, et al. Synergistic innate and adaptive immune response to combination immunotherapy with anti-tumor antigen antibodies and extended serum half-life IL-2. *Cancer Cell*. 2015;27(4):489-501.
41. Moynihan KD, Opel CF, Szeto GL, et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. *Nat Med*. 2016;22(12):1402-1410.
42. Chulpanova DS, Kitaeva K V, Green AR, Rizvanov AA, Solovyeva V V. Molecular Aspects and Future Perspectives of Cytokine-Based Anti-cancer Immunotherapy. *Front Cell Dev Biol*. 2020;8.

43. Rosenberg SA, Packard BS, Aebersold PM, et al. Use of Tumor-Infiltrating Lymphocytes and Interleukin-2 in the Immunotherapy of Patients with Metastatic Melanoma. *N Engl J Med*. 1988;319(25):1676-1680.
44. Mehta HM, Malandra M, Corey SJ. G-CSF and GM-CSF in Neutropenia. *J Immunol*. 2015;195(4):1341-1349.
45. Morstyn G, Souza LM, Keech J, et al. Effect of granulocyte colony stimulating factor on neutropenia induced by cytotoxic chemotherapy. *The Lancet*. 1988;331(8587):667-672.
46. Ribatti D. The discovery of plasma cells: An historical note. *Immunol Lett*. 2017;188:64-67.
47. Mankarious S, Lee M, Fischer S, et al. The half-lives of IgG subclasses and specific antibodies in patients with primary immunodeficiency who are receiving intravenously administered immunoglobulin. *J Lab Clin Med*. 1988;112(5):634-640.
48. Bentler T. Naked, conjugated, bispecific: mAbs explained. Published August 31, 2017. Accessed January 10, 2023. https://news.sanfordhealth.org/cancer/mabs/
49. Strohl WR. Current progress in innovative engineered antibodies. *Protein Cell*. 2018;9(1):86-120.
50. Pierpont TM, Limper CB, Richards KL. Past, Present, and Future of Rituximab—The World's First Oncology Monoclonal Antibody Therapy. *Front Oncol*. 2018;8.
51. Tabiasco J, Espinosa E, Hudrisier D, Joly E, Fournie JJ, Vercellone A. Active trans-synaptic capture of membrane fragments by natural killer cells. *Eur J Immunol*. 2002;32(5):1502-1508.
52. Metelitsa LS, Gillies SD, Super M, Shimada H, Reynolds CP, Seeger RC. Antidisialoganglioside/granulocyte macrophage-colony-stimulating factor fusion protein facilitates neutrophil antibody-dependent cellular cytotoxicity and depends on FcgammaRII (CD32) and Mac-1 (CD11b/CD18) for enhanced effector cell adhesion and azurophil granule exocytosis. *Blood*. 2002;99(11):4166-4173.
53. Treffers LW, ten Broeke T, Rosner T, et al. IgA-Mediated Killing of Tumor Cells by Neutrophils Is Enhanced by CD47-SIRPalpha Checkpoint Inhibition. *Cancer Immunol Res*. 2020;8(1):120-130.
54. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell*. 2011;144(5):646-674.
55. Cameron F, Whiteside G, Perry C. Ipilimumab. *Drugs*. 2011;71(8):1093-1104.
56. Schnell A, Bod L, Madi A, Kuchroo VK. The yin and yang of co-inhibitory receptors: toward anti-tumor immunity without autoimmunity. *Cell Res*. 2020;30(4):285-299.
57. van Rees DJ, Szilagyi K, Kuijpers TW, Matlung HL, van den Berg TK. Immunoreceptors on neutrophils. *Semin Immunol*. 2016;28(2):94-108.
58. Matlung HL, Szilagyi K, Barclay NA, van den Berg TK. The CD47-SIRPalpha signaling axis as an innate immune checkpoint in cancer. *Immunol Rev*. 2017;276(1):145-164.
59. Willingham SB, Volkmer JP, Gentles AJ, et al. The CD47-signal regulatory protein alpha (SIRPα) interaction is a therapeutic target for human solid tumors. *Proc Natl Acad Sci U S A*. 2012;109(17):6662-6667.
60. Zhao XW, van Beek EM, Schornagel K, et al. CD47-signal regulatory protein-α (SIRPα) interactions form a barrier for antibody-mediated tumor cell destruction. *Proc Natl Acad Sci U S A*. 2011;108(45):18342-18347.
61. Zhao XW, Matlung HL, Kuijpers TW, van den Berg TK. On the mechanism of CD47 targeting in cancer. *Proc Natl Acad Sci U S A*. 2012;109(42):E2843-E2843.
62. Ring NG, Herndler-Brandstetter D, Weiskopf K, et al. Anti-SIRPα antibody immunotherapy enhances neutrophil and macrophage antitumor activity. *Proc Nat Acad Sci U S A*. 2017;114(49):110578-110585.

63. Kim D, Wang J, Willingham SB, Martin R, Wernig G, Weissman IL. Anti-CD47 antibodies promote phagocytosis and inhibit the growth of human myeloma cells. *Leukemia*. 2012;26(12):2538-2545.
64. Weiskopf K. Cancer immunotherapy targeting the CD47/SIRPα axis. *Eur J Cancer*. 2017;76:100-109.
65. van den Berg TK, Valerius T. Myeloid immune-checkpoint inhibition enters the clinical stage. *Nat Rev Clin Oncol*. 2019;16(5):275-276.
66. Advani R, Flinn I, Popplewell L, et al. CD47 Blockade by Hu5F9-G4 and Rituximab in Non-Hodgkin's Lymphoma. *N Engl J Med*. 2018;379(18):1711-1721.
67. Sikic BI, Lakhani N, Patnaik A, et al. First-in-Human, First-in-Class Phase I Trial of the Anti-CD47 Antibody Hu5F9-G4 in Patients With Advanced Cancers. *J Clin Oncol*. 2019;37(12):946-953.
68. McCracken MN, Cha AC, Weissman IL. Molecular Pathways: Activating T Cells after Cancer Cell Phagocytosis from Blockade of CD47 "Don't Eat Me" Signals. *Clin Cancer Res*. 2015;21(16):3597-3601.
69. Eshhar Z, Waks T, Gross G, Schindler DG. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci U S A*. 1993;90(2):720-724.
70. Sievers NM, Dörrie J, Schaft N. CARs: Beyond T Cells and T Cell-Derived Signaling Domains. *Int J Mol Sci*. 2020;21(10).
71. NIH. CAR T-Cell Therapy Approved for Some Children and Young Adults with Leukemia. Published September 11, 2017. Accessed January 16, 2023. https://www.cancer.gov/news-events/cancer-currents-blog/2017/tisagenlecleucel-fda-childhood-leukemia
72. FDA. FDA approves CAR-T cell therapy to treat adults with certain types of large B-cell lymphoma. Published October 18, 2018. Accessed January 23, 2023. https://www.fda.gov/news-events/press-announcements/fda-approves-car-t-cell-therapy-treat-adults-certain-types-large-b-cell-lymphoma
73. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J*. 2021;11(4):69.
74. Martinez M, Moon EK. CAR T Cells for Solid Tumors: New Strategies for Finding, Infiltrating, and Surviving in the Tumor Microenvironment. *Front Immunol*. 2019;10:128.
75. D'Aloia MM, Zizzari IG, Sacchetti B, Pierelli L, Alimandi M. CAR-T cells: the long and winding road to solid tumors. *Cell Death Dis*. 2018;9(3):282.
76. Offord C. Making CAR T-Cell Therapy safer. The Scientist. Published April 1, 2017. Accessed January 23, 2023. https://www.the-scientist.com/bio-business/making-car-t-cell-therapy-safer-31730
77. Harrer DC, Dörrie J, Schaft N. Chimeric Antigen Receptors in Different Cell Types: New Vehicles Join the Race. *Hum Gene Ther*. 2018;29(5):547-558.
78. Klichinsky M, Ruella M, Shestova O, et al. Human chimeric antigen receptor macrophages for cancer immunotherapy. *Nat Biotechnol*. 2020;38(8):947-953.
79. De Oliveira SN, Ryan C, Giannoni F, et al. Modification of Hematopoietic Stem/Progenitor Cells with CD19-Specific Chimeric Antigen Receptors as a Novel Approach for Cancer Immunotherapy. *Hum Gene Ther*. 2013;24(10):824-839.
80. Roberts MR, Cooke KS, Tran AC, et al. Antigen-specific cytolysis by neutrophils and NK cells expressing chimeric immune receptors bearing zeta or gamma signaling domains. *J Immunol*. 1998;161(1):375-384.

81. Chang Y, Syahirah R, Wang X, et al. Engineering chimeric antigen receptor neutrophils from human pluripotent stem cells for targeted cancer immunotherapy. *Cell Rep.* 2022;40(3):111128.
82. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol.* 2004;172(5):2731-2738.
83. Zschaler J, Schlorke D, Arnhold J. Differences in Innate Immune Response between Man and Mouse. *Crit Rev Immunol.* 2014;34(5):433-454.
84. Haley PJ. Species differences in the structure and function of the immune system. *Toxicology.* 2003;188(1):49-71.
85. Shay T, Jojic V, Zuk O, et al. Conservation and divergence in the transcriptional programs of the human and mouse immune systems. *Proc Natl Acad Sci U S A.* 2013;110(8):2946-2951.
86. Zöllner O, Lenter MC, Blanks JE, et al. L-Selectin from Human, but Not from Mouse Neutrophils Binds Directly to E-Selectin. *J Cell Biol.* 1997;136(3):707-716.
87. Otten MA, Rudolph E, Dechant M, et al. Immature neutrophils mediate tumor cell killing via IgA but not IgG Fc receptors. *J Immunol.* 2005;174(9):5472-5480.
88. Aleyd E, Heineke MH, van Egmond M. The era of the immunoglobulin A Fc receptor FcαRI; its function and potential as target in disease. *Immunol Rev.* 2015;268(1):123-138.
89. Tecchio C, Micheletti A, Cassatella MA. Neutrophil-derived cytokines: facts beyond expression. *Front Immunol.* 2014;5:508.
90. Tamassia N, Zimmermann M, Castellucci M, et al. Cutting Edge: An Inactive Chromatin Configuration at the IL-10 Locus in Human Neutrophils. *J Immunol.* 2013;190(5):1921-1925.
91. Bagaitkar J, Matute JD, Austin A, Arias AA, Dinauer MC. Activation of neutrophil respiratory burst by fungal particles requires phosphatidylinositol 3-phosphate binding to p40phox in humans but not in mice. *Blood.* 2012;120(16):3385-3387.
92. Condliffe AM, Davidson K, Anderson KE, et al. Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils. *Blood.* 2005;106(4):1432-1440.
93. Xue Q, Yan Y, Zhang R, Xiong H. Regulation of iNOS on Immune Cells and Its Role in Diseases. *Int J Mol Sci.* 2018;19(12):3805.
94. Eisenhauer PB, Lehrer RI. Mouse neutrophils lack defensins. *Infect Immun.* 1992;60(8):3446-3447.
95. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol.* 2003;3(9):710-720.
96. Hajjar E, Broemstrup T, Kantari C, Witko-Sarsat V, Reuter N. Structures of human proteinase 3 and neutrophil elastase – so similar yet so different. *FEBS J.* 2010;277(10):2238-2254.
97. Rausch PG, Moore TG. Granule Enzymes of Polymorphonuclear Neutrophils: A Phylogenetic Comparison. *Blood.* 1975;46(6):913-919.
98. Munder M, Mollinedo F, Calafat J, et al. Arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity. *Blood.* 2005;105(6):2549-2556.
99. Schreiber RD, Old LJ, Smyth MJ. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. *Science (1979).* 2011;331(6024):1565-1570.
100. Eruslanov EB, Singhal S, Albelda SM. Mouse versus Human Neutrophils in Cancer: A Major Knowledge Gap. *Trends Cancer.* 2017;3(2):149-160.
101. Sagiv JY, Michaeli J, Assi S, et al. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep.* 2015;10(4):562-573.

102. Mishalian I, Bayuh R, Levy L, Zolotarov L, Michaeli J, Fridlender ZG. Tumor-associated neutrophils (TAN) develop pro-tumorigenic properties during tumor progression. *Cancer Immunol Immunother*. 2013;62(11):1745-1756.
103. Fujiwara S. Humanized mice: A brief overview on their diverse applications in biomedical research. *J Cell Physiol*. 2018;233(4):2889-2901.
104. Martinov T, McKenna KM, Tan WH, et al. Building the Next Generation of Humanized Hemato-Lymphoid System Mice. *Front Immunol*. 2021;12:643852.
105. Lang J, Weiss N, Freed BM, Torres RM, Pelanda R. Generation of hematopoietic humanized mice in the newborn BALB/c-Rag2nullIl2rγnull mouse model: A multivariable optimization approach. *Clin Immunol*. 2011;140(1):102-116.
106. Ito M, Hiramatsu H, Kobayashi K, et al. NOD/SCID/γcnull mouse: an excellent recipient mouse model for engraftment of human cells. *Blood*. 2002;100(9):3175-3182.
107. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol*. 2007;7(2):118-130.
108. Manz MG. Human-hemato-lymphoid-system mice: opportunities and challenges. *Immunity*. 2007;26(5):537-541.
109. Rongvaux A, Willinger T, Martinek J, et al. Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol*. 2014;32(4):364-372.
110. Nicola NA, Begley CG, Metcalf D. Identification of the human analogue of a regulator that induces differentiation in murine leukaemic cells. *Nature*. 1985;314(6012):625-628.
111. Zsebo KM, Cohen AM, Murdock DC, et al. Recombinant Human Granulocyte Colony Stimulating Factor: Molecular and Biological Characterization. *Immunobiology*. 1986;172(3):175-184.
112. Guo Y, Luan L, Patil NK, Sherwood ER. Immunobiology of the IL-15/IL-15Rα complex as an antitumor and antiviral agent. *Cytokine Growth Factor Rev*. 2017;38:10-21.
113. Sippel TR, Radtke S, Olsen TM, Kiem HP, Rongvaux A. Human hematopoietic stem cell maintenance and myeloid cell development in next-generation humanized mouse models. *Blood Adv*. 2019;3(3):268-274.

CHAPTER 2

PLASTICITY IN PRO- AND ANTI-TUMOR ACTIVITY OF NEUTROPHILS: SHIFTING THE BALANCE

Charita Furumaya*, Paula Martinez-Sanz*, Panagiota Bouti, Taco W. Kuijpers, Hanke L. Matlung

* Authors contributed equally

Frontiers in Immunology 2020 Sep 2; 11:2100

ABSTRACT

Over the last decades, cancer immunotherapies such as checkpoint blockade and adoptive T cell transfer have been a game changer in many aspects and have improved the treatment for various malignancies considerably. Despite the clinical success of harnessing the adaptive immunity to combat the tumor, the benefits of immunotherapy are still limited to a subset of patients and cancer types. In recent years, neutrophils, the most abundant circulating leukocytes, have emerged as promising targets for anti-cancer therapies. Traditionally regarded as the first line of defense against infections, neutrophils are increasingly recognized as critical players during cancer progression. Evidence shows the functional plasticity of neutrophils in the tumor microenvironment, allowing neutrophils to exert either pro-tumor or anti-tumor effects. This review describes the tumor-promoting roles of neutrophils, focusing on their myeloid-derived suppressor cell activity, as well as their role in tumor elimination, exerted mainly via antibody-dependent cellular cytotoxicity. We will discuss potential approaches to therapeutically target neutrophils in cancer. These include strategies in humans to either silence the pro-tumor activity of neutrophils, or to activate or enhance their anti-tumor functions. Redirecting neutrophils seems a promising approach to harness innate immunity to improve treatment for cancer patients.

INTRODUCTION

The new immunotherapies aimed at targeting of immunosuppressive checkpoint receptors, using antibodies against the inhibitory programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) pathway or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) pathway, have vigorously changed the landscape of anti-cancer immunotherapy in the last decades[1]. This way of harnessing capacities of the immune system to combat cancer has recently delivered the first unprecedented clinical successes in the treatment of difficult-to-treat cancers, such as advanced stage metastatic melanoma[2] and non-small cell lung carcinoma[3], as well as more recently in other cancer types[4–6]. Despite the encouraging efficacy seen for some cancer patients, where durable responses are observed for several years, others either fail to respond to these therapies or acquire resistance over time[7]. Another recent and successful approach of cancer immunotherapy consists of the use of modified T cells with chimeric antigen receptors, the so-called CAR T cells. These have shown promising results for patients with advanced B-cell cancers in the early days[8] and more recently also in the case of other previously incurable malignancies, due to their specificity for a cell-surface antigen[9]. Yet, these novel therapies still raise certain general concerns to clinicians, as they have been associated with serious toxicities[10]. Although the current focus of cancer immunotherapies is on targeting adaptive immunity to fight the tumor, these therapies are limited to specific cancer types or need more efficient therapeutic control to reduce adverse effects. Besides, the cytotoxic anti-tumor capacities, as well as CAR T cell expansion, can easily be hampered by the interaction with other immune cells present in the tumor microenvironment (TME).

A different approach to immunotherapy in cancer focuses on the innate immune system. Cells belonging to the innate compartment are endowed with the capacity of reacting fast to invading pathogens that enter the body by recognizing sets of repeated patterns, as well as damage signals released from tissue injuries. In this way, innate immune cells have the outstanding talent of distinguishing self from non-self and respond to the latter in an appropriate manner. In addition, such cells are also able to distinguish self from "altered-self", and this is the case for cancer. Innate immune cells such as natural killer (NK) cells, dendritic cells (DC), neutrophils and macrophages are first-line effectors for the elimination of cancer cells before an adaptive immune response is mounted. In this way, NK cell-based therapies (*i.e.* used in the form of adoptive cell transfer or as CAR NK cells)[11], as well as DC-based therapies (*i.e.* used for vaccination against cancer)[12,13] are currently being exploited for the treatment of cancer. However, the clinical application and efficacy of these therapies can be hampered as these approaches are still in early stages of development. Alternatively, neutrophils or polymorphonuclear (PMN) leukocytes, as the most abundant cell type among the circulating white blood cells in humans, can also be considered as compelling cells to address therapeutically.

NEUTROPHILS: FROM INNATE EFFECTOR TO MYELOID SUPPRESSOR CELLS

Neutrophils are commonly short-lived cells with a half-life in the circulation of less than 24 h and one or more days in the tissues depending on the extravascular milieu. Thus these cells are renewed in the bone marrow at an estimated rate of 10^{11} cells per day. The percentage of neutrophils in blood in healthy adults ranges from 50% to 70%[14], although these numbers may differ under pathological conditions[15]. The mobilization of neutrophils from the bone marrow into the circulation is defined by the secretion of various stimuli from the site of injury or disease, to which they respond, resulting in variations in neutrophil numbers in blood. For example, granulocyte-colony stimulating factor (G-CSF), which is frequently produced by certain tumors or by cell types surrounding the tumor[16,17], is able to skew the neutrophil retention/release balance in the bone marrow and ultimately lead to an increased release of neutrophils into the circulation[18]. Moreover, chemokines, such as IL-8, can also be produced by tumor cells and attract myeloid cells, including neutrophils, to the TME, thereby affecting the number of neutrophils in the tumor[19]. To date, many studies have looked at the neutrophil-to-lymphocyte ratio (NLR) to determine the correlation between the number of circulating neutrophils and cancer prognosis. In all cases, a high NLR has been recognized as a bad prognostic marker for all cancer types and stages of cancer[20–22]. Moreover, the extent of intra-tumoral neutrophils also seems to have an unfavorable prognostic value in several cancer types[23–25]. On the other hand, low neutrophil counts in blood has been proven advantageous for survival[26]. Nonetheless, the option of persistent neutrophil depletion cannot be regarded in the context of cancer since prolonged neutropenia is considered a life-threatening condition due to the indispensable role of neutrophils in the protection of the host in the natural (mucosal) barriers against incoming microbial pathogens.

In fact, neutrophils in cancer may represent a heterogeneous population of cells, which can display different phenotypes and perform opposing functions. In mice, neutrophils found in tumor tissue have been referred to as tumor-associated neutrophils (TANs), which were classified as either anti-tumor neutrophils (N1) or tumor-promoting neutrophils (N2)[27]. In experimental models, several factors of the TME have been suggested to determine the polarization of the recruited neutrophils in the tumor towards one or the other phenotype *in vivo*. Supportive evidence of such neutrophil subsets in human cancer tissue is, however, lacking to date. Studies in cancer patients have reported that circulating neutrophils can be classified into different subpopulations according to their densities upon isolation and centrifugation. Normal or high density neutrophils (HDNs) are associated with anti-tumor activity, whereas neutrophils in the low-density fraction (LDNs) are believed to expand in malignancy and display myeloid-derived suppressor cell (MDSC) and other pro-tumor activity. Similar to TANs, HDNs are capable of switching to LDNs in response to

factors in the surrounding environment[28]. Unfortunately, the current understanding of neutrophil subpopulations is still limited and debated due to the lack of specific molecular markers, non-uniform study approaches and variable expertise. Nonetheless, it is clear that neutrophils are being increasingly recognized as important players in cancer, and that they can carry both pro- and anti-tumoral properties depending on cancer type, stage and location of the disease[29].

In the following chapters, we aim to provide a summary of the current knowledge and latest findings on pro- and anti-tumor activities of neutrophils by describing the main mechanisms of action and highlight some conceivable ways by which we could silence tumor-promoting activity or redirect the activity of pro-tumor neutrophils into cytotoxic effector cells to help combat cancer.

ROLE OF NEUTROPHILS IN TUMOR PROGRESSION

Promotion of proliferation, angiogenesis, invasion and metastasis by neutrophils

Neutrophils are capable of promoting tumor growth and progression, and their presence is often associated with poor clinical outcome[22]. Mechanisms by which neutrophils have been shown to mediate tumor progression include enhancing proliferation, angiogenesis, invasion, metastasis and immune suppression[30]. Neutrophils in the TME have the ability to directly induce the proliferation of cancer cells, for example via the serine protease neutrophil elastase (NE)[31,32]. NE also plays a role in the migration and invasion of cancer cells[33]. Other neutrophil granule components such as matrix metalloproteinase-9 (MMP-9) have been described to mediate angiogenesis and tumor cell invasion via degradation of the basement membrane[34]. Additionally, a large body of literature demonstrates a pro-metastatic function of neutrophils. In a mouse breast cancer model, neutrophil-derived factors were shown to drive cancer spread[35]. Furthermore, neutrophils have been suggested to promote cancer cell adherence, which was shown to be dependent on neutrophil Mac-1 (αMβ2 or CD11b/CD18), and thereby mediate metastasis in a murine model of liver metastasis[36]. Concordantly, human neutrophils were shown to induce tumor cell migration and to interact with melanoma cells via β2 integrin[37].

Also the involvement of neutrophil extracellular traps (NETs) in cancer cell migration and extravasation is being investigated. Upon activation, neutrophils form NETs composed of released chromatin and granular proteins which trap and kill microbes[38]. The neutrophil chemoattractant IL-8, which is produced in the TME, has been suggested to induce NET formation[39]. The presence of NETs in the TME of patients with metastatic disease has been demonstrated, and additional studies in murine models have further suggested their role in cancer progression[40]. NETs promoted cancer cell migration, invasion and angiogenesis

in vitro[41]. Multiple studies illustrated the trapping of circulating murine tumor cells in NETs, which facilitated their extravasation and metastasis[42–45].Increased levels of NETs were also observed in patients suffering from different types of locally infiltrating cancer[45,46], which was associated with adverse patient outcomes in colorectal cancer[47].

Immunosuppression by neutrophils

In mice, myeloid-derived suppressor cells (MDSCs) represent a heterogeneous group of pathologically activated immature myeloid cells with immunosuppressive properties[48]. MDSCs accumulate under inflammatory conditions, including experimental cancer, and are divided into two major subsets depending on their lineage, either granulocytic (PMN-MDSCs) or monocytic (M-MDSCs)[49]. The presence of PMN-MDSCs in patients has been shown to be associated with poor prognosis in different types of cancer[50–52]. In mice, PMN-MDSCs are characterized as $CD11b^+Ly6G^+$ cells, while in humans the surface marker definition is $CD11b^+CD15^+CD14^-CD33^+CD66b^+HLA\text{-}DR^-$[53]. However, based on these cell surface markers, PMN-MDSCs overlap with all circulating neutrophils, making an accurate discrimination between PMN-MDSCs and neutrophils impossible. Also other markers proposed to be more specific in identifying PMN-MDSCs, such as LOX-1 or CD10[54,55], have not been confirmed to discriminate circulating PMN-MDSCs in cancer patients[56].

While PMN-MDSCs were originally described as a subpopulation of immature myeloid cells capable of suppressing immune responses, mature neutrophils also have the ability to limit T cell activity and promote immune evasion[28,57], but only upon cellular activation[56,58]. Thus the functional similarities between PMN-MDSCs and neutrophils further complicate the differentiation between the two populations. Functional plasticity of neutrophils suggests that a shift in neutrophil phenotype occurs, depending on signals from the TME, which lead to the acquisition of immunosuppressive activity or other pro-tumorigenic functions. To avoid confusion, we will mostly refer to these cells as immunosuppressive neutrophils. Such mature neutrophils with a T cell suppressive phenotype have been identified in various human cancers and are also associated with accelerated tumor progression and worse clinical outcomes[49,58], illustrating their clinical relevance as potential targets to improve cancer immunotherapy.

Activation of neutrophil immunosuppressive activity

Tumor cells and other cell types in the TME produce a wide range of inflammatory mediators, many of which have been demonstrated to contribute to the generation and recruitment of neutrophils with pro-tumor activity. High levels of the colony stimulating factor G-CSF released by tumors corresponds with the expansion of immunosuppressive neutrophils in cancer patients[50]. Likewise, mature neutrophils of G-CSF-treated donors have been reported to display an activated immunosuppressive phenotype[55]. Other signals

implicated in the pathological activation of neutrophils include GM-CSF, TNFα, IL-1β, VEGF, IL-6, and IL-8[59]. However, our latest experiments in human neutrophils demonstrated that only fMLF, TLR ligands such as LPS, and TNFα act as activators of T cell suppressive activity in neutrophils[56,60]. The presence of soluble factors in ascites and malignant effusions from cancer patients was shown to induce a suppressive phenotype of neutrophils in the TME, which was dependent on complement factor C3[58].

Mechanisms of neutrophil immunosuppressive activity

In order to limit T cell mediated anti-tumor immune responses, suppressive neutrophils rely on several effector functions originally linked to their role as killers of invading pathogens. Degranulation refers to the process by which neutrophils release various factors stored in intracellular granules into phagosomes or the extracellular environment[61]. Immunosuppression by neutrophils has been linked to the metabolism of L-arginine, which is converted into L-ornithine by arginase-1, an enzyme present in gelatinase granules[62,63]. Elevated arginase-1 plasma levels were observed in cancer patients, and the modulation of T cell responses was shown to be dependent on arginase-1[50,64,65] via the depletion of L-arginine, an amino acid crucial for the expression of the T cell receptor ζ chain, which is in turn needed for T cell activation[66–68]. Additionally, L-arginine shortage prevents the successful formation of immunological synapses due to impaired dephosphorylation of cofilin, which is an important player in the modulation of the actin cytoskeleton and the formation of an immunological synapse[69,70]. The dependence on arginase-1 and its regulation of the T cell receptor ζ chain was demonstrated using PBMCs from healthy donors[71,72] and in cancer patients[64,73]. Accordingly, suppression of T cell mediated responses by activated human neutrophils was shown to depend on degranulation, which was elegantly confirmed using neutrophils from rare familial hemophagocytic lymphohistiocytosis (FHL)-5 patients, which show defective granule mobilization due to mutations in the *STXBP2* gene[56,74–76].

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex generates oxidative stress by the production of reactive oxygen species (ROS) upon cellular activation, a process which is upregulated in different types of cancer[77]. Hydrogen peroxide (H_2O_2) produced by the conversion of superoxide (O_2^-) suppresses T cell activation and proliferation via several mechanism[78,79]. H_2O_2 can induce T cell apoptosis, or inhibit T cell activation by blocking NF-kB activation or affecting the availability of the T cell receptor ζ chain, which is essential for T cell activation, as described above[80,81]. T cell activation is additionally impaired by the interference of ROS with the regulation of cofilin[82,83], as was also described as an effect of L-arginine deficiency. While L-arginine deficiency leads to impaired dephosphorylation of cofilin, ROS induce a conformational change in cofilin through its oxidation, both resulting in an inhibition of T cell response[69,82,83]. Furthermore, H_2O_2 could be involved in preventing the metabolic switch from mitochondrial respiration to

aerobic glycolysis that is required for T cell clonal expansion and cytokine production[84]. Our data also indicate that ROS production is required for the suppression of T cell activation by human neutrophils[56]. This was substantiated using neutrophils from patients with chronic granulomatous disease (CGD), a rare genetic defect in which neutrophils are incapable of ROS production due to a mutation in a subunit of the NADPH oxidase complex[85,86].

T cell function could additionally be impaired by suppressive neutrophils via the interaction between T cell PD-1 and PD-L1 on neutrophils, which is known to block T cell proliferation and cytokine production[87]. Crosstalk between PD-1 and PD-L1 plays an important role in T cell suppression in cancer[88]. Neutrophil expression of the immune checkpoint surface molecule PD-L1 can be induced by IFNγ and GM-CSF or hypoxic conditions[89–91]. Increased PD-L1 expression as well as suppression of T cell proliferation by neutrophils were demonstrated in patients with hepatocellular carcinoma[92]. Moreover, inhibition of T cell function by neutrophils was dependent on PD-1 expression by T cells and PD-L1 expression by neutrophils. PD-L1^{+} neutrophils suppress T cell function by interacting with PD-1 on T cells, thus limiting the anti-tumor activity of T cells. This immune tolerance via neutrophil PD-L1 appears to contribute to human gastric cancer growth and progression[93].

In order for neutrophils to suppress T cell responses, the formation of close cell-cell contact via the expression of integrin Mac-1 on neutrophils is required[94]. Singel *et al.* recently demonstrated the requirement of cell-cell contact for T cell suppression by human neutrophils activated by ovarian cancer ascites[58]. This is in line with our findings, which demonstrate that suppression of T cells by neutrophils is dependent on CD11b-mediated interactions[56]. Consistently, neutrophils from a patient with leukocyte adhesion deficiency type 1 (LAD-1), which lack the expression of β2 integrins, including Mac-1, were not capable of suppressing T cell proliferation[56]. Blocking ICAM-1 on T cells decreased, but did not fully abrogate T cell suppression by activated neutrophils, indicating that additional ligands of Mac-1 on T cells are important for the interaction with neutrophils[56]. In tumor-bearing mice, neutrophils were shown to induce T cell apoptosis in a contact-dependent manner[95]. Similarly, an immunosuppressive myeloid cell population induced apoptosis of activated T cells in breast cancer patients[96]. In our studies, live-cell imaging revealed intercellular contacts between neutrophils and T cells resulting in neutrophils containing pieces of T cell membrane[56], a process known as trogocytosis[97]. During co-culture of activated neutrophils and T cells, a population of smaller T cells appeared, which could no longer be activated to proliferate. In contrast to earlier findings with respect to apoptosis induction, these small T cells displayed neither of the two common cell death mechanisms, *i.e.* apoptosis or necroptosis. Thus, the exact steps leading to T cell death upon suppression by neutrophils remain to be further investigated.

In addition to their own direct and suppressive effect on T cells, neutrophils can also inhibit NK cell activation in tumor-bearing mice[98,99]. Neutrophils suppressed NK cell cytotoxicity, which resulted in defective antitumor responses and promoted metastasis in mice[100,101]. Finally, further contribution of neutrophils to the immunosuppressive microenvironment within tumor tissue is made via the development and induction of regulatory T cells (Tregs)[102]. Treg enrichment occurs due to reduced sensitivity of Tregs to oxidative stress in the TME[103,104], which ultimately accounts for their preferential outgrowth and selection. Neutrophils also recruit Tregs through the secretion of CCL17, as was shown in murine tumors[105]. Additionally, a positive feedback loop between neutrophils and Tregs, mediated by the anti-inflammatory cytokine IL-10, could support the immune suppression[106,107]. Induction of Tregs by suppressive neutrophils was demonstrated in patients with bladder cancer[108].

In summary, neutrophils can directly and indirectly mediate tumor progression via the promotion of proliferation, angiogenesis, invasion, and metastasis, in which mechanisms such as the release of granule components and NET formation are important. Furthermore, neutrophils inhibit anti-tumor responses by limiting T cell activation, which is dependent on degranulation, ROS production and cell-cell contact. Alternatively, neutrophils contribute to an immunosuppressive TME by affecting other immune cells, such as NK cells and Tregs. Apart from the pro-tumorigenic mechanisms by which neutrophils are activated to 'adopt' their tumor-enhancing, immunosuppressive role, neutrophils can also exert strong anti-tumor activity.

ROLE OF NEUTROPHILS IN TUMOR ELIMINATION

The vast majority of studies support the positive correlation of neutrophils with cancer progression by the mechanisms described above. Nevertheless, recent data also point into the opposite direction in which neutrophils can act as effector cells and combat cancer leading to the eradication of tumor cells[30,109,110]. In fact, there are sufficient reasons to think of neutrophils as promising effector cells against cancer. One of the main advantages of this cell type is that they are found in high levels in blood under normal conditions, and in addition, their number can be incremented further upon *in vivo* treatment with G-CSF and GM-CSF cytokines[111,112]. Another feature that makes them unique is that neutrophils do not need *ex vivo* culturing and expansion for a later infusion into the patient, compared to most other immune cells used therapeutically, such as T cells, NK cells or DCs[113]. Siders *et al.* showed that increasing the number of circulating neutrophils by a mere injection of G-CSF turned them into excellent cytotoxic cells that were able to kill alemtuzumab-opsonized cells in a xenograft model of a $CD52^+$ tumor[114]. This, however, must be regarded with caution as such effect may only be valid in the context of antibody therapy, as was

also shown in other studies[115,116]. In other circumstances, the therapeutic effect of G-CSF treatment may mainly mobilize neutrophils of an immunosuppressive phenotype which are beneficial to the tumor, as discussed earlier in this review[50,55].

Direct killing properties of neutrophils against cancer

The mechanisms by which anti-tumor neutrophils induce cytotoxicity of the tumor cells are, however, not yet clear. Few studies demonstrated that the secretion of ROS through the respiratory burst comes into play once neutrophils are in direct contact with the tumor cells[117–119]. Specifically, neutrophil secretion of H_2O_2 induced a lethal influx of Ca^{2+}, mediated by the TRMP2 channel which ultimately killed the tumor cell. Furthermore, a mechanism was identified by which neutrophils isolated from healthy donors induced apoptotic cell death of tumor cells upon physical contact mediated through Fas ligand/Fas interaction[120]. Although these contact-dependent processes can, under specific circumstances, induce tumor cell killing, the best well-established anti-tumor mechanism by which neutrophils mediate tumor cell death is through Fc receptor-dependent cytotoxicity against antibody-opsonized cells.

Antibody-mediated killing properties of neutrophils against cancer

Therapeutic monoclonal antibodies (mAbs) have become available for the treatment of cancer in the last decades and have provided significant improvement in the treatment outcome for a number of cancer subtypes. These mAbs can initiate direct tumor cell killing, through the $F(ab')_2$ domains of the immunoglobulin (Ig) interfering with the intrinsic function of the target cell[121,122], or upon binding of the Fc part to the C1q component of the complement system inducing complement-dependent cytotoxicity (CDC)[123]. Besides the direct mechanisms, the Fc region of the mAb can also bind to activating Fc receptors on innate immune cells which will in turn elicit indirect-mediated killing via antibody-dependent cellular cytotoxicity/phagocytosis (ADCC/P) of the opsonized cells[124–128].

The contribution of neutrophils in ADCC to eradicate antibody-opsonized tumor cells *in vitro* was already demonstrated some decades ago by Gale and Zighelboim[129]. A few years later, Barker and colleagues showed a prominent role of neutrophils isolated from neuroblastoma patients in mediating the *ex vivo* lysis of neuroblastoma cells opsonized with anti-GD2 antibody. Interestingly, the cytotoxicity induced by neutrophils was in all cases higher than was seen for NK cells from the same donor[130]. More recently, a number of studies have demonstrated the cytotoxic capacity of neutrophils in the context of antibody therapy in an *in vivo* setting of tumor-bearing mice which showed once again the indispensable role of neutrophils in achieving efficient antibody therapy responses[114,131,132]. Most importantly, a considerable amount of literature has suggested the relevance of the anti-tumor capacities of neutrophils in a clinical setting. More specifically, different

polymorphic variants found on the IgG Fc receptor IIa (FcγRIIa) explained the variability seen in the clinical outcome of breast cancer[133,134], colorectal cancer[135], and neuroblastoma patients[136] in response to antibody treatment.

IgG isotypes and Fcγ receptor involvement

Despite the fact that several Ig isotypes exist, as of yet, all approved therapeutic antibodies for cancer treatment in the clinic are IgG-based, particularly IgG1, IgG2 and IgG4 isotypes. The relevance of the IgG backbone used for such antibodies has to do with the different binding affinities to the several Fcγ receptors that are in turn differentially expressed on a number of immune cells[137,138]. This results in the induction of extremely diverse and highly regulated antibody responses, as the distinct affinities can also convey stronger or weaker effector functions by the different Fcγ receptor-expressing cells[139].

Six classical Fcγ receptors can be expressed by the human innate immune cells: FcγRI (CD64), FcγRIIa (CD32a), FcγRIIb (CD32b), FcγRIIc (CD32c), FcγRIIIa (CD16a) and FcγRIIIb (CD16b). From these, neutrophils constitutively express FcγRIIa and FcγRIIIb, and the FcγRI only upon activation. Depending on ethnic background, 15% of the population also expresses low levels of neutrophil FcγRIIc[140]. All these Fcγ receptors on neutrophils can bind to the IgG opsonizing cancer cells[138,141] and have their own capacity to contribute to ADCC activity. FcγRI and FcγRIIa/c are activating receptors and their signals are transduced by an immunoreceptor tyrosine-based activation motif (ITAM) that is either associated with the common FcRγ chain in the case of FcγRI, or present in the cytoplasmic tail of the Fcγ receptor itself for FcγRIIa/c. Subsequently, Syk tyrosine kinases can bind to these activating motifs and activate downstream signaling pathways, initiating ADCC, phagocytosis, cell migration, and degranulation processes[141,142]. FcγRIIIb, instead, is anchored to the cell membrane through a glycosyl phosphatidylinositol (GPI) molecule and lacks both transmembrane and cytoplasmic domains, restricting it from having signaling capacities[143]. As mentioned above, resting neutrophils constitutively express both low- and intermediate-affinity FcγRIIa and FcγRIIIb in high levels[141]. However, the high-affinity activating FcγRI is not detectable on resting neutrophils but can be upregulated upon activation of cells with G-CSF and IFNγ as demonstrated by us[30,144] and others[145,146]. In addition, FcγRIIIb is actively shed from the surface of activated neutrophils when stimulated by various stimuli, including G-CSF and IFNγ[144,147].

The specific contribution of each Fcγ receptor of neutrophils to mediate ADCC, however, has been found to differ per cancer type. With the use of blocking antibodies for the different Fcγ receptors, several studies have reported FcγRIIa to be the dominant receptor triggering neutrophil ADCC in solid tumors. This was seen for $EGFR^+$ cancer cells opsonized with cetuximab[148], as well as for trastuzumab-coated $HER2/neu^+$ human breast

cancer SKBR3 cells[144,149]. Of interest, although FcγRI is the high-affinity receptor for IgG1 antibodies, no effect was found when monovalent Fc fragments were used for blockade. This might be explained by the incapacity to fully block the receptor by use of these monovalent Fc fragments or due to the relatively low expression levels on the surface of neutrophils[150]. Nevertheless, contradicting results were described regarding the potential of FcγRI on neutrophils in mediating ADCC. It was shown that such receptor can very well contribute to tumor killing in a number of tumor types[151–154]. With regard to the involvement of FcγRIIIb in the killing of tumor cells in the context of antibody therapy, we and others have shown this receptor to be a negative regulator of neutrophil ADCC as it scavenges available therapeutic antibody due to its high expression on the neutrophil surface[144,148]. For hematological tumors the evidence is slightly different. An *in vivo* study testing the efficacy of a FcγRI bispecific antibody in a mouse model of a rituximab-treated B-cell lymphoma reported the clearance of the tumor cells by G-CSF stimulated neutrophils[155], indicating that FcγRI may be the main receptor mediating neutrophil ADCC in such context. Another level of complexity of this receptor family comes from the several Fcγ receptor polymorphisms described in humans that could affect the degree of ADCC responses (extensively reviewed by Bruhns and Jonsson[138]), which ultimately can influence clinical responses to antibody therapy. It is however relevant to acknowledge that Fcγ receptors are not the only feature that makes neutrophils capable of killing cancer cells. Although falling outside the scope of this review, integrins, Mac-1 in particular, have also been shown to be indispensable in mediating ADCC processes[149].

Neutrophil-mediated antibody-dependent killing mechanisms

Until recently, the mechanism by which ADCC leads to cell death remained largely unclear. A large body of evidence has reported that neutrophils have the ability to trogocytose, mainly in the context of antibody therapy[156]. In 2002, trogocytosis was identified as an active mechanism that involves the transfer of plasma membrane and their associated molecules from a donor cell to an acceptor cell during intercellular contact[157]. Although the purpose of the process has remained unclear[97], more recently, new evidence has accumulated to determine the importance of trogocytosis. In the context of infection, neutrophils were able to kill serum-opsonized *Trichomonas vaginalis* parasite using trogocytosis[158]. Additionally, the tumoricidal effect of trogocytosis was shown in the study of Velmurugan *et al*, where they demonstrated that macrophage-trogocytosis led to efficient tumor cell death of trastuzumab-opsonized breast cancer cells[159]. Most recently, we reported a direct association between neutrophil-mediated trogocytosis and tumor cell killing in antibody-opsonized solid cancer cells[149], by which the neutrophil takes 'bites' from the plasma membrane of the cancer cells. Hereby, the neutrophils cause membrane damage eventually leading to a necrotic type of cancer cell death. Furthermore, neutrophil-mediated antibody-dependent destruction of cancer cells was found not to depend on

their classic antimicrobial effector mechanisms, such as granule exocytosis (degranulation) and NADPH oxidase activity. In particular, neutrophils from FHL-5 patients, as well as neutrophils from patients with CGD, showed unexpectedly intact killing of HER2/neu$^+$ breast cancer cells in the presence of the therapeutic antibody trastuzumab[149]. Together, these findings support the idea of trogocytosis as a most relevant process involved in tumor killing in the context of antibody therapy.

Conversely, trogocytosis has also been described as a mechanism to escape ADCC, ADCP or CDC, mainly in hematological cancers, as it involves the shaving of the target antigen from tumor cells by the effector cells. This was shown not only for chronic lymphocytic leukemia (CLL) upon rituximab treatment, where a partial loss of CD20 from CLL B cells accompanied trogocytosis events by neutrophils[160], but also anti-CD38 therapy with daratumumab directed against multiple myeloma (MM) which led to monocyte- and neutrophil-mediated shaving of CD38 from MM cells[161]. Moreover, neutrophil-mediated trogocytosis might be the reason behind the significant reduction in HER2/neu expression, seen in a cohort of breast cancer in women treated with trastuzumab[162,163]. Therefore, depending on the circumstances, or perhaps tumor type, trogocytosis can be regarded either as a process initiating tumor killing or as a way for the tumor cells to evade immune activity.

Role of neutrophils regulating adaptive immune response

For cancer therapy, it has now become clear that initiating potent adaptive immune responses is fundamental to establish long-term anti-tumor immunity. Although neutrophils have historically been regarded as strict innate cells with end-stage effector functions, new evidence has emerged manifesting their involvement in modulating the adaptive immune compartment[164,165]. At sites of infection, neutrophils were found to act as danger sensors by communicating the presence of inflammation or damage to DCs, which induces DC maturation, triggering in turn strong proliferation and T_h1 polarization of naive CD4$^+$ T cells[166,167]. In addition, neutrophils can also act as APCs themselves. *In vitro*, activated neutrophils stimulated with GM-CSF and IFNγ were able to present antigens to memory CD4$^+$ T cells due to the acquisition of MHC-II molecule expression, as well as costimulatory molecules such as CD86, OX40L and 4-1BBL at early stages of tumorigenesis[168–170]. Moreover, both human and mouse neutrophils were found to cross-present exogenous antigens to naive CD8$^+$ T cells thereby turning them into cytotoxic T cells[171]. Lastly, recent evidence suggests that upon antigen capture at the periphery, neutrophils can migrate to the lymph nodes in a CC-chemokine receptor 7 (CCR7)-dependent manner, under certain circumstances, as their presence has been found in lymphoid organs *in vivo* [165,172,173].

In the context of cancer, however, neutrophils can mediate opposite adaptive immune responses depending on the TME or stage of the tumor *in vivo* For some cancer mouse models, neutrophil depletion led to a decrease in CD4 and CD8 T cell activation, thereby enhancing tumor growth[174], while in others their presence was able to suppress CD8 T cells and promote metastasis[175]. The evidence for the role of neutrophils in inducing adaptive immune responses after antibody therapy of cancer is, unfortunately, more scarce. Yet, neutrophils were able to boost T-cell activation when combining a Fc/IL-2+TA99 antibody with adoptive T-cell transfer in a B16F10 melanoma model achieving significant tumor control[176]. In general, neutrophils can not only play a role in innate immunity but also guide and support adaptive immune responses through cellular crosstalk.

TARGETING NEUTROPHIL ACTIVITY IN CANCER THERAPY

The different functions that neutrophils acquire in the context of cancer highlight their plasticity and ability to respond towards various targets within and outside of the TME. In this review, we have focused on the role of neutrophils in tumor progression or tumor elimination and we have described the major mechanisms that neutrophils utilize to achieve an efficient response. Although mechanistically not yet completely understood, some reports[28,177] suggest TANs in several mouse models of cancer to display a gradual change during tumor progression, shifting from anti-tumor properties at the early stages towards pro-tumorigenic properties during the course of the disease.

As reviewed above, both immunosuppressive as well as anti-tumor neutrophils share some common characteristics, *i.e.* their need for activation through specific stimuli to be able to optimally exert their function[27,50,59,178]. Another necessity for neutrophils to perform their MDSC and anti-tumor function is Mac-1-mediated close contact, either between neutrophil and T cell in case of MDSC activity, or between neutrophil and antibody-opsonized tumor cell in case of ADCC[56,58,94,149]. At the same time, the fact that classical anti-microbial killing mechanisms, *i.e.* degranulation and ROS production, are essential for MDSC function of neutrophils, yet dispensable for neutrophil ADCC, shows that at least some of the required cellular mechanisms for MDSC and ADCC neutrophil activity are directly opposing[56,149].

Together, in the following part of this review we will elaborate on ways to modulate pro-tumorigenic function, further enhance the anti-tumor response, or even redirect tumor-promoting neutrophils towards anti-tumor neutrophils (**Figure 1**). Interestingly, the first *in vitro* study showing a way to polarize human neutrophils towards the distinct N1 or N2 phenotype has recently been published[178].

Limiting pro-tumorigenic capacity of neutrophils

Reducing neutrophil numbers

The first possibility to interfere with the tumor-promoting role of neutrophils in cancer is the neutralization of these cells. Antibody-mediated depletion of neutrophils resulted in decreased metastasis in an intrasplenic model of liver metastasis and in a metastatic breast cancer mouse model[36,174]. Similarly, targeting of $Ly6G^+$ cells in a murine model of pancreatic ductal adenocarcinoma increased intra-tumoral T cell accumulation and inhibited cancer progression[179].

The described promising results of antibody targeting in mice called for a similar approach to be undertaken in humans, in order to promote anti-cancer T cell responses. Based on immunophenotyping and functional assays to detect T cell suppression, the presence of suppressive neutrophils was determined in blood obtained from patients with different types of cancer, including prostate, lung, head and neck, and breast. The immunotoxin Gemtuzumab ozogamicin was used to deplete cells expressing CD33, identified as a surface marker on suppressive cells across cancer types. This depletion restored T cell proliferation, enhanced CAR T cell responses and tumor cell death[180].

Furthermore, a small-molecule receptor tyrosine kinase inhibitor has proven to successfully modulate the immunosuppressive TME. Sunitinib inhibits signaling through multiple receptor tyrosine kinases, including vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRα and PDGFRβ), stem cell factor receptor (c-Kit) and colony-stimulating factor-1 receptor (CSF-1R)[181]. Treatment with sunitinib decreased the number of suppressive cells, enhanced CD8 and CD4 cell tumor infiltration and improved survival in tumor-bearing mice[182]. In renal cell carcinoma patients, sunitinib also reversed T cell suppression and reduced Tregs at the tumor site[183]. Moreover, treatment with sunitinib in human renal cell carcinoma improved the expansion of tumor-infiltrating lymphocytes[184].

Since neutrophils were shown to upregulate PD-L1, neutrophils may also be affected by therapies targeting immune checkpoints. For example ipilimumab treatment led to reduced PMN-MDSC numbers and less immunosuppressive activity in melanoma patients, which correlated with improved clinical outcome[185–187]. Thus, increasing clinical evidence supports the notion that reducing neutrophil numbers in cancer could be beneficial to patients.

Targeting neutrophil recruitment and activation of MDSC activity

An alternative approach to targeting the pro-tumor activity of neutrophils is to inhibit their recruitment or activation. For instance, IL-8 secreted by tumor cells is responsible for the chemotactic recruitment of neutrophils to the TME via the receptors CXCR1

and CXCR2[39]. Already applied in patients suffering from other inflammatory diseases, inhibition of CXCR2 prevents the recruitment of immunosuppressive neutrophils[188,189]. Recent studies in mouse models of cancer have also shown promising effects of CXCR2 inhibition, which increased effector T cell accumulation in tumors and enhanced responses to immunotherapy, slowing tumorigenesis or preventing metastasis[190–192]. The effect of the CXCR1 and CXCR2 inhibitors reparixin and SX-682 are currently being tested in clinical trials in metastatic breast cancer patients (NCT02370238, NCT03161431)[193].

Another example of the stimulation of anti-cancer responses achieved by limiting neutrophil recruitment is the inhibition of the receptor tyrosine kinase cMET[194]. cMET is a receptor for hepatocyte growth factor (HGF), which shows increased levels and association with poor clinical outcome in human cancer[195]. Limiting neutrophil recruitment by blocking cMET promotes the efficacy of adoptive T cell transfer and checkpoint therapy in murine melanoma[196]. Antagonists of the HGF/cMET pathway have been developed and are being tested in multiple types of human cancer[197].

In addition, targeting pathways leading to the pathological activation of neutrophils could represent a strategy to limit their tumor-promoting effects. IL-17 production by γδ T cells was shown to induce G-CSF release in mice, resulting in the accumulation of neutrophils with a T cell-suppressive phenotype[174,198]. In human breast cancer, IL-17 and γδ T cells have also been described as poor prognostic factors[199,200]. Depletion of IL-17, G-CSF or γδ T cells resulted in decreased T cell suppression, and the absence of γδ T cells or neutrophils reduced metastases in a murine breast cancer model[174]. Currently, IL-17 specific antibodies are being tested in psoriasis patients, but more preclinical studies are needed before this could also be applied in cancer patients[201].

In humans, G-CSF-mobilized neutrophils display immunosuppressive activity[202]. Interestingly, we reported that neutrophil mobilization for granulocyte transfusion purposes silenced the ability of neutrophils to suppress T cell responses[202].The lack of MDSC activity was identified while our findings demonstrated unaltered anti-microbial effector activities, including motility, ROS formation, degranulation and killing of bacteria and fungi[202,203]. This important finding suggests that MDSC activity comprises common effector functions combined with a distinct and unique activity that is (only) involved upon MDSC induction. This finding raises the possibility of using a selective approach in patients to silence immunosuppressive neutrophils and to thereby enhance T cell activity in tumors. Alternatively, proteins of the complement system represent potential targets to block the immunomodulatory and pro-tumor activity of neutrophils, since the importance of C3 activation in T cell suppression by neutrophils from cancer patients was nicely illustrated[58].

Targeting additional pro-tumor function of neutrophils

As described in previous sections, neutrophils rely on several mechanisms to exert their pro-tumorigenic function. Interfering with these downstream tumor-enhancing effects could provide therapeutic benefit. For instance, increased uptake of fatty acids by murine PMN-MDSCs was demonstrated to support their immunosuppressive activity[204]. Accordingly, PMN-MDSCs of patients with head and neck, lung, or breast cancer displayed lipid accumulation, along with increased expression of FATP2, a fatty acid transporting protein[205]. FATP2 deletion in mice resulted in the loss of the ability to suppress T cell responses leading to a delay in tumor progression. However, most findings from these experimental studies were obtained in mouse models, which by definition cannot be easily extrapolated to human cancer[206].

Other studies have focused on targeting NE, which contributes to cancer progression through enhanced proliferation, invasion and metastasis[31–33,35–37,207,208]. Suppression of NE activity by the small molecule sivelestat resulted in reduced tumor growth in murine models of colorectal and prostate cancer[207,208]. The NE inhibitor sivelestat sodium hydrate has been successfully applied in esophageal cancer patients, although this study did not focus on tumor progression[209]. Furthermore, NETs may represent a target for reducing the pro-tumor effects of neutrophils since their elimination by the DNA degrading enzyme DNase I is a method that has been established and is tested in clinical trials (https://clinicaltrials.gov/), though not yet in cancer[210]. More clinical studies are necessary to investigate the potential of targeting NE or NETs in human cancer patients. The potential ways to limit pro-tumorigenic activity of neutrophils are summarized in **Figure 1; 1-4**.

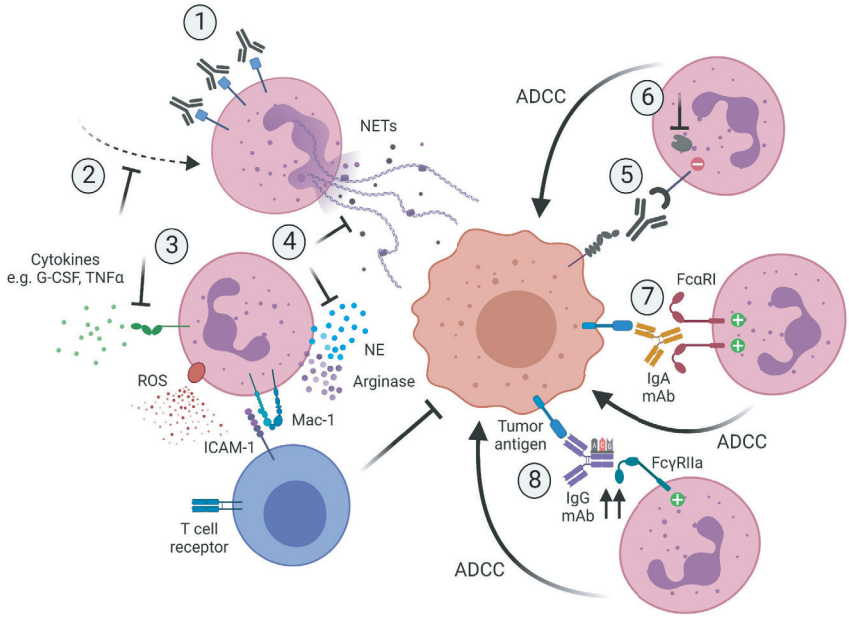


Figure 1. Potential ways to therapeutically target neutrophils in cancer by blocking their pro-tumor activity (1-4) or promoting their anti-tumor capacities (5-8). *1) Reduction of neutrophil numbers in the TME, for example by using antibodies targeting CD33 present on immunosuppressive neutrophils. 2) Blocking the recruitment of pro-tumor neutrophils to the TME, for example by inhibiting the chemokine receptor CXCR2. 3) Blocking of activation signals such as G-CSF or TNFα necessary for neutrophils to acquire a pro-tumor or immunosuppressive phenotype. 4) Neutrophils require ROS production and degranulation to exert their immunosuppressive role, which is dependent on close contact with T cells via Mac-1. Also, neutrophils rely on mechanisms such as NET formation and the release of granule components such as NE to exert their pro-tumor activity. Targeting these downstream mechanisms would limit the pro-tumorigenic activity of neutrophils. 5) Interfering with innate immune inhibitory checkpoints would restore antibody-mediated anti-tumor capabilities of neutrophils. 6) Targeting the recruitment and function of downstream regulators of the inhibitory receptor would further enhance antibody-mediated anti-tumorigenic capacities of neutrophils towards tumor cells. 7) The use of IgA-based therapeutic mAbs that can bivalently bind FcαRI on the neutrophil would induce stronger anti-tumor cytotoxic responses. 8) The use of protein engineering techniques to modify the Fc region of IgG therapeutic antibodies would increase the affinity to the activating FcγRIIa, resulting in more potent ADCC responses towards the opsonized tumor cells. Created with BioRender.com.*

Promoting anti-tumor capacity of neutrophils

Releasing the off-switch

Although the above-mentioned approaches to modulate MDSC activity or other pro-tumorigenic effector functions of neutrophils are very promising, redirecting the toxic MDSC activity against T cells towards an anti-tumorigenic role might contribute and steer the effector functions away from neutrophils as suppressor cells to effective tumor-killing in the TME. We have already described the efficacy of neutrophils attacking antibody-opsonized tumor cells, and thereby we could speculate that the presence of a mAb could already shift MDSC activity to a certain extent by binding to the Fcγ receptor on their surface and subsequently inducing a cytotoxic response.

Recent research has been focusing on further augmenting the anti-tumor responses of neutrophils, *i.e.* by trying to switch off the brakes. Neutrophils express a variety of inhibitory receptors on their cell surface[211] providing potential therapeutic targets for checkpoint-blockade therapy. One well-established example of successful checkpoint-blockade on neutrophils is CD47-signal regulatory protein alpha (SIRPα) disruption[212]. We and others have already shown the potency of blocking the interaction between CD47 and SIRPα on neutrophils, thereby enhancing the neutrophil's ADCC capacity against both solid and hematological tumors *in vitro* and *in vivo*[213–215]. Antibodies targeting either SIRPα or CD47 have been recently described, showing high efficacy and minimal to moderate toxicity effects[216–218] and a number of clinical trials with these CD47-SIRPα-interfering agents are ongoing (https://clinicaltrials.gov/). In particular, Hu5F9-G4 against CD47 was tested in combination with rituximab for the treatment of patients with Non-Hodgkin's lymphoma in a phase 1b clinical trial[219] inducing durable complete responses while no clinically significant toxicity events were observed. At the moment, Hu5F9-G4 is tested in combination with cetuximab for the treatment of colorectal cancers in a phase 1b/2 clinical trial (NCT02953782). Pre-clinical data further showed that blockade of the CD47-SIRPα checkpoint on innate cells eventually activates an anti-tumor response in T cells, bridging the innate with the adaptive immunity[220]. Indeed, macrophages were shown to function as APCs and thereby activate the $CD8^+$ T cell population while decreasing priming of $CD4^+$ T cells after anti-CD47-induced phagocytosis of tumor cells[221]. Also DCs are able to contribute to the therapeutic effect of anti-CD47 treatment through cross-priming of $CD8^+$ T cells[222]. Results from additional future clinical studies will demonstrate whether targeting the CD47-SIRPα axis in a clinical setting indeed will activate both innate as well as adaptive anti-tumor immunity.

Overall, most current studies considered macrophages or NK cells to be the major effector cells in innate checkpoint-blockade antibody therapy[223–225]. Given that neutrophils are endowed with similar inhibitory receptors, they can potentially acquire a supporting anti-

tumor response. Recent studies have elucidated that Sialic acid-binding immunoglobulin-like lectins or Siglec-blockade can augment the anti-tumor response both by reinvigorating the innate cells and by depleting MDSCs from the TME, thereby unleashing the T cells of the adaptive immune system[180,226–228]. In another perspective, targeting hypersialylation on tumor cells, a trait related to tumor progression and therapy resistance, could also inhibit the Siglec-sialoglycan axis and re-inforce the anti-tumor response[229]. The role of Siglec checkpoint-blockade has been highlighted in neutrophils, where research on Siglec-9 or its murine equivalent Siglec-E showed upregulation of the protein receptor in the cytotoxic synapse formation between neutrophils and carcinoma cells, while incubation of neutrophils with anti-Siglec-9 mAbs resulted in significantly increased tumor cell killing by neutrophils[230–233].

Enhancement of the anti-tumor response of neutrophils can also be achieved by targeting signaling partners downstream of the inhibitory receptors. Even better, combination of ligand-receptor interaction disruption and simultaneous blockade of a protein functioning downstream, could have a substantial impact[234,235]. Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) are two important regulators of immune cell responses. As they bind directly to the ITIM motif of the cytoplasmic tails of inhibitory receptors, drugs targeting their function could potentially amplify the neutrophil effector function against tumor cells[236]. Despite the above-mentioned promising data on neutrophil effector function, evidence from *in vivo* experiments will shed more light on the complexity of neutrophil anti-tumor response.

Augmenting the on-switch

A different perspective that could redirect pro-tumor and immunosuppressive activity of neutrophils to improve their cytotoxic responses towards cancer in the context of antibody therapy, could be the use of alternative antibody isotypes compared to the classical IgG1. Particularly, IgA antibodies, which specifically bind the IgA Fcα receptor I (FcαRI or CD89) present on cells of the myeloid lineage, including neutrophils[237], are currently considered as a promising approach in immunotherapy against cancer because of their superior ability to induce neutrophil-mediated ADCC. This was reported for a number of tumor-associated antigens such as Ep-CAM for colon carcinoma, HER2/neu for breast carcinoma, EGFR for epithelial, colorectal and renal cell carcinoma, HLA class II, CD20 and CD30 for B-cell lymphoma, and carcinoembryonic antigen as shown in *in vitro* studies[127,128,238–242]. The induction of such stronger cytotoxic responses upon an IgA engagement of human neutrophils could be explained by the higher avidity of FcαRI which binds bivalently to IgA, and hence recruit more ITAMs to initiate a more robust signaling to activate effector functions[243]. Noteworthy, immature neutrophils that were mobilized from the bone marrow after G-CSF treatment triggered a more efficient *ex vivo* tumor cell lysis in

the presence of an IgA antibody compared to IgG[244,245]. Based on this we speculate that the use of IgA antibodies could unleash the cytotoxic potential of G-CSF mobilized immature neutrophils in antibody therapy of cancer that otherwise would be immunosuppressive, or even trigger re-polarization of PMN-MDSCs, due to FcαRI constitutive expression[128]. In an *in vivo* setting, however, the use of IgA tumor-targeting antibodies is restricted due to the lack of an FcαRI homologue in mice. Nevertheless, the existing genetic engineering techniques have allowed the creation of FcαRI transgenic mouse models, which have been used to confirm the powerful capacity of IgA-mediated tumor killing by myeloid cells in a few studies for EGFR$^+$ tumors[246,247]. An important limitation of IgA antibodies *in vivo* as well as in humans is the short half-life compared to that of IgG isotypes (15 hours versus 4 days, respectively in mice, and 5-6 days versus 21 days, respectively in humans)[237,248]. Attempts to extend the half-life of such promising antibodies or combine them with immune checkpoint blockade therapy are currently being studied[249,250] and will bring new insights for human application in the near future.

Alternatively, several other approaches to increase ADCC activity of therapeutic antibodies are currently being studied. The majority of these approaches involve glyco- and protein-engineering of IgG1-Fc portions to improve the binding affinities to the activating Fcγ receptors on immune effector cells. On the one hand, it is now firmly established that the glycosylation patterns of the IgG-Fc region are essential for the activation of downstream biological mechanisms of the molecule. Consequently, interfering with such post-translational modifications can drastically influence the effector functions of the immune cells binding to it[137,251]. Specifically, core-fucosylation modifications of the IgG-Fc part are the ones showing a more significant effect[252], although Fc galactosylation and sialylation can have an influence as well[253,254]. In particular, the removal of the core fucose from Fc glycans of IgG1 was shown to increase the binding affinity to FcγRIIIa on NK cells, which resulted in a significant enhancement of ADCC activity for this particular effector cell type[255–257]. In the case of neutrophils as effector cells, a reduction of the fucose content of the mAb actually abolished anti-tumor activity instead, indicating that antibody fucosylation differentially impacts cytotoxicity mediated by human NK cells and neutrophils[148]. A similar finding was described upon deglycosylation of alemtuzumab[114]. These observations may be explained by the fact that human neutrophils only express the decoy receptor FcγRIIIb, which was found to bind with high affinity to low-fucose antibodies, thereby impeding antibody efficacy. A better approach to specifically enhance neutrophil-mediated ADCC responses could be achieved by interfering with the amino acid sequences of the Fc region of the targeting antibody. Specific mutations in this region lead to a higher affinity of anti-EGFR mAbs to the activating FcγRIIa on neutrophils rather than to the decoy FcγRIIIb, which resulted in a restored ADCC activity by purified human neutrophils[257]. This approach should be considered to enable the successful development of “next-generation”

antibodies when targeting neutrophils as promising effector cells. The most encouraging ways to enhance the anti-tumorigenic activity of neutrophils are depicted in **Figure 1; 5-8**.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Over time, neutrophils have been identified as important players in cancer, having the unique capacity to either promote or suppress tumor progression. In the present review, we have first provided an overview of such opposing functions of neutrophils that neutrophils can perform. Cancer progression is mediated by neutrophils via several mechanisms, such as the promotion of proliferation, angiogenesis, invasion and metastasis, as well as via suppression of anti-tumor T cell responses. Potential ways to limit the pro-tumor activity of neutrophils include the reduction of neutrophil numbers, or the inhibition of the recruitment or activation of immunosuppressive neutrophils. Conversely, neutrophils can efficiently act as effector cells towards cancer when triggered in the presence of a therapeutic antibody opsonizing the cancer cells, leading to tumor elimination. By releasing the brakes that suppress neutrophils (*i.e.* interfering with immune checkpoints) or by augmenting the affinity to the opsonizing antibody of interest, the anti-tumorigenic capacities of neutrophils can be significantly enhanced. As research focuses more and more on exploiting neutrophils against cancer, we anticipate that the aforementioned approaches will prove to be highly valuable to suppress the pro-tumor capacities of neutrophils and consequently fully unleash the anti-tumor potential of neutrophils. However, the similarity of these yet distinguished populations, may make the neutrophil-specific targeting difficult to accomplish *in vivo*. The coming years of neutrophil-related research will help understanding neutrophil behavior, while by using new developments we might witness a new era on harnessing neutrophil function against tumor progression.

ACKNOWLEDGEMENTS

We would like to thank Dr. R. van Bruggen for critically reading the manuscript.

AUTHOR CONTRIBUTIONS

HLM and TWK are the principle investigators who designed and supervised the work. CF, PMS, and PB wrote the manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST DISCLOSURE

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FUNDING

CF is supported by the Louise Vehmeijer Foundation. HM is supported by the Dutch Cancer Society (grant #11537, awarded to HM).

REFERENCES

1. Sharma P, Allison JP. The future of immune checkpoint therapy. *Science (1979).* 2015;348(6230):56-61.
2. Eggermont AMM, Chiarion-Sileni V, Grob JJ, et al. Adjuvant ipilimumab versus placebo after complete resection of high-risk stage III melanoma (EORTC 18071): a randomised, double-blind, phase 3 trial. *Lancet Oncol.* 2015;16(5):522-530.
3. Borghaei H, Paz-Ares L, Horn L, et al. Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer. *N Engl J Med.* 2015;373(17):1627-1639.
4. Hu B, Jacobs R, Ghosh N. Checkpoint Inhibitors Hodgkin Lymphoma and Non-Hodgkin Lymphoma. *Curr Hematol Malig Rep.* 2018;13(6):543-554.
5. Teo MY, Rosenberg JE. Nivolumab for the treatment of urothelial cancers. *Expert Rev Anticancer Ther.* 2018;18(3):215-221.
6. Planes-Laine G, Rochigneux P, Bertucci F, et al. PD-1/PD-L1 Targeting in Breast Cancer: The First Clinical Evidences Are Emerging. A Literature Review. *Cancers (Basel).* 2019;11(7):1033.
7. O'Donnell JS, Long G V, Scolyer RA, Teng MW, Smyth MJ. Resistance to PD1/PDL1 checkpoint inhibition. *Cancer Treat Rev.* 2017;52:71-81.
8. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med.* 2011;365(8):725-733.
9. Singh AK, McGuirk JP. CAR T cells: continuation in a revolution of immunotherapy. *Lancet Oncol.* 2020;21(3):e168-e178.
10. Neelapu SS, Tummala S, Kebriaei P, et al. Chimeric antigen receptor T-cell therapy - assessment and management of toxicities. *Nat Rev Clin Oncol.* 2018;15(1):47-62.
11. Shin MH, Kim J, Lim SA, Kim J, Kim SJ, Lee KM. NK Cell-Based Immunotherapies in Cancer. *Immune Netw.* 2020;20(2):e14.
12. Kalinski P, Muthuswamy R, Urban J. Dendritic cells in cancer immunotherapy: vaccines and combination immunotherapies. *Expert Rev Vaccines.* 2013;12(3):285-295.
13. Melero I, Gaudernack G, Gerritsen W, et al. Therapeutic vaccines for cancer: an overview of clinical trials. *Nat Rev Clin Oncol.* 2014;11(9):509-524.
14. Heifets L. Centennial of Metchnikoff's discovery. *J Reticuloendothel Soc.* 1982;31(5):381-391.
15. Palmer C, Diehn M, Alizadeh AA, Brown PO. Cell-type specific gene expression profiles of leukocytes in human peripheral blood. *BMC Genomics.* 2006;7:115.
16. Chen C, Duckworth CA, Zhao Q, Pritchard DM, Rhodes JM, Yu LG. Increased circulation of galectin-3 in cancer induces secretion of metastasis-promoting cytokines from blood vascular endothelium. *Clin Cancer Res.* 2013;19(7):1693-1704.
17. Jablonska J. Sionov R.V. and Granot Z. LS. The regulation of pre-metastatic niche formation by neutrophils. *Oncotarget.* 2007;8(67):112132-112144.
18. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. *Nat Rev Cancer.* 2016;16(7):431-446.
19. Gonzalez-Aparicio M, Alfaro C. Significance of the IL-8 pathway for immunotherapy. *Hum Vaccin Immunother.* 2020;16(10):2312-2317.
20. Guthrie GJ, Charles KA, Roxburgh CS, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: experience in patients with cancer. *Crit Rev Oncol Hematol.* 2013;88(1):218-230.

21. Templeton AJ, McNamara MG, Seruga B, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst*. 2014;106(6):dju124.
22. Grecian R, Whyte MKB, Walmsley SR. The role of neutrophils in cancer. *Br Med Bull*. 2018;128(1):5-14.
23. Caruso RA, Bellocco R, Pagano M, Bertoli G, Rigoli L, Inferrera C. Prognostic value of intratumoral neutrophils in advanced gastric carcinoma in a high-risk area in northern Italy. *Mod Pathol*. 2002;15(8):831-837.
24. Reid MD, Basturk O, Thirabanjasak D, et al. Tumor-infiltrating neutrophils in pancreatic neoplasia. *Mod Pathol*. 2011;24(12):1612-1619.
25. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, von der Maase H. Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. *J Clin Oncol*. 2009;27(28):4709-4717.
26. Shitara K, Matsuo K, Oze I, et al. Meta-analysis of neutropenia or leukopenia as a prognostic factor in patients with malignant disease undergoing chemotherapy. *Cancer Chemother Pharmacol*. 2011;68(2):301-307.
27. Fridlender ZG, Sun J, Kim S, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell*. 2009;16(3):183-194.
28. Sagiv JY, Michaeli J, Assi S, et al. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep*. 2015;10(4):562-573.
29. Shaul ME, Fridlender ZG. Cancer-related circulating and tumor-associated neutrophils - subtypes, sources and function. *FEBS J*. 2018;285(23):4316-4342.
30. Treffers LW, Hiemstra IH, Kuijpers TW, van den Berg TK, Matlung HL. Neutrophils in cancer. *Immunol Rev*. 2016;273(1):312-328.
31. Houghton AM, Rzymkiewicz DM, Ji H, et al. Neutrophil elastase–mediated degradation of IRS-1 accelerates lung tumor growth. *Nat Med*. 2010;16(2):219-223.
32. Nawa M, Osada S, Morimitsu K, et al. Growth Effect of Neutrophil Elastase on Breast Cancer: Favorable Action of Sivelestat and Application to Anti-HER2 Therapy. *Anticancer Res*. 2012;32(1):13.
33. Gaida MM, Steffen TG, Günther F, et al. Polymorphonuclear neutrophils promote dyshesion of tumor cells and elastase-mediated degradation of E-cadherin in pancreatic tumors. *Eur J Immunol*. 2012;42(12):3369-3380.
34. Kuang DM, Zhao Q, Wu Y, et al. Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. *J Hepatol*. 2011;54(5):948-955.
35. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature*. 2015;528(7582):413-417.
36. Spicer JD, McDonald B, Cools-Lartigue JJ, et al. Neutrophils Promote Liver Metastasis via Mac-1–Mediated Interactions with Circulating Tumor Cells. *Cancer Res*. 2012;72(16):3919-3927.
37. Huh SJ, Liang S, Sharma A, Dong C, Robertson GP. Transiently Entrapped Circulating Tumor Cells Interact with Neutrophils to Facilitate Lung Metastasis Development. *Cancer Res*. 2010;70(14):6071-6082.
38. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil Extracellular Traps Kill Bacteria. *Science (1979)*. 2004;303(5663):1532-1535.

39. Alfaro C, Teijeira A, Oñate C, et al. Tumor-Produced Interleukin-8 Attracts Human Myeloid-Derived Suppressor Cells and Elicits Extrusion of Neutrophil Extracellular Traps (NETs). *Clin Cancer Res*. 2016;22(15):3924-3936.
40. Berger-Achituv S, Brinkmann V, Abu-Abed U, et al. A proposed role for neutrophil extracellular traps in cancer immunoediting. *Front Immunol*. 2013;4:48.
41. Jung HS, Gu J, Kim JE, Nam Y, Song JW, Kim HK. Cancer cell–induced neutrophil extracellular traps promote both hypercoagulability and cancer progression. *PLoS One*. 2019;14(4):e0216055-. https://doi.org/10.1371/journal.pone.0216055
42. Cools-Lartigue J, Spicer J, McDonald B, et al. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. *J Clin Invest*. 2013;123(8):3446-3458.
43. Lee W, Ko SY, Mohamed MS, Kenny HA, Lengyel E, Naora H. Neutrophils facilitate ovarian cancer premetastatic niche formation in the omentum. *J Exp Med*. 2019;216(1):176-194.
44. Park J, Wysocki RW, Amoozgar Z, et al. Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. *Sci Transl Med*. 2016;8(361):361ra138.
45. Tohme S, Yazdani HO, Al-Khafaji AB, et al. Neutrophil Extracellular Traps Promote the Development and Progression of Liver Metastases after Surgical Stress. *Cancer Res*. 2016;76(6):1367-1380.
46. Oklu R, Sheth RA, Wong KHK, Jahromi AH, Albadawi H. Neutrophil extracellular traps are increased in cancer patients but does not associate with venous thrombosis. *Cardiovasc Diagn Ther*. 2017;7(Suppl 3):S140-S149.
47. Richardson JJR, Hendrickse C, Gao-Smith F, Thickett DR. Neutrophil Extracellular Trap Production in Patients with Colorectal Cancer In Vitro. *Int J Inflam*. 2017;2017:4915062.
48. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9(3):162-174.
49. Solito S, Marigo I, Pinton L, Damuzzo V, Mandruzzato S, Bronte V. Myeloid-derived suppressor cell heterogeneity in human cancers. *Ann N Y Acad Sci*. 2014;1319(1):47-65.
50. Raychaudhuri B, Rayman P, Ireland J, et al. Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. *Neuro Oncol*. 2011;13(6):591-599.
51. Solito S, Falisi E, Diaz-Montero CM, et al. A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. *Blood*. 2011;118(8):2254-2265.
52. Yamauchi Y, Safi S, Blattner C, et al. Circulating and Tumor Myeloid-derived Suppressor Cells in Resectable Non–Small Cell Lung Cancer. *Am J Respir Crit Care Med*. 2018;198(6):777-787.
53. Mandruzzato S, Brandau S, Britten CM, et al. Toward harmonized phenotyping of human myeloid-derived suppressor cells by flow cytometry: results from an interim study. *Cancer Immunol Immunother*. 2016;65(2):161-169.
54. Condamine T, Dominguez GA, Youn JI, et al. Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients. *Sci Immunol*. 2016;1(2):aaf8943-aaf8943.
55. Marini O, Costa S, Bevilacqua D, et al. Mature CD10(+) and immature CD10(-) neutrophils present in G-CSF-treated donors display opposite effects on T cells. *Blood*. 2017;129(10):1343-1356.
56. Aarts CEM, Hiemstra IH, Béguin EP, et al. Activated neutrophils exert myeloid-derived suppressor cell activity damaging T cells beyond repair. *Blood Adv*. 2019;3(22):3562-3574.

57. Hock BD, Taylor KG, Cross NB, Kettle AJ, Hampton MB, McKenzie JL. Effect of activated human polymorphonuclear leucocytes on T lymphocyte proliferation and viability. *Immunology*. 2012;137(3):249-258.
58. Singel KL, Emmons TR, Khan ANH, et al. Mature neutrophils suppress T cell immunity in ovarian cancer microenvironment. *JCI Insight*. 2019;4(5).
59. Lechner MG, Liebertz DJ, Epstein AL. Characterization of Cytokine-Induced Myeloid-Derived Suppressor Cells from Normal Human Peripheral Blood Mononuclear Cells. *J Immunol*. 2010;185(4):2273-2284.
60. Aarts CEM, Kuijpers TW. Neutrophils as myeloid-derived suppressor cells. *Eur J Clin Invest*. 2018;48(S2):e12989.
61. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. 2013;13(3):159-175.
62. Jacobsen LC, Theilgaard-Mönch K, Christensen EI, Borregaard N. Arginase 1 is expressed in myelocytes/metamyelocytes and localized in gelatinase granules of human neutrophils. *Blood*. 2006;109(7):3084-3087.
63. Ochoa AC, Zea AH, Hernandez C, Rodriguez PC. Arginase, Prostaglandins, and Myeloid-Derived Suppressor Cells in Renal Cell Carcinoma. *Clin Cancer Res*. 2007;13(2):721s-726s.
64. Rodriguez PC, Ernstoff MS, Hernandez C, et al. Arginase I–Producing Myeloid-Derived Suppressor Cells in Renal Cell Carcinoma Are a Subpopulation of Activated Granulocytes. *Cancer Res*. 2009;69(4):1553-1560.
65. Sippel TR, White J, Nag K, et al. Neutrophil Degranulation and Immunosuppression in Patients with GBM: Restoration of Cellular Immune Function by Targeting Arginase I. *Clin Cancer Res*. 2011;17(22):6992-7002.
66. Taheri F, Ochoa J, Faghiri Z, et al. L-Arginine regulates the expression of the T-cell receptor zeta chain (CD3zeta) in Jurkat cells. *Clin Cancer Res*. 2001;7(3 Suppl):958s-9965.
67. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I Production in the Tumor Microenvironment by Mature Myeloid Cells Inhibits T-Cell Receptor Expression and Antigen-Specific T-Cell Responses. *Cancer Res*. 2004;64(16):5839-5849.
68. Paulo CR, Arnold HZ, Kirk SC, Jovanny Z, Juan BO, Augusto CO. Regulation of T cell receptor CD3zeta chain expression by L-arginine. *J Biol Chem*. 2002;277(24):21123-21129.
69. Burkhardt JK, Carrizosa E, Shaffer MH. The Actin Cytoskeleton in T Cell Activation. *Annu Rev Immunol*. 2008;26(1):233-259.
70. Feldmeyer N, Wabnitz G, Leicht S, et al. Arginine deficiency leads to impaired cofilin dephosphorylation in activated human T lymphocytes. *Int Immunol*. 2012;24(5):303-313.
71. Zea AH, Rodriguez PC, Culotta KS, et al. l-Arginine modulates CD3ζ expression and T cell function in activated human T lymphocytes. *Cell Immunol*. 2004;232(1):21-31.
72. Munder M, Schneider H, Luckner C, et al. Suppression of T-cell functions by human granulocyte arginase. *Blood*. 2006;108(5):1627-1634.
73. Liu CY, Wang YM, Wang CL, et al. Population alterations of l-arginase- and inducible nitric oxide synthase-expressed CD11b+/CD14−/CD15+/CD33+ myeloid-derived suppressor cells and CD8+ T lymphocytes in patients with advanced-stage non-small cell lung cancer. *J Cancer Res Clin Oncol*. 2010;136(1):35-45.
74. de Saint Basile G, Menasche G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat Rev Immunol*. 2010;10(8):568-579.

75. Meeths M, Entesarian M, Al-Herz W, et al. Spectrum of clinical presentations in familial hemophagocytic lymphohistiocytosis type 5 patients with mutations in STXBP2. *Blood*. 2010;116(15):2635-2643.
76. Zhao XW, Gazendam RP, Drewniak A, et al. Defects in neutrophil granule mobilization and bactericidal activity in familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) syndrome caused by STXBP2/Munc18-2 mutations. *Blood*. 2013;122(1):109-111.
77. Corzo CA, Cotter MJ, Cheng P, et al. Mechanism Regulating Reactive Oxygen Species in Tumor-Induced Myeloid-Derived Suppressor Cells1. *J Immunol*. 2009;182(9):5693-5701.
78. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res*. 2001;61(12):4756-4760.
79. Szuster-Ciesielska A, Hryciuk-Umer E, Stepulak A, Kupisz K, Kandefer-Szerszeń M. Reactive oxygen species production by blood neutrophils of patients with laryngeal carcinoma and antioxidative enzyme activity in their blood. *Acta Oncol (Madr)*. 2004;43(3):252-258.
80. Malmberg KJ, Arulampalam V, Ichihara F, et al. Inhibition of Activated/Memory (CD45RO+) T Cells by Oxidative Stress Associated with Block of NF-κB Activation1. *J Immunol*. 2001;167(5):2595-2601.
81. Whiteside TL. Down-regulation of ζ-chain expression in T cells: a biomarker of prognosis in cancer? *Cancer Immunol Immunother*. 2004;53(10):865-878.
82. Klemke M, Wabnitz GH, Funke F, Funk B, Kirchgessner H, Samstag Y. Oxidation of Cofilin Mediates T Cell Hyporesponsiveness under Oxidative Stress Conditions. *Immunity*. 2008;29(3):404-413.
83. Wabnitz GH, Goursot C, Jahraus B, et al. Mitochondrial translocation of oxidized cofilin induces caspase-independent necrotic-like programmed cell death of T cells. *Cell Death Dis*. 2010;1(7):e58-e58.
84. Kramer PA, Prichard L, Chacko B, et al. Inhibition of the lymphocyte metabolic switch by the oxidative burst of human neutrophils. *Clin Sci*. 2015;129(6):489-504.
85. Roos D. The Genetic Basis of Chronic Granulomatous Disease. *Immunol Rev*. 1994;138(1):121-157.
86. Segal AW. How neutrophils kill microbes. *Annu Rev Immunol*. 2005;23:197-223.
87. Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation. *J Exp Med*. 2000;192(7):1027-1034.
88. Zitvogel L, Kroemer G. Targeting PD-1/PD-L1 interactions for cancer immunotherapy. *Oncoimmunology*. 2012;1(8):1223-1225.
89. Bankey PE, Banerjee S, Zucchiatti A, et al. Cytokine induced expression of programmed death ligands in human neutrophils. *Immunol Lett*. 2010;129(2):100-107.
90. de Kleijn S, Langereis JD, Leentjens J, et al. IFN-γ-Stimulated Neutrophils Suppress Lymphocyte Proliferation through Expression of PD-L1. *PLoS One*. 2013;8(8):e72249-.
91. Noman MZ, Desantis G, Janji B, et al. PD-L1 is a novel direct target of HIF-1α, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. *J Exp Med*. 2014;211(5):781-790.
92. He G, Zhang H, Zhou J, et al. Peritumoural neutrophils negatively regulate adaptive immunity via the PD-L1/PD-1 signalling pathway in hepatocellular carcinoma. *J Exp Clin Cancer Res*. 2015;34(1):141.

93. Wang T ting, Zhao Y liang, Peng L sheng, et al. Tumour-activated neutrophils in gastric cancer foster immune suppression and disease progression through GM-CSF-PD-L1 pathway. *Gut*. 2017;66(11):1900.
94. Pillay J, Kamp VM, van Hoffen E, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. 2012;122(1):327-336.
95. Michaeli J, Shaul ME, Mishalian I, et al. Tumor-associated neutrophils induce apoptosis of non-activated CD8 T-cells in a TNFα and NO-dependent mechanism, promoting a tumor-supportive environment. *Oncoimmunology*. 2017;6(11):e1356965.
96. Yu J, Du W, Yan F, et al. Myeloid-Derived Suppressor Cells Suppress Antitumor Immune Responses through IDO Expression and Correlate with Lymph Node Metastasis in Patients with Breast Cancer. *J Immunol*. 2013;190(7):3783-3797.
97. Joly E, Hudrisier D. What is trogocytosis and what is its purpose? *Nat Immunol*. 2003;4(9):815.
98. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine Selectively Eliminates Splenic Gr-1+/CD11b+ Myeloid Suppressor Cells in Tumor-Bearing Animals and Enhances Antitumor Immune Activity. *Clin Cancer Res*. 2005;11(18):6713-6721.
99. Li H, Han Y, Guo Q, Zhang M, Cao X. Cancer-Expanded Myeloid-Derived Suppressor Cells Induce Anergy of NK Cells through Membrane-Bound TGF-β11. *J Immunol*. 2009;182(1):240-249.
100. Sceneay J, Chow MT, Chen A, et al. Primary Tumor Hypoxia Recruits CD11b+/Ly6Cmed/Ly6G+ Immune Suppressor Cells and Compromises NK Cell Cytotoxicity in the Premetastatic Niche. *Cancer Res*. 2012;72(16):3906-3911.
101. Spiegel A, Brooks MW, Houshyar S, et al. Neutrophils Suppress Intraluminal NK Cell–Mediated Tumor Cell Clearance and Enhance Extravasation of Disseminated Carcinoma Cells. *Cancer Discov*. 2016;6(6):630-649.
102. Chaudhary B, Elkord E. Regulatory T Cells in the Tumor Microenvironment and Cancer Progression: Role and Therapeutic Targeting. *Vaccines (Basel)*. 2016;4(3):28.
103. Mougiakakos D, Johansson CC, Jitschin R, Böttcher M, Kiessling R. Increased thioredoxin-1 production in human naturally occurring regulatory T cells confers enhanced tolerance to oxidative stress. *Blood*. 2011;117(3):857-861.
104. Mougiakakos D, Johansson CC, Kiessling R. Naturally occurring regulatory T cells show reduced sensitivity toward oxidative stress–induced cell death. *Blood*. 2009;113(15):3542-3545.
105. Mishalian I, Bayuh R, Eruslanov E, et al. Neutrophils recruit regulatory T-cells into tumors via secretion of CCL17—A new mechanism of impaired antitumor immunity. *Int J Cancer*. 2014;135(5):1178-1186.
106. Lewkowicz N, Klink M, Mycko MP, Lewkowicz P. Neutrophil – CD4+CD25+ T regulatory cell interactions: A possible new mechanism of infectious tolerance. *Immunobiology*. 2013;218(4):455-464.
107. Lewkowicz N, Mycko MP, Przygodzka P, et al. Induction of human IL-10-producing neutrophils by LPS-stimulated Treg cells and IL-10. *Mucosal Immunol*. 2016;9(2):364-378.
108. Eruslanov E, Neuberger M, Daurkin I, et al. Circulating and tumor-infiltrating myeloid cell subsets in patients with bladder cancer. *Int J Cancer*. 2012;130(5):1109-1119.
109. Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? *Carcinogenesis*. 2012;33(5):949-955.
110. Rakic A, Beaudry P, Mahoney DJ. The complex interplay between neutrophils and cancer. *Cell Tissue Res*. 2018;371(3):517-529.

111. Lieschke GJ, Burgess AW. Granulocyte Colony-Stimulating Factor and Granulocyte-Macrophage Colony-Stimulating Factor. *N Engl J Med*. 1992;327(1):28-35.
112. Carulli G. Effects of recombinant human granulocyte colony-stimulating factor administration on neutrophil phenotype and functions. *Haematologica*. 1997;82(5):606-616.
113. Gorabi AM, Hajighasemi S, Sathyapalan T, Sahebkar A. Cell transfer-based immunotherapies in cancer: A review. *IUBMB Life*. 2020;72(4):790-800.
114. Siders WM, Shields J, Garron C, et al. Involvement of neutrophils and natural killer cells in the anti-tumor activity of alemtuzumab in xenograft tumor models. *Leuk Lymphoma*. 2010;51(7):1293-1304.
115. Cornet S, Mathe D, Chettab K, et al. Pegfilgrastim Enhances the Antitumor Effect of Therapeutic Monoclonal Antibodies. *Mol Cancer Ther*. 2016;15(6):1238-1247.
116. Torka P, Patel P, Tan W, et al. A Phase II Trial of Rituximab Combined With Pegfilgrastim in Patients With Indolent B-cell Non-Hodgkin Lymphoma. *Clin Lymphoma Myeloma Leuk*. 2018;18(1):e51-e60.
117. Zivkovic M, Poljak-Blazi M, Zarkovic K, Mihaljevic D, Schaur RJ, Zarkovic N. Oxidative burst of neutrophils against melanoma B16-F10. *Cancer Lett*. 2007;246(1-2):100-108.
118. Yan J, Kloecker G, Fleming C, et al. Human polymorphonuclear neutrophils specifically recognize and kill cancerous cells. *Oncoimmunology*. 2014;3(7):e950163.
119. Gershkovitz M, Fainsod-Levi T, Zelter T, Sionov R V, Granot Z. TRPM2 modulates neutrophil attraction to murine tumor cells by regulating CXCL2 expression. *Cancer Immunol Immunother*. 2019;68(1):33-43.
120. Sun B, Qin W, Song M, et al. Neutrophil Suppresses Tumor Cell Proliferation via Fas /Fas Ligand Pathway Mediated Cell Cycle Arrested. *Int J Biol Sci*. 2018;14(14):2103-2113.
121. Glennie MJ, French RR, Cragg MS, Taylor RP. Mechanisms of killing by anti-CD20 monoclonal antibodies. *Mol Immunol*. 2007;44(16):3823-3837.
122. Hudis C. Trastuzumab-Mechanism of Action and Use in Clinical Practice. *N Engl J Med*. 2007;357(1):39-51.
123. Gelderman KA, Tomlinson S, Ross GD, Gorter A. Complement function in mAb-mediated cancer immunotherapy. *Trends Immunol*. 2004;25(3):158-164.
124. Nimmerjahn F, Ravetch J V. Fc-Receptors as Regulators of Immunity. *Adv Immunol*. 2007;96:179-204.
125. Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. *Nat Rev Immunol*. 2010;10(5):301-316.
126. van Egmond M, Bakema JE. Neutrophils as effector cells for antibody-based immunotherapy of cancer. *Semin Cancer Biol*. 2013;23(3):190-199.
127. Bakema JE, van Egmond M. Fc receptor-dependent mechanisms of monoclonal antibody therapy of cancer. *Curr Top Microbiol Immunol*. 2014;382:373-392.
128. Heemskerk N, van Egmond M. Monoclonal antibody-mediated killing of tumour cells by neutrophils. *Eur J Clin Invest*. 2018;48 Suppl 2:e12962.
129. Gale RP, Zighelboim J. Polymorphonuclear leukocytes in antibody-dependent cellular cytotoxicity. *J Immunol*. 1975;114(3):1047-1051.
130. Barker E, Mueller BM, Handgretinger R, Herter M, Yu AL, Reisfeld RA. Effect of a chimeric anti-ganglioside GD2 antibody on cell-mediated lysis of human neuroblastoma cells. *Cancer Res*. 1991;51(1):144-149.

131. Hernandez-Ilizaliturri FJ, Jupudy V, Ostberg J, et al. Neutrophils Contribute to the Biological Antitumor Activity of Rituximab in a Non-Hodgkin's Lymphoma Severe Combined Immunodeficiency Mouse Model. *Clin Cancer Res*. 2003;9(16):5866-5873.
132. Albanesi M, Mancardi DA, Jonsson F, et al. Neutrophils mediate antibody-induced antitumor effects in mice. *Blood*. 2013;122(18):3160-3164.
133. Musolino A, Naldi N, Bortesi B, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol*. 2008;26(11):1789-1796.
134. Tamura K, Shimizu C, Hojo T, et al. FcgammaR2A and 3A polymorphisms predict clinical outcome of trastuzumab in both neoadjuvant and metastatic settings in patients with HER2-positive breast cancer. *Ann Oncol*. 2011;22(6):1302-1307.
135. Zhang W, Gordon M, Schultheis AM, et al. FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. *J Clin Oncol*. 2007;25(24):3712-3718.
136. Cheung NK V., Sowers R, Vickers AJ, Cheung IY, Kushner BH, Gorlick R. FCGR2A polymorphism is correlated with clinical outcome after immunotherapy of neuroblastoma with anti-GD2 antibody and granulocyte macrophage colony-stimulating factor. *J Clin Oncol*. 2006;24(18):2885-2890.
137. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol*. 2014;5:520.
138. Bruhns P, Jonsson F. Mouse and human FcR effector functions. *Immunol Rev*. 2015;268(1):25-51.
139. Chen X, Song X, Li K, Zhang T. FcgammaR-Binding Is an Important Functional Attribute for Immune Checkpoint Antibodies in Cancer Immunotherapy. *Front Immunol*. 2019;10:292.
140. Nagelkerke SQ, Tacke CE, Breunis WB, et al. Extensive Ethnic Variation and Linkage Disequilibrium at the FCGR2/3 Locus: Different Genetic Associations Revealed in Kawasaki Disease. *Front Immunol*. 2019;10:185.
141. Wang Y, Jonsson F. Expression, Role, and Regulation of Neutrophil Fcgamma Receptors. *Front Immunol*. 2019;10:1958.
142. Blank U Benhamou M, Monteiro RC. LP. Inhibitory ITAMs as novel regulators of immunity. *Immunol Rev*. 2009;232(1):59-71.
143. Fleit HB Unkeless JC. WSD. Human neutrophil Fc gamma receptor distribution and structure. *Proc Natl Acad Sci USA*. 1982;79(10):3275-3279.
144. Treffers LW, van Houdt M, Bruggeman CW, et al. FcgammaRIIIb Restricts Antibody-Dependent Destruction of Cancer Cells by Human Neutrophils. *Front Immunol*. 2018;9:3124.
145. Repp R, Valerius T, Sendler A, et al. Neutrophils express the high affinity receptor for IgG (Fc gamma RI, CD64) after in vivo application of recombinant human granulocyte colony- stimulating factor. *Blood*. 1991;78(4):885-889.
146. van de Winkel JG, Capel PJ. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. *Immunol Today*. 1993;14(5):215-221.
147. Middelhoven PJ, van Buul JD, Kleijer M, Roos D, Hordijk PL. Actin Polymerization Induces Shedding of FcγRIIIb (CD16) from Human Neutrophils. *Biochem Biophys Res Commun*. 1999;255(3):568-574.
148. Peipp M, Lammerts van Bueren JJ, Schneider-Merck T, et al. Antibody fucosylation differentially impacts cytotoxicity mediated by NK and PMN effector cells. *Blood*. 2008;112(6):2390-2399.

149. Matlung HL, Babes L, Zhao XW, et al. Neutrophils Kill Antibody-Opsonized Cancer Cells by Trogoptosis. *Cell Rep*. 2018;23(13):3946-3959 e6.
150. Treffers LW, Zhao XW, van der Heijden J, et al. Genetic variation of human neutrophil Fcgamma receptors and SIRPalpha in antibody-dependent cellular cytotoxicity towards cancer cells. *Eur J Immunol*. 2018;48(2):344-354.
151. Valerius T, Repp R, de Wit TP, et al. Involvement of the high-affinity receptor for IgG (Fc gamma RI; CD64) in enhanced tumor cell cytotoxicity of neutrophils during granulocyte colony-stimulating factor therapy. *Blood*. 1993;82(3):931-939.
152. Michon J, Moutel S, Barbet J, et al. In vitro killing of neuroblastoma cells by neutrophils derived from granulocyte colony-stimulating factor-treated cancer patients using an anti-disialoganglioside/anti-Fc gamma RI bispecific antibody. *Blood*. 1995;86(3):1124-1130.
153. Keler T, Mandal A, Wallace P, et al. Bispecillc Antibody-dependent Cellular Cytotoxicity of HER2/neu-overexpressing Tumor Cells by Fcy Receptor Type I-expressing Effector Cells. *Cancer Res*. 1997;57(18):4008-4014.
154. Heijnen I, van de Winkel J, Glennie M. Lysis of murine B lymphoma cells by transgenic phagocytes via a human FcgRI murine MHC class II bispecific antibody. *Cancer Immunol Immunother*. 1997;45(3-4):166-170.
155. Honeychurch J, Valerius T, Heijnen I, Van De Winkel J, Glennie M, Tutt A. Therapeutic efficacy of FcgammaRI/CD64-directed bispecific antibodies in B-cell lymphoma. *Blood*. 2000;96(10):3544-3552.
156. Taylor RP, Lindorfer MA. Fcgamma-receptor-mediated trogocytosis impacts mAb-based therapies: historical precedence and recent developments. *Blood*. 2015;125(5):762-766.
157. Tabiasco J, Espinosa E, Hudrisier D, Joly E, Fournie JJ, Vercellone A. Active trans-synaptic capture of membrane fragments by natural killer cells. *Eur J Immunol*. 2002;32(5):1502-1508.
158. Mercer F, Ng SH, Brown TM, Boatman G, Johnson PJ. Neutrophils kill the parasite Trichomonas vaginalis using trogocytosis. *PLoS Biol*. 2018;16(2):e2003885.
159. Velmurugan R, Challa DK, Ram S, Ober RJ, Ward ES. Macrophage-Mediated Trogocytosis Leads to Death of Antibody-Opsonized Tumor Cells. *Mol Cancer Ther*. 2016;15(8):1879-1889.
160. Valgardsdottir R, Cattaneo I, Klein C, Introna M, Figliuzzi M, Golay J. Human neutrophils mediate trogocytosis rather than phagocytosis of CLL B cells opsonized with anti-CD20 antibodies. *Blood*. 2017;129(19):2636-2644.
161. Krejcik J, Frerichs KA, Nijhof IS, et al. Monocytes and Granulocytes Reduce CD38 Expression Levels on Myeloma Cells in Patients Treated with Daratumumab. *Clin Cancer Res*. 2017;23(24):7498-7511.
162. Strizova Z, Vachtenheim Jr. J, Bartunkova J. The potential role of neutrophil trogocytosis and G-CSF in the loss of HER2 expression. *Breast Cancer Res Treat*. 2019;178(1):247-248.
163. Ignatov T, Gorbunow F, Eggemann H, Ortmann O, Ignatov A. Loss of HER2 after HER2-targeted treatment. *Breast Cancer Res Treat*. 2019;175(2):401-408.
164. van Gisbergen KP, Sanchez-Hernandez M, Geijtenbeek TB, van Kooyk Y. Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *J Exp Med*. 2005;201(8):1281-1292.
165. Megiovanni AM, Sanchez F, Robledo-Sarmiento M, Morel C, Gluckman JC, Boudaly S. Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *J Leukoc Biol*. 2006;79(5):977-988.

166. Sandilands GP, Ahmed Z, Perry N, Davison M, Lupton A, Young B. Cross-linking of neutrophil CD11b results in rapid cell surface expression of molecules required for antigen presentation and T-cell activation. *Immunology*. 2005;114(3):354-368.
167. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, et al. Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer. *J Clin Invest*. 2014;124(12):5466-5480.
168. Eruslanov EB. Phenotype and function of tumor-associated neutrophils and their subsets in early-stage human lung cancer. *Cancer Immunol Immunother*. 2017;66(8):997-1006.
169. Beauvillain C, Delneste Y, Scotet M, et al. Neutrophils efficiently cross-prime naive T cells in vivo. *Blood*. 2007;110(8):2965-2973.
170. Maletto BA, Ropolo AS, Alignani DO, et al. Presence of neutrophil-bearing antigen in lymphoid organs of immune mice. *Blood*. 2006;108(9):3094-3102.
171. Beauvillain C, Cunin P, Doni A, et al. CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood*. 2011;117(4):1196-1204.
172. Voisin MB, Nourshargh S. Neutrophil trafficking to lymphoid tissues: physiological and pathological implications. *J Pathol*. 2019;247(5):662-671.
173. Suttmann H, Riemensberger J, Bentien G, et al. Neutrophil granulocytes are required for effective Bacillus Calmette-Guerin immunotherapy of bladder cancer and orchestrate local immune responses. *Cancer Res*. 2006;66(16):8250-8257.
174. Coffelt SB, Kersten K, Doornebal CW, et al. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature*. 2015;522(7556):345-348.
175. Zhu EF, Gai SA, Opel CF, et al. Synergistic innate and adaptive immune response to combination immunotherapy with anti-tumor antigen antibodies and extended serum half-life IL-2. *Cancer Cell*. 2015;27(4):489-501.
176. Mishalian I Levy L, Zolotarov L, Michaeli J, Fridlender ZG. BR. Tumorassociated neutrophils (TAN) develop protumorigenic properties during tumor progression. *Cancer Immunol Immunother*. 2013;62:1745–1756.
177. Pylaeva E, Lang S, Jablonska J. The Essential Role of Type I Interferons in Differentiation and Activation of Tumor-Associated Neutrophils. *Front Immunol*. 2016;7:629.
178. Ohms M, Möller S, Laskay T. An Attempt to Polarize Human Neutrophils Toward N1 and N2 Phenotypes in vitro. *Front Immunol*. 2020;11:532.
179. Stromnes IM, Brockenbrough JS, Izeradjene K, et al. Targeted depletion of an MDSC subset unmasks pancreatic ductal adenocarcinoma to adaptive immunity. *Gut*. 2014;63(11):1769.
180. Fultang L, Panetti S, Ng M, et al. MDSC targeting with Gemtuzumab ozogamicin restores T cell immunity and immunotherapy against cancers. *EBioMedicine*. 2019;47:235-246.
181. Roskoski R. Sunitinib: A VEGF and PDGF receptor protein kinase and angiogenesis inhibitor. *Biochem Biophys Res Commun*. 2007;356(2):323-328.
182. Ozao-Choy J, Ma G, Kao J, et al. The Novel Role of Tyrosine Kinase Inhibitor in the Reversal of Immune Suppression and Modulation of Tumor Microenvironment for Immune-Based Cancer Therapies. *Cancer Res*. 2009;69(6):2514-2522.
183. Ko JS, Zea AH, Rini BI, et al. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. *Clin Cancer Res*. 2009;15(6):2148-2157.
184. Guislain A, Gadiot J, Kaiser A, et al. Sunitinib pretreatment improves tumor-infiltrating lymphocyte expansion by reduction in intratumoral content of myeloid-derived suppressor cells in human renal cell carcinoma. *Cancer Immunol Immunother*. 2015;64(10):1241-1250.

185. Gebhardt C, Sevko A, Jiang H, et al. Myeloid Cells and Related Chronic Inflammatory Factors as Novel Predictive Markers in Melanoma Treatment with Ipilimumab. *Clin Cancer Res*. 2015;21(24):5453-5459.
186. de Coaña YP, Poschke I, Gentilcore G, et al. Ipilimumab Treatment Results in an Early Decrease in the Frequency of Circulating Granulocytic Myeloid-Derived Suppressor Cells as well as Their Arginase1 Production. *Cancer Immunol Res*. 2013;1(3):158-162.
187. Sade-Feldman M, Kanterman J, Klieger Y, et al. Clinical Significance of Circulating CD33+CD11b+HLA-DR− Myeloid Cells in Patients with Stage IV Melanoma Treated with Ipilimumab. *Clin Cancer Res*. 2016;22(23):5661-5672.
188. De Soyza A, Pavord I, Elborn JS, et al. A randomised, placebo-controlled study of the CXCR2 antagonist AZD5069 in bronchiectasis. *Eur Respir J*. 2015;46(4):1021.
189. Rennard SI, Dale DC, Donohue JF, et al. CXCR2 Antagonist MK-7123. A Phase 2 Proof-of-Concept Trial for Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med*. 2015;191(9):1001-1011.
190. Kumar V, Donthireddy L, Marvel D, et al. Cancer-Associated Fibroblasts Neutralize the Anti-tumor Effect of CSF1 Receptor Blockade by Inducing PMN-MDSC Infiltration of Tumors. *Cancer Cell*. 2017;32(5):654-668.e5.
191. Steele CW, Karim SA, Leach JDG, et al. CXCR2 Inhibition Profoundly Suppresses Metastases and Augments Immunotherapy in Pancreatic Ductal Adenocarcinoma. *Cancer Cell*. 2016;29(6):832-845.
192. Sun L, Clavijo PE, Robbins Y, et al. Inhibiting myeloid-derived suppressor cell trafficking enhances T cell immunotherapy. *JCI Insight*. 2019;4(7).
193. Schott AF, Goldstein LJ, Cristofanilli M, et al. Phase Ib Pilot Study to Evaluate Reparixin in Combination with Weekly Paclitaxel in Patients with HER-2–Negative Metastatic Breast Cancer. *Clin Cancer Res*. 2017;23(18):5358-5365.
194. Patnaik A, Swanson KD, Csizmadia E, et al. Cabozantinib Eradicates Advanced Murine Prostate Cancer by Activating Antitumor Innate Immunity. *Cancer Discov*. 2017;7(7):750-765.
195. Wislez M, Rabbe N, Marchal J, et al. Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: role in tumor progression and death. *Cancer Res*. 2003;63(6):1405-1412.
196. Glodde N, Bald T, van den Boorn-Konijnenberg D, et al. Reactive Neutrophil Responses Dependent on the Receptor Tyrosine Kinase c-MET Limit Cancer Immunotherapy. *Immunity*. 2017;47(4):789-802.e9.
197. De Silva DM, Roy A, Kato T, et al. Targeting the hepatocyte growth factor/Met pathway in cancer. *Biochem Soc Trans*. 2017;45(4):855-870.
198. Wu P, Wu D, Ni C, et al. GammadeltaT17 Cells Promote the Accumulation and Expansion of Myeloid-Derived Suppressor Cells in Human Colorectal Cancer. *Immunity*. 2014;40(5):785-800.
199. Chen WC, Lai YH, Chen HY, Guo HR, Su IJ, Chen HHW. Interleukin-17-producing cell infiltration in the breast cancer tumour microenvironment is a poor prognostic factor. *Histopathology*. 2013;63(2):225-233.
200. Ma C, Zhang Q, Ye J, et al. Tumor-Infiltrating γδ T Lymphocytes Predict Clinical Outcome in Human Breast Cancer. *J Immunol*. 2012;189(10):5029-5036.
201. Gaffen SL, Jain R, Garg A V, Cua DJ. The IL-23–IL-17 immune axis: from mechanisms to therapeutic testing. *Nat Rev Immunol*. 2014;14(9):585-600.

202. Aarts CEM, Hiemstra IH, Furumaya C, van Bruggen R, Kuijpers TW. Different MDSC Activity of G-CSF/Dexamethasone Mobilized Neutrophils: Benefits to the Patient? *Front Oncol*. 2020;10:1110.
203. Drewniak A, Boelens J, Vrielink H, et al. Granulocyte concentrates: prolonged functional capacity during storage in the presence of phenotypic changes. *Haematologica*. 2008;93(7):1058-1067.
204. Al-Khami AA, Zheng L, Del Valle L, et al. Exogenous lipid uptake induces metabolic and functional reprogramming of tumor-associated myeloid-derived suppressor cells. *Oncoimmunology*. 2017;6(10):e1344804.
205. Veglia F, Tyurin VA, Blasi M, et al. Fatty acid transport protein 2 reprograms neutrophils in cancer. *Nature*. 2019;569(7754):73-78.
206. Eruslanov EB, Singhal S, Albelda SM. Mouse versus Human Neutrophils in Cancer: A Major Knowledge Gap. *Trends Cancer*. 2017;3(2):149-160.
207. Ho A, Chen C, Cheng C, et al. Neutrophil elastase as a diagnostic marker and therapeutic target in colorectal cancers. *Oncotarget*. 2014;5:473-480.
208. Lerman I, Garcia-Hernandez M de la L, Rangel-Moreno J, et al. Infiltrating Myeloid Cells Exert Protumorigenic Actions via Neutrophil Elastase. *Mol Cancer Res*. 2017;15(9):1138-1152.
209. Suda K, Kitagawa Y, Ozawa S, et al. Neutrophil elastase inhibitor improves postoperative clinical courses after thoracic esophagectomy. *Dis Esophagus*. 2007;20(6):478-486.
210. Hawes MC, Wen F, Elquza E. Extracellular DNA: A Bridge to Cancer. *Cancer Res*. 2015;75(20):4260-4264.
211. van Rees DJ, Szilagyi K, Kuijpers TW, Matlung HL, van den Berg TK. Immunoreceptors on neutrophils. *Semin Immunol*. 2016;28(2):94-108.
212. Zhang W, Huang Q, Xiao W, et al. Advances in Anti-Tumor Treatments Targeting the CD47/SIRPalpha Axis. *Front Immunol*. 2020;11:18.
213. Zhao XW, van Beek EM, Schornagel K, et al. CD47-signal regulatory protein-α (SIRPα) interactions form a barrier for antibody-mediated tumor cell destruction. *Proc Natl Acad Sci U S A*. 2011;108(45):18342-18347.
214. Ring NG, Herndler-Brandstetter D, Weiskopf K, et al. Anti-SIRPα antibody immunotherapy enhances neutrophil and macrophage antitumor activity. *Proc Natl Acad Sci U S A*. 2017;114(49):E10578-e10585.
215. Horrigan SK, Reproducibility Project: Cancer B. Replication Study: The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Elife*. 2017;6.
216. Sim J, Sockolosky JT, Sangalang E, et al. Discovery of high affinity, pan-allelic, and pan-mammalian reactive antibodies against the myeloid checkpoint receptor SIRPalpha. *MAbs*. 2019;11(6):1036-1052.
217. Voets E, Parade M, Lutje Hulsik D, et al. Functional characterization of the selective pan-allele anti-SIRPalpha antibody ADU-1805 that blocks the SIRPalpha-CD47 innate immune checkpoint. *J Immunother Cancer*. 2019;7(1):340.
218. Sikic BI, Lakhani N, Patnaik A, et al. First-in-Human, First-in-Class Phase I Trial of the Anti-CD47 Antibody Hu5F9-G4 in Patients With Advanced Cancers. *J Clin Oncol*. 2019;37(12):946-953.
219. Advani R, Flinn I, Popplewell L, et al. CD47 Blockade by Hu5F9-G4 and Rituximab in Non-Hodgkin's Lymphoma. *N Engl J Med*. 2018;379(18):1711-1721.
220. McCracken MN, Cha AC, Weissman IL. Molecular Pathways: Activating T Cells after Cancer Cell Phagocytosis from Blockade of CD47 "Don't Eat Me" Signals. *Clin Cancer Res*. 2015;21(16):3597-3601.

221. Tseng D, Volkmer JP, Willingham SB, et al. Anti-CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective antitumor T-cell response. *Proc Natl Acad Sci U S A*. 2013;110(27):11103-11108.
222. Liu X, Pu Y, Cron K, et al. CD47 blockade triggers T cell-mediated destruction of immunogenic tumors. *Nat Med*. 2015;21(10):1209-1215.
223. Meyaard L, Adema GJ, Chang C, et al. LAIR-1, a Novel Inhibitory Receptor Expressed on Human Mononuclear Leukocytes. *Immunity*. 1997;7(2):283-290.
224. Kubagawa H, Burrows PD, Cooper MD. A novel pair of immunoglobulin-like receptors expressed by B cells and myeloid cells. *Proc Natl Acad Sci*. 1997;94(10):5261-5266.
225. Yi X, Zhang J, Zhuang R, et al. Silencing LAIR-1 in human THP-1 macrophage increases foam cell formation by modulating PPARgamma and M2 polarization. *Cytokine*. 2018;111:194-205.
226. Chang YC, Nizet V. The interplay between Siglecs and sialylated pathogens. *Glycobiology*. 2014;24(9):818-825.
227. Hudak JE, Canham SM, Bertozzi CR. Glycocalyx engineering reveals a Siglec-based mechanism for NK cell immunoevasion. *Nat Chem Biol*. 2014;10(1):69-75.
228. Haas Q, Boligan KF, Jandus C, et al. Siglec-9 Regulates an Effector Memory CD8(+) T-cell Subset That Congregates in the Melanoma Tumor Microenvironment. *Cancer Immunol Res*. 2019;7(5):707-718.
229. Bull C, Stoel MA, den Brok MH, Adema GJ. Sialic acids sweeten a tumor's life. *Cancer Res*. 2014;74(12):3199-3204.
230. von Gunten S, Schaub A, Vogel M, Stadler BM, Miescher S, Simon HU. Immunologic and functional evidence for anti-Siglec-9 autoantibodies in intravenous immunoglobulin preparations. *Blood*. 2006;108(13):4255-4259.
231. Laubli H, Alisson-Silva F, Stanczak MA, et al. Lectin galactoside-binding soluble 3 binding protein (LGALS3BP) is a tumor-associated immunomodulatory ligand for CD33-related Siglecs. *J Biol Chem*. 2014;289(48):33481-33491.
232. McMillan SJ, Sharma RS, Richards HE, Hegde V, Crocker PR. Siglec-E promotes beta2-integrin-dependent NADPH oxidase activation to suppress neutrophil recruitment to the lung. *J Biol Chem*. 2014;289(29):20370-20376.
233. Adams OJ, Stanczak MA, von Gunten S, Laubli H. Targeting sialic acid-Siglec interactions to reverse immune suppression in cancer. *Glycobiology*. 2018;28(9):640-647.
234. Chen S, Song Z, Zhang A. Small-Molecule Immuno-Oncology Therapy: Advances, Challenges and New Directions. *Curr Top Med Chem*. 2019;19(3):180-185.
235. Kerr WG, Chisholm JD. The Next Generation of Immunotherapy for Cancer: Small Molecules Could Make Big Waves. *J Immunol*. 2019;202(1):11-19.
236. Dempke WCM, Uciechowski P, Fenchel K, Chevassut T. Targeting SHP-1, 2 and SHIP Pathways: A Novel Strategy for Cancer Treatment? *Oncology*. 2018;95(5):257-269.
237. Monteiro RC, Van De Winkel JG. IgA Fc receptors. *Annu Rev Immunol*. 2003;21:177-204.
238. Dechant M VT. IgA antibodies for cancer therapy. *Crit Rev Oncol Hematol*. 2001;39:69-77.
239. Lohse S, Derer S, Beyer T, et al. Recombinant dimeric IgA antibodies against the epidermal growth factor receptor mediate effective tumor cell killing. *J Immunol*. 2011;186(6):3770-3778.
240. Lohse S, Loew S, Kretschmer A, et al. Effector mechanisms of IgA antibodies against CD20 include recruitment of myeloid cells for antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. *Br J Haematol*. 2018;181(3):413-417.

241. Aleyd E, Heineke MH, van Egmond M. The era of the immunoglobulin A Fc receptor FcαRI; its function and potential as target in disease. *Immunol Rev*. 2015;268(1):123-138.
242. Borrok MJ, Luheshi NM, Beyaz N, et al. Enhancement of antibody-dependent cell-mediated cytotoxicity by endowing IgG with FcalphaRI (CD89) binding. *MAbs*. 2015;7(4):743-751.
243. Brandsma AM, Bondza S, Evers M, et al. Potent Fc Receptor Signaling by IgA Leads to Superior Killing of Cancer Cells by Neutrophils Compared to IgG. *Front Immunol*. 2019;10:704.
244. Otten MA, Rudolph E, Dechant M, et al. Immature neutrophils mediate tumor cell killing via IgA but not IgG Fc receptors. *J Immunol*. 2005;174(9):5472-5480.
245. Otten MA, Leusen JH, Rudolph E, et al. FcR gamma-chain dependent signaling in immature neutrophils is mediated by FcalphaRI, but not by FcgammaRI. *J Immunol*. 2007;179(5):2918-2924.
246. Boross P, Lohse S, Nederend M, et al. IgA EGFR antibodies mediate tumour killing in vivo. *EMBO Mol Med*. 2013;5(8):1213-1226.
247. Lohse S, Meyer S, Meulenbroek LA, et al. An Anti-EGFR IgA That Displays Improved Pharmacokinetics and Myeloid Effector Cell Engagement In Vivo. *Cancer Res*. 2016;76(2):403-417.
248. Kim J, Hayton WL, Robinson JM, Anderson CL. Kinetics of FcRn-mediated recycling of IgG and albumin in human: pathophysiology and therapeutic implications using a simplified mechanism-based model. *Clin Immunol*. 2007;122(2):146-155.
249. Meyer S, Nederend M, Jansen JH, et al. Improved in vivo anti-tumor effects of IgA-Her2 antibodies through half-life extension and serum exposure enhancement by FcRn targeting. *MAbs*. 2016;8(1):87-98.
250. Treffers LW, ten Broeke T, Rosner T, et al. IgA-Mediated Killing of Tumor Cells by Neutrophils Is Enhanced by CD47-SIRPalpha Checkpoint Inhibition. *Cancer Immunol Res*. 2020;8(1):120-130.
251. Jefferis R. Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. *Trends Pharmacol Sci*. 2009;30(7):356-362.
252. Pereira NA, Chan KF, Lin PC, Song Z. The "less-is-more" in therapeutic antibodies: Afucosylated anti-cancer antibodies with enhanced antibody-dependent cellular cytotoxicity. *MAbs*. 2018;10(5):693-711.
253. Scallon BJ, Tam SH, McCarthy SG, Cai AN, Raju TS. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. *Mol Immunol*. 2007;44(7):1524-1534.
254. Thomann M, Schlothauer T, Dashivets T, et al. In vitro glycoengineering of IgG1 and its effect on Fc receptor binding and ADCC activity. *PLoS One*. 2015;10(8):e0134949.
255. Shields RL, Lai J, Keck R, et al. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J Biol Chem*. 2002;277(30):26733-26740.
256. Ferrara C, Grau S, Jager C, et al. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcgammaRIII and antibodies lacking core fucose. *Proc Natl Acad Sci U S A*. 2011;108(31):12669-12674.
257. Derer S, Glorius P, Schlaeth M, et al. Increasing FcγRIIa affinity of an FcγRIII-optimized anti-EGFR antibody restores neutrophil-mediated cytotoxicity. *MAbs*. 2014;6(2):409-421.

CHAPTER 3

G-CSF AS A SUITABLE ALTERNATIVE TO GM-CSF TO BOOST DINUTUXIMAB-MEDIATED NEUTROPHIL CYTOTOXICITY IN NEUROBLASTOMA TREATMENT

Paula Martinez-Sanz*, Dieke J. van Rees*, Lieke M. J. van Zogchel, Bart Klein, Panagiota Bouti, Hugo Olsman, Karin Schornagel, Ivana Kok, Ali Sunak, Kira Leeuwenburg, Ilse Timmerman, Miranda P. Dierselhuis, Waleed M. Kholosy, Jan J. Molenaar, Robin van Bruggen, Timo K. van den Berg, Taco W. Kuijpers, Hanke L. Matlung, Godelieve A. M. Tytgat, Katka Franke

* Authors contributed equally

Journal for ImmunoTherapy of Cancer 2021 May 28; 9(5):e002259

ABSTRACT

Background: Current immunotherapy for patients with high-risk neuroblastoma involves the therapeutic antibody dinutuximab that targets GD2, a ganglioside expressed on the majority of neuroblastoma tumors. Opsonized tumor cells are killed through antibody-dependent cellular cytotoxicity (ADCC), a process mediated by various immune cells, including neutrophils. The capacity of neutrophils to kill dinutuximab-opsonized tumor cells can be further enhanced by granulocyte-macrophage colony-stimulating factor (GM-CSF), which has been shown in the past to improve responses to anti-GD2 immunotherapy. However, access to GM-CSF (sargramostim) is limited outside of Northern America, creating a high clinical need for an alternative method to stimulate dinutuximab responsiveness in the treatment of neuroblastoma. In this *in vitro* study, we have investigated whether clinically well-established granulocyte colony-stimulating factor (G-CSF) can be a potentially suitable alternative for GM-CSF in the dinutuximab immunotherapy regimen of patients with neuroblastoma.

Methods: We compared the capacity of neutrophils stimulated either *in vitro* or *in vivo* with GM-CSF or G-CSF to kill dinutuximab-opsonized GD2-positive neuroblastoma cell lines and primary patient tumor material. Blocking experiments with antibodies inhibiting either respective Fc gamma receptors (FcγR) or neutrophil integrin CD11b/CD18 demonstrated the involvement of these receptors in the process of ADCC. Flow cytometry and live cell microscopy were used to quantify and visualize neutrophil-neuroblastoma interactions.

Results: We found that G-CSF was as potent as GM-CSF in enhancing the killing capacity of neutrophils towards neuroblastoma cells. This was observed with *in vitro* stimulated neutrophils, and with *in vivo* stimulated neutrophils from both patients with neuroblastoma and healthy donors. Enhanced killing due to GM-CSF or G-CSF stimulation was consistent regardless of dinutuximab concentration, tumor-to-neutrophil ratio and concentration of the stimulating cytokine. Both GM-CSF and G-CSF stimulated neutrophils required FcγRIIa and CD11b/CD18 integrin to perform ADCC, and this was accompanied by trogocytosis of tumor material by neutrophils and tumor cell death in both stimulation conditions.

Conclusions: Our preclinical data support the use of G-CSF as an alternative stimulating cytokine to GM-CSF in the treatment of high-risk neuroblastoma with dinutuximab, warranting further testing of G-CSF in a clinical setting.

INTRODUCTION

Neuroblastoma, a tumor originating from the early embryonic neural crest, is the most common extracranial solid tumor diagnosed in children. The median age at diagnosis is 19 months and it accounts for almost 15% of all cancer-related mortality in children[1]. The prognosis and treatment options for this neuroendocrine tumor, generally arising in the adrenal glands and sympathetic ganglia, are determined by the stage of the disease. For very low-risk, low-risk and intermediate-risk categories—as classified by the International Neuroblastoma Risk Group, which uses molecular, pathological as well as clinical criteria for patient classification[2] – the risk of recurrence is minimal. However, the prognosis for high-risk neuroblastoma remains poor despite intensive multimodal treatment comprising surgery, chemotherapy, myeloablative therapy with stem cell rescue and radiotherapy[3,4]. In 2015, the Food and Drug Administration approved the addition of the therapeutic antibody dinutuximab to the maintenance phase of the treatment protocol for patients with high-risk neuroblastoma (**Suppl. Figure 1**). This combination of dinutuximab with the existing multimodal treatment increased the survival rate from high-risk neuroblastoma to 50%[5]. Nonetheless, still half of the patients relapse and succumb to the tumor. Increasing the efficacy of dinutuximab is therefore of utmost importance.

The chimeric monoclonal antibody dinutuximab binds GD2, a ganglioside present on the surface of neuroblastoma cells. Upon binding, dinutuximab marks the cells for immune-mediated destruction via antibody-dependent cellular cytotoxicity (ADCC) by Fc gamma receptor (FcγR) expressing immune cells[6,7]. Natural killer (NK) cells and macrophages play a prominent role in mediating ADCC in diverse cancer types. In neuroblastoma, however, neutrophils have been described as the major cell population involved in dinutuximab-mediated killing of neuroblastoma cells *in vitro*[8]. The cytotoxic activity of dinutuximab can be significantly enhanced when given in combination with cytokines that specifically stimulate the activity of immune cells. Particularly, granulocyte-macrophage colony-stimulating factor (GM-CSF), stimulating neutrophils and macrophages, and interleukin-2 (IL-2), stimulating NK cells, were demonstrated to positively contribute to the efficacy preclinically[8–10] and also in clinical trials[11,12].

Notably, a partially randomized phase III trial showed a survival benefit for patients with high-risk neuroblastoma treated during alternating cycles of dinutuximab combined with GM-CSF or IL-2, and isotretinoin, as compared with patients treated with isotretinoin alone (COG ANBL0032 study)[12]. This trial led to the standardization of this combination regimen for the maintenance phase in the treatment protocol for patients with high-risk neuroblastoma in the USA (**Suppl. Figure 1**)[5,13]. However, the addition of IL-2 has later been shown to bring minimal clinical improvement for dinutuximab-treated patients with neuroblastoma[14,15], and access to GM-CSF for clinical use outside of Northern America is

limited[16]. Therefore, the immunotherapy regimen for patients with high-risk neuroblastoma in Europe is not fully defined with regard to the cytokine administration, resulting in a potentially increased risk of suboptimal treatment.

In this study, we aim at providing preclinical evidence for the use of an alternative stimulating cytokine if GM-CSF is unavailable, to ultimately increase dinutuximab responsiveness in the treatment protocol of neuroblastoma. Based on the known ability of granulocyte colony-stimulating factor (G-CSF) to enhance neutrophil-mediated ADCC[17–20], we propose the use of this clinically well-established, and broadly available cytokine. Furthermore, we investigated the potentially negative effect of G-CSF on neuroblastoma cells as an additional safety measure, since several studies suggested G-CSF treatment of chemotherapy-induced neutropenia to cause alterations in tumor cell phenotype, promoting neuroblastoma tumorigenicity[21–23]. Our preclinical data on G-CSF efficacy in neutrophil activity against neuroblastoma support the clinical use of G-CSF as it potentiates neutrophil-mediated ADCC of dinutuximab-opsonized neuroblastoma cells to the same extent as GM-CSF.

MATERIALS AND METHODS

Patients, controls and samples

For *in vivo* GM-CSF stimulated neutrophils, remnant heparinized blood was used from patients with high-risk neuroblastoma at the Princess Máxima Center during the different GM-CSF cycles of the dinutuximab treatment protocol (specified in **Suppl. Table 1** and **Suppl. Figure 1**), for which Biobank approval was obtained. These patients received GM-CSF as part of the immunotherapy regimen, which was given as maintenance therapy after induction and consolidation phases of the treatment protocol, according to the COG ANBL0032 study. Here, GM-CSF (250 μg/m^2/day, sargramostim, Leukine) was administered subcutaneously in course 1, 3 and 5 for 14 days. After the first three consecutive GM-CSF doses, the blood was sampled prior to the fourth dose of GM-CSF and before dinutuximab treatment started. As control, healthy unrelated donor neutrophils were used.

For *in vivo* G-CSF stimulated neutrophils, heparinized blood was collected at Sanquin from granulocyte transfusion donors ~30 hour after subcutaneous injection of 10 μg/kg clinical grade G-CSF (filgrastim, Neupogen). As control, heparinized blood was collected from healthy unrelated volunteers, as well as at least 3 weeks later (when G-CSF is cleared from circulation)

Heparinized peripheral blood samples from healthy volunteers were obtained at Sanquin and this was approved by the Sanquin Research Institutional Ethical Committee.

For *in vivo* G-CSF stimulated neutrophils, heparinized blood was collected at Sanquin from granulocyte transfusion donors ~30 hour after subcutaneous injection of 10 µg/kg clinical grade G-CSF (filgrastim, Neupogen). As control, heparinized blood was collected from healthy unrelated volunteers, as well as at least 3 weeks later (when G-CSF is cleared from circulation)[24–27] from the same G-CSF injected healthy donor.

Ethics approval

The parts of the study involving human participants were reviewed and approved by Sanquin Research Institutional Ethical Committee and Princess Maxima Center Biobank. All blood samples were obtained after informed consent and according to the Declaration of Helsinki 1964. The patients and participants provided informed consent to participate in this study.

Neutrophil isolation and *in vitro* stimulation

Heparinized peripheral blood was diluted 1:2 with phosphate-buffered saline (PBS)+10% trisodium citrate and separated by density gradient centrifugation over isotonic Percoll (1.076 g/mL, GE Healthcare). The pellet fraction, containing both erythrocytes and granulocytes, underwent erythrocyte lysis with ice cold hypotonic ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in water). After isolation, 5×10^6/mL neutrophils were resuspended in 4-(2-hydroxyethyl)−1-piperazineethanesulfonic acid supplemented with 5 g/L human albumin (Albuman, Sanquin Plasma Products), 1 mM CaCl and 5.5 mM glucose[28].

Neutrophils were either used directly after isolation (referred to as unstimulated neutrophils) or were stimulated overnight at 37°C and 5% CO_2 with either 10 ng/mL recombinant human GM-CSF (Peprotech) or 10 ng/mL clinical grade G-CSF (Neupogen), unless otherwise specified. After overnight incubation, the percentage of apoptotic cells was determined using Annexin V staining (BD Biosciences) to correct for the number of viable neutrophils (the percentage of apoptotic neutrophils typically ranged between 10% and 30%) prior to any experiments.

Cell culture

The human neuroblastoma cell lines NMB, IMR-32 and LAN-1 were obtained from the Leibniz Institute, Germany. These cell lines were routinely cultured at 37°C and 5% CO_2 and maintained in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 20% of heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (further referred to as IMDM complete medium) for a maximum of 30 passages. NMB cells were harvested using trypsin; IMR-32 and LAN-1 cells were harvested by tapping the culture flask and resuspending the culture medium. The human

neuroblastoma cell lines SHEP-2, SK-N-AS, SH-SY5Y and SK-N-BE (kindly provided by the Department of Oncogenomics, Amsterdam UMC) were routinely cultured at 37°C and 5% CO_2 and maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) completed with 20% of FCS, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin for maximum of 30 passages. These cells grew adherent as well as in suspension and were harvested by collecting supernatant as well as by using trypsin.

Primary patient-derived neuroblastoma cells

The primary patient-derived neuroblastoma spheroid line AMC691B (further referred to as 691B) was derived from a bone marrow metastasis (B) of patient 691[29]. 691B cells grow in spheroids and were cultured and maintained in DMEM with low glucose and sodium pyruvate (Invitrogen) supplemented with 25% Ham's F12 nutrient mixture (Invitrogen), 1× B-27 supplement minus vitamin A (50×, Gibco), 1× N-2 supplement (100×, Gibco), 20 ng/mL animal-free human epidermal growth factor (Peprotech), 40 ng/mL human basic fibroblast growth factor (Peprotech), 200 ng/mL human insulin-like growth factor (Peprotech), 10 ng/mL human platelet-derived growth factor (PDGF)-AA (Peprotech), 10 ng/mL human PDGF-BB (Peprotech), 100 units/mL penicillin and 100 µg/mL streptomycin for maximum of 24 passages. To obtain a single-cell suspension, cells were treated with Accutase solution for 5 min (Sigma-Aldrich).

Chromium-based ADCC assay

Target cells (1×10^6) were labeled with 100 µCi ^{51}Cr (PerkinElmer) for 90 min at 37°C and washed with PBS. Chromium-labeled target cells (5×10^3) were co-incubated with neutrophils in a 96-well U-bottom plate (Corning) in the absence or presence of dinutuximab (Unituxin, Ch14.18, United Therapeutics) in the appropriate culture medium for 4 hours at 37°C and 5% CO_2. A target:effector (T:E) ratio of 1:50 (*i.e.* 5000:250,000 cells) and a final concentration of 0.5 µg/mL of dinutuximab were used, unless specified otherwise. Spontaneous and maximum ^{51}Cr release were determined by incubating the target cells without effector cells and by treating the target cells with a 0.1% Triton X-100 solution in culture medium, respectively. After incubation, 30 µL of supernatant was subsequently transferred to Lumaplates (PerkinElmer). The plates were dried overnight at room temperature and analyzed in a MicroBeta^2 plate reader (PerkinElmer). The percentage of cytotoxicity was calculated as: [(experimental counts per minute (CPM))−spontaneous CPM)/(maximum CPM−spontaneous CPM)]×100%. All conditions were performed in triplicate.

For Fcγ receptor blocking experiments, $F(ab')_2$ fragments against FcγRIIa (CD32, clone 7.3, Ancell) or FcγRIIIb (CD16, clone 3G8, Ancell) were used and compared with isotype control

mIgG1 F(ab')$_2$ fragments (clone MOPC 31C, Ancell). Using purified human IgG Fc fragments (Bethyl, USA), we aimed to saturate the high-affinity FcγRI. Blocking reagents were pre-incubated with neutrophils at 10 µg/mL for 45 min at room temperature. Subsequently, the effector cells were used in the chromium-based ADCC assay.

For integrin blocking experiments, F(ab')$_2$ fragments against CD18 (clone IB4, Ancell) were pre-incubated with neutrophils at 10 µg/mL for 15 min at room temperature, after which the cells were used in the chromium-based ADCC assay.

Trogocytosis assay

The trogocytosis of neuroblastoma cells by neutrophils was quantified using flow cytometry and measured by the uptake of tumor cell membrane by the neutrophils. Tumor cells were stained with 2 µM lipophilic membrane dye 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO, Invitrogen); neutrophils were labeled with 0.625 ng Calcein Violet-AM (Invitrogen) for 30 min at 37°C. After labeling, populations were washed twice with PBS. Cells were co-incubated at a T:E ratio of 1:5 (*i.e.* 50,000:250,000 cells) in the absence or presence of 0.5 µg/mL dinutuximab in a U-bottom 96-well plate (Greiner Bio-One) for 60 min at 37°C and 5% CO_2 in IMDM complete medium. After incubation, cells were fixed with STOPbuffer (PBS containing 20 mM sodium fluoride, 0.5% paraformaldehyde (PFA) and 1% bovine serum albumin) and analyzed using flow cytometer Canto II (BD Biosciences). The neutrophil population (all Calcein Violet-AM$^+$ events) was assessed for the mean fluorescence intensity of membrane dye DiO. Data were analyzed with FlowJo software (V.10.6.1, Becton Dickinson, Ashland, Oregon, USA).

Live cell imaging

NMB target cells labeled with 5 µM lipophilic membrane dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD, Invitrogen) and 2.5 nM cytoplasmic dye Calcein Red-Orange-AM (ThermoFisher) were co-incubated with unstained neutrophils at a T:E ratio of 1:5 in glass chambered coverslips (Ibidi) of 9.4×10.7×6.8 mm^3 well dimensions. Two drops of the DNA-binding Nuc-Green dye were added in the extracellular medium before imaging. Cells were co-incubated for periods up to 4 hours at 37°C and 5% CO_2 in the presence of 0.5 µg/mL dinutuximab in IMDM complete medium. Imaging was performed within 5 min after co-incubation of tumor cells and neutrophils and lasted up to 210 min using a Leica TCM SP8 confocal microscope (Leica).

Flow cytometry staining

For neutrophil characterization, cells were stained with 10 µg/mL fluorescein isothiocyanate (FITC)-labeled antibodies: anti-FcγRI (CD64, clone 10.1, Bio-Rad), anti-FcγRII (CD32, clone

AT10, Bio-Rad), anti-FcγRIII (CD16, clone 3G8, BD Biosciences), anti-CD11b (clone CLB-mon-gran/1, B2, Sanquin Reagents) and anti-CD18 (clone MEM48, Diaclone).

For target cell characterization, human anti-GD2 antibody dinutuximab (Unituxin, Ch14.18, United Therapeutics) was used to quantify GD2 expression by titrating dinutuximab from 10 µg/mL to 0.001 µg/mL. Secondary antibody Alexa Fluor 647 goat antihuman IgG $F(ab')_2$ fragment (Jackson ImmunoResearch) was used for detection. To determine expression of G-CSF receptor on neuroblastoma cells, 20 µg/mL of anti-CD114 PE-Cyanine7 (BD Biosciences) was used. Cell viability of tumor cells was determined using Hoechst 33342 solution (Invitrogen). All incubations took place for 20 min on ice in the dark. The appropriately labeled IgG isotypes were used to correct for any potential background. After washing, cells were resuspended in PBS supplemented with 6 g/L human albumin (Albuman, Sanquin Plasma Products) and fluorescence was measured on a Canto II flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (V.10.6.1).

Effect of G-CSF on JAK/STAT3 pathway

Tumor cell samples were exposed to 10 ng/mL G-CSF (Neupogen) for 0, 5, 10 and 20 min. Hereafter, cells were fixed with 4% PFA, permeabilized with ice-cold 90% methanol and stained with fluorescently labeled antibodies for total STAT3-PerCp-Cyanine5.5 (BD Biosciences) and phospho-STAT3-PE (pSTAT3, BD Biosciences) as previously described[30]. Neutrophils were used as control in this setting. Fluorescence was measured on a Canto II flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (V.10.6.1).

Effect of G-CSF on neuroblastoma cell proliferation rates

Tumor cells were cultured in the presence or absence of 10 ng/mL clinical grade G-CSF (Neupogen) in the appropriate culture medium for 1–3 weeks. The medium supplemented with cytokine was refreshed twice a week where applicable; 0.3×10^6 IMR-32 cells or 0.5×10^6 691B cells were plated in each well of a 6-well plate (Corning) and the proliferation rate of these cultures was determined by counting the cells using a CASY Cell Counter (Roche Innovatis). The population doubling time of G-CSF-treated cultures was calculated with a doubling time calculator (www.doubling-time.com/compute.php).

RNA isolation, cDNA synthesis and RT-quantitative PCR

Total RNA was extracted from neuroblastoma cell lines on days 0, 7, 14 and 21 after G-CSF exposure by using the QIAamp RNA Blood Mini Kit (Qiagen) according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 2 to 3 µg RNA, using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems), as described previously[31]. Quantitative PCR for reference gene beta-glucoronidase (*GUSB*), adrenergic neuroblastoma markers[32,33] paired-like homeobox 2b (*PHOX2B*), cholinergic receptor nicotinic alpha

3 (*CHRNA3*), dopamine beta hydroxylase (*DBH*) and tyrosine hydroxylase (*TH*), and mesenchymal neuroblastoma markers[34] paired related homeobox 1 (*PRRX1*) and periostin (*POSTN*) was performed using the Viia7 (Applied Biosystems) as previously described[31]. Normalization for expression was based on the expression of *GUSB* with the equation: normalized threshold cycle (dCt)=(Ct_{marker}−Ct_{GUSB}). All reactions were performed in triplicate (except *GUSB*, which was performed in duplicate) and mean values were used. As a positive control, a calibration curve of neuroblastoma cell line IMR-32 was used for the adrenergic markers, plasmids were used for *GUSB* and the mesenchymal panel to establish the PCR efficiency.

Statistical analysis

Differences between groups were assessed using GraphPad Prism 8. Specific test and number of individual biological replicates (n) are indicated in figure legends for each experiment. When p values were ≤ 0.05, differences were deemed significant; error bars indicate the SEM.

RESULTS

GM-CSF and G-CSF equally enhance neutrophil-mediated ADCC of neuroblastoma cells

To compare the effect of GM-CSF with G-CSF on the tumor cell killing capacity of neutrophils, we performed ADCC experiments with various GD2-positive and GD2-negative neuroblastoma cells (all cell line characteristics are summarized in **Suppl. Table 1**, titration of dinutuximab depicted in **Suppl. Figure 2A–C**) using neutrophils that were unstimulated or stimulated overnight with the respective cytokines *in vitro*. Unstimulated neutrophils were not able to kill dinutuximab-opsonized GD2-positive neuroblastoma cell lines NMB, IMR-32 and LAN-1, whereas stimulation of neutrophils either with GM-CSF or G-CSF enhanced the cytotoxicity levels significantly, with neither cytokine being superior to the other (**Figure 1A**). The effect of either GM-CSF or G-CSF stimulation on the killing efficacy remained equal, irrespective of the concentration of dinutuximab tested (**Figure 1B** and **Suppl. Figure 3A**), different T:E ratios (**Figure 1C** and **Suppl. Figure 3B**) or the various concentrations of GM-CSF and G-CSF used to stimulate neutrophils (**Figure 1D** and **Suppl. Figure 3C**). The high concentrations of dinutuximab (**Figure 1B**, exceeding 5 µg/mL) led to decreased cytotoxicity, probably due to the formation of immune complexes. We observed no killing of GD2-negative neuroblastoma cell lines SH-SY5Y and SK-N-BE in the presence of dinutuximab, regardless of the stimulating cytokine used (**Suppl. Figure 2D**). Neuroblastoma cell lines SHEP-2 and SK-N-AS, expressing lower levels of GD2 (**Suppl. Figure 2B**), were also killed by stimulated neutrophils irrespective of the cytokine used (**Figure 1E**), although at lower levels than the GD2-positive cell lines NMB, IMR-32 and LAN-1 (**Figure 1A**). To test the ability of both cytokines to promote neutrophil-mediated

killing of primary patient material, we used GM-CSF and G-CSF stimulated neutrophils in an ADCC assay with GD2 expressing 691B cells derived from bone marrow metastasis of a patient with high-risk neuroblastoma (characteristics in **Suppl. Figure 2C** and **Suppl. Table 1**). Both stimuli induced effective killing of primary tumor cells and no differences were seen between the two cytokines (**Figure 1F**). Together, these observations show that the *in vitro* stimulating effect of G-CSF is as effective as GM-CSF in boosting neutrophil-mediated ADCC of neuroblastoma cells.

GM-CSF and G-CSF both mediate neutrophil ADCC through FcγRIIa and CD11b/CD18 integrins

In order to perform ADCC, neutrophils need expression of Fcγ receptors (neutrophils can express FcγRI, FcγRIIa/c and FcγRIIIb) and CD11b/CD18 integrins[35,36]. As stimulation with GM-CSF or G-CSF enhances tumor cell killing (**Figure 1**), we explored whether the killing of tumor cells by differently stimulated neutrophils required presence of these molecules in a similar fashion. Freshly isolated, unstimulated, neutrophils constitutively express low-affinity and intermediate-affinity FcγRIIa or FcγRIIc (CD32a/c) and FcγRIIIb (CD16b), respectively. It has been shown that on stimulation with G-CSF neutrophils upregulate the expression of FcγRI (CD64) and shed FcγRIIIb[18,37,38]. We showed that overnight stimulation of neutrophils with either GM-CSF or G-CSF *in vitro* significantly lowered FcγRIIIb expression compared with unstimulated neutrophils, whereas FcγRIIa expression remained unaltered on stimulation. FcγRI expression increased slightly but this was not statistically significant. No differences in neutrophil Fcγ receptor levels were observed between GM-CSF and G-CSF stimulation (**Figure 2A-B**). Next to this, we investigated which Fcγ receptor(s) neutrophils need to facilitate killing of neuroblastoma cells. Previous studies showed that FcγRIIa is the major Fcγ receptor that mediates the killing of antibody-opsonized solid cancer cells[35,36,39]. Indeed, blocking FcγRIIa using $F(ab')_2$ fragments reduced ADCC of dinutuximab-opsonized NMB cells for both GM-CSF and G-CSF overnight stimulated neutrophils, while blocking of FcγRI or FcγRIIIb had no effect on the killing capacity of neutrophils, regardless of the stimulating cytokine used (**Figure 2C**).

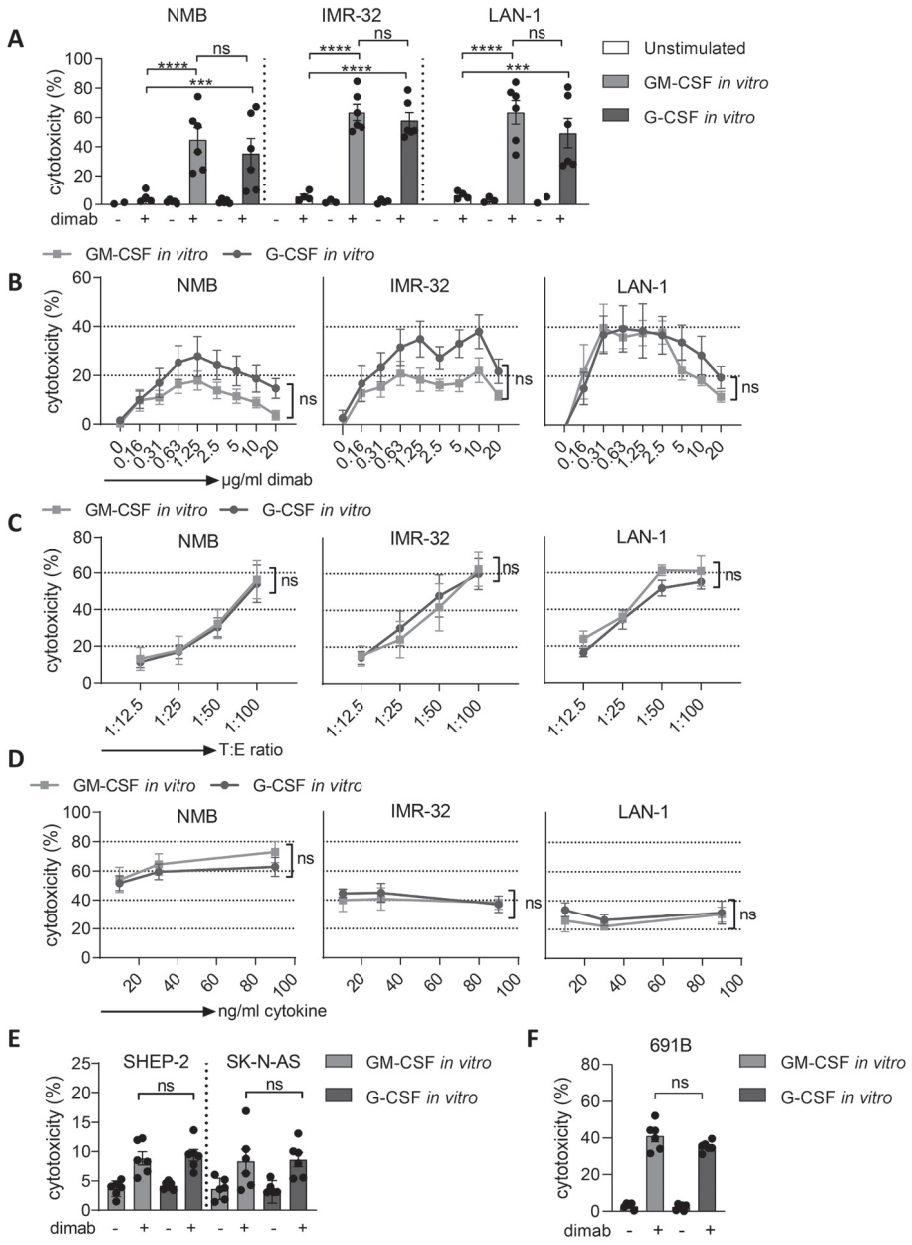


Figure 1. GM-CSF and G-CSF equally enhance neutrophil-mediated ADCC of neuroblastoma cells.
(A) *ADCC of NMB, IMR-32 and LAN-1 cells opsonized with (+) or without (–) dinutuximab (dimab) by unstimulated neutrophils (white bars) or stimulated in vitro with GM-CSF (light gray bars) or G-CSF (dark gray bars). NMB n=6, IMR-32 n=4–6, LAN-1 n=4–6 of three individual experiments. Statistical significance*

was tested with an ordinary one-way ANOVA with post hoc Sidak test. **(B)** *ADCC of NMB, IMR-32 and LAN-1 cells opsonized with increasing concentrations of dinutuximab, from 0 to 20 µg/mL, by neutrophils stimulated in vitro with GM-CSF (light gray squares) or G-CSF (dark gray circles). NMB n=4, IMR-32 n=6, LAN-1 n=4 of three individual experiments. Statistical differences were assessed with an unpaired T-test on AUC;* **Suppl. Figure 3A***).* **(C)** *ADCC of dinutuximab-opsonized NMB, IMR-32 and LAN-1 cells by neutrophils stimulated in vitro with GM-CSF (light gray squares) or G-CSF (dark gray circles) at different T:E ratios ranging from 1:12.5 to 1:100. NMB n=4, IMR-32 n=4, LAN-1 n=6 of three individual experiments. Statistical differences were tested with an unpaired T-test on AUC (***Suppl. Figure 3B***).* **(D)** *ADCC of dinutuximab-opsonized NMB, IMR-32 and LAN-1 cells by neutrophils stimulated in vitro with increasing concentrations of GM-CSF (light gray squares) or G-CSF (dark gray circles), from 10 to 90 ng/mL. NMB n=4, IMR-32 n=4, LAN-1 n=4 of two individual experiments. Statistical significance was assessed with an unpaired T-test on AUC (***Suppl. Figure 3C***).* **(E)** *ADCC of SHEP-2 and SK-N-AS cells opsonized with (+) or without (–) dinutuximab (dimab) by neutrophils stimulated in vitro with GM-CSF (light gray bars) or G-CSF (dark gray bars). SHEP-2 n=6, SK-N-AS n=6 of three individual experiments. Statistical differences were assessed with an unpaired T-test.* **(F)** *ADCC of primary patient-derived 691B cells opsonized with (+) or without (–) dinutuximab (dimab) by neutrophils stimulated in vitro with GM-CSF (light gray bars) or G-CSF (dark gray bars). N=6 of three individual experiments. Statistical significance was tested with an unpaired T-test. ADCC, antibody-dependent cellular cytotoxicity; ANOVA, analysis of variance; AUC, areas under curve; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; ns, not significant. ***p<0.001; ****p<0.0001.*

In addition to Fcγ receptors, neutrophils need functional expression of the heterodimer integrin CD11b/CD18 to perform ADCC[35,38,40]. After overnight *in vitro* stimulation with either GM-CSF or G-CSF, the expression of CD11b and CD18 remained similar compared to unstimulated neutrophils (**Figure 2A-B**). When blocking CD18 using $F(ab')_2$ fragments, known to inhibit CD11b/CD18 integrin function[35], tumor cell killing was abolished similarly for both GM-CSF and G-CSF stimulated neutrophils (**Figure 2D**), suggesting that both cytokines stimulate neutrophils to kill neuroblastoma cells through functional CD11b/CD18 integrins.

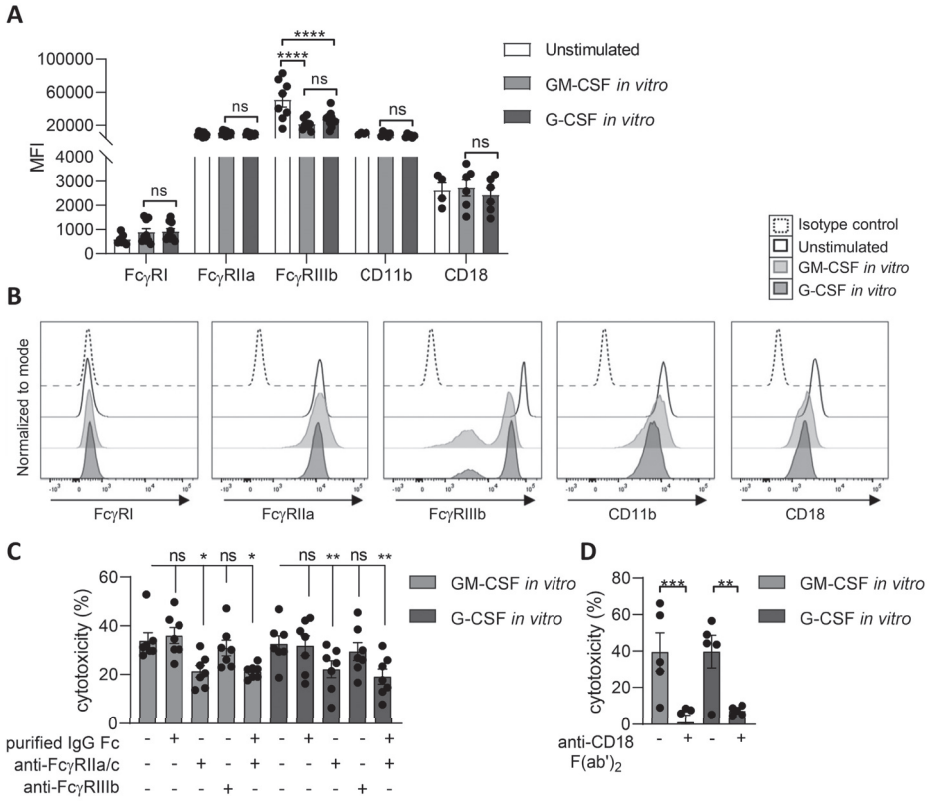


Figure 2. GM-CSF and G-CSF both mediate neutrophil ADCC through FcγRIIa and CD11b/CD18 integrins. (A) *Fcγ receptor and CD11b/CD18 integrin expression (expressed as MFI) on neutrophils after in vitro GM-CSF (light gray bars) or G-CSF (dark gray bars) stimulation compared with unstimulated neutrophils (white bars). FcγRI n=7–9, FcγRIIa n=8–10, FcγRIIIb n=8–10, CD11b n=4–6, CD18 n=4–6 of five individual experiments. Statistical differences were tested with ordinary one-way ANOVA with post hoc Dunnett test.* **(B)** *Representative histogram of flow cytometry analysis of (from left to right) FcγRI, FcγRIIa, FcγRIIIb, CD11b and CD18 expression on unstimulated neutrophils (in white), and neutrophils stimulated in vitro with GM-CSF (in light gray) or G-CSF (in dark gray). The dashed line depicts an isotype control.* **(C)** *ADCC of dinutuximab-opsonized NMB cells by in vitro stimulated neutrophils with GM-CSF (light gray bars) or G-CSF (dark gray bars). Fcγ receptors are blocked or saturated (indicated with +) using F(ab')2 fragments against FcγRIIIb and FcγRIIa or purified IgG Fc tails, respectively. N=7 of four individual experiments. Statistical significance was tested with ordinary one-way ANOVA with post hoc Sidak test.* **(D)** *ADCC of dinutuximab-opsonized NMB cells by in vitro stimulated with GM-CSF (light gray bars) or G-CSF (dark gray bars) without (–) or with (+) CD18 integrin block with F(ab')2 fragments. N=5 of three individual experiments. Statistical differences with ordinary one-way ANOVA with post hoc Sidak test. ADCC, antibody-dependent cellular cytotoxicity; ANOVA, analysis of variance; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MFI, mean fluorescence intensity; ns, not significant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.*

GM-CSF and G-CSF stimulation induce trogocytosis of neuroblastoma cells by neutrophils and is accompanied by tumor cell death

The ability of immune cells to perform trogocytosis, an active mechanism involving the uptake of plasma membrane from a donor cell, is well-known[41]. In the recent years, it has become clear that trogocytosis can also be a cytotoxic mechanism, at least in the context of antibody-dependent tumor cell killing by myeloid cells[42]. More specifically, neutrophil-mediated trogocytosis in which the neutrophil takes 'bites' from the plasma membrane of antibody-opsonized cancer cells can result in cancer cell lysis, also known as trogoptosis. This has been described for trastuzumab-opsonized breast cancer cells and it has been shown to be dependent on functional FcγRIIa and CD11b/CD18 integrins[36]. To investigate whether GM-CSF and G-CSF stimulated neutrophils could trogocytose neuroblastoma cells, we performed a FACS-based trogocytosis assay where we labeled overnight stimulated neutrophils with Calcein Violet-AM and freshly harvested NMB cells with the membrane dye DiO (**Figure 3A**). In conditions with dinutuximab, neutrophils stimulated with either GM-CSF or G-CSF became positive for the membrane dye DiO, indicative of trogocytosis (**Figure 3A-B**), although this was significantly higher in G-CSF stimulated neutrophils. In an attempt to demonstrate whether trogocytic events by neutrophils coincided with tumor cell death, we performed live cell confocal imaging on dinutuximab-opsonized cells. We labeled NMB cells with membrane dye DiD and cytoplasmic dye Calcein Red-Orange-AM, which were co-incubated with stimulated neutrophils. As a live/dead indicator, a membrane-impermeable DNA-binding dye was added to the extracellular medium during imaging. During co-incubation of tumor cells with neutrophils stimulated overnight with either GM-CSF or G-CSF, we observed that neutrophils became positive for the neuroblastoma membrane dye, but not the cytoplasmic dye (which would indicate phagocytosis; **Figure 3C**). In addition, we found that neutrophil trogocytic interactions were followed by tumor cell death as appreciated by the staining of nuclear material exposed to the extracellular medium upon membrane disruption induced by the attacking neutrophils (**Figure 3C**). Collectively, these data support a link between trogocytic events and subsequent tumor cell death.

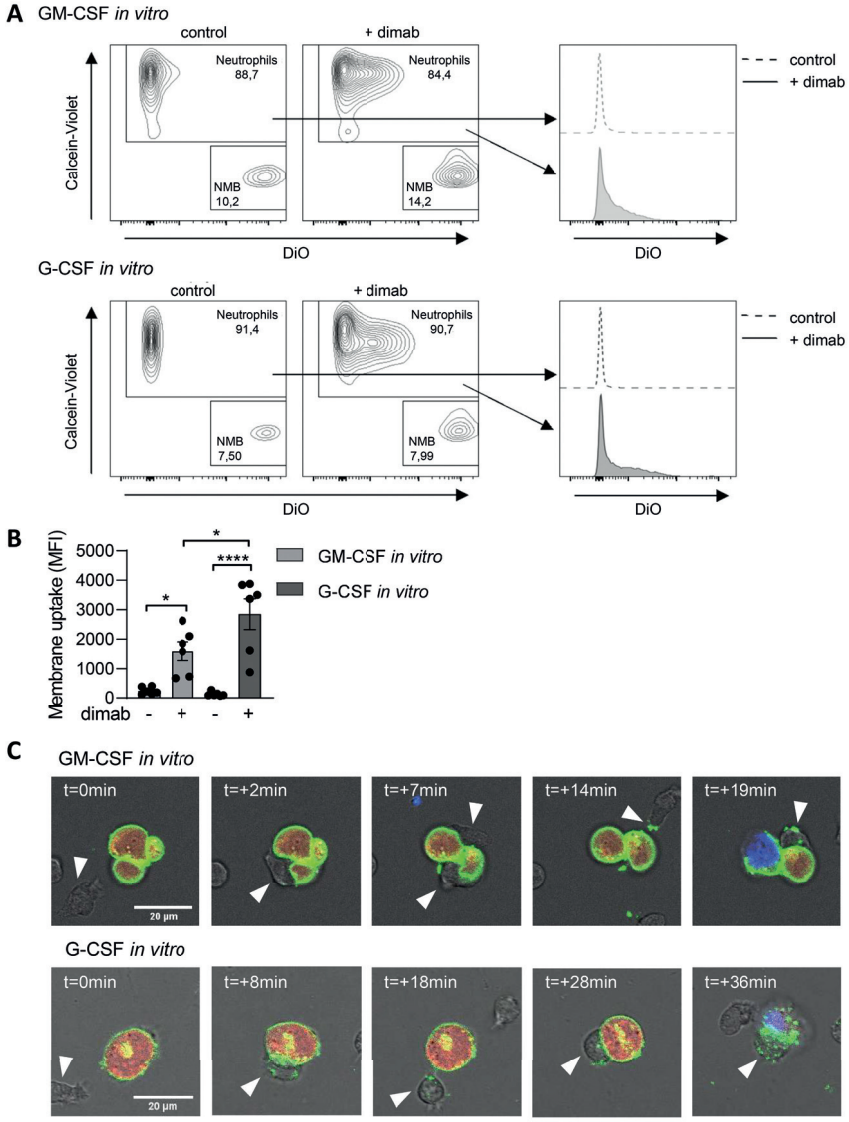


Figure 3. GM-CSF and G-CSF stimulation induce trogocytosis of neuroblastoma cells by neutrophils. (A) *Representative flow cytometry plots (left) and histogram (right) of a trogocytosis experiment, where Calcein Violet-AM-labeled neutrophils are distinguished from DiO-labeled tumor cells. Note the increase for membrane dye DiO in the neutrophils in conditions with dinutuximab (+dimab in flow cytometry plots and continuous line in histogram) as compared with conditions without dinutuximab (control in flow cytometry plots and dashed line in histogram). In vitro GM-CSF stimulated neutrophils are depicted in the top panels; in vitro G-CSF stimulated neutrophils are depicted in the bottom panels. Numbers indicated are the percentages of the mentioned populations.* **(B)** *NMB neuroblastoma membrane uptake by neutrophils*

in vitro stimulated with GM-CSF (light gray bars) or G-CSF (dark gray bars) in the presence or absence of dinutuximab (dimab). Data depict the MFI of membrane dye DiO in the neutrophil population. N=6 of three individual experiments. Statistical differences were tested with ordinary one-way ANOVA with post hoc Sidak test. **(C)** *Live cell confocal imaging stills showing neutrophils (white arrows) stimulated with GM-CSF (upper panels) or G-CSF (lower panels) in vitro taking up pieces of membrane of DiD (green) and Calcein Red-Orange-AM-labeled (orange) NMB cells, opsonized with dinutuximab. Note the uptake of the membrane label DiD only, and no uptake of the orange cytoplasmic dye by neutrophils. As an indication of cell death, note how neutrophil trogocytic interactions are directly followed by tumor cell membrane permeabilization and NucGreen staining of nuclear material (blue) once exposed to the extracellular medium. Time (min) is set to 0 from the moment a neutrophil approached the tumor cell and is indicated in the upper left corner of each still. Imaging took place within 210 min from start of the recording for all movies. Scale bar represents 20 μM. ANOVA, analysis of variance; DiD, 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate salt; DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MFI, mean fluorescence intensity.*p<0.05; ****p<0.0001.*

G-CSF treatment does not alter neuroblastoma cell phenotype *in vitro*

Currently, G-CSF is used to treat chemotherapy-induced neutropenia, often occurring in patients with high-risk neuroblastoma[43], which was shown to be an advantageous addition to the treatment protocol[44]. However, although not generally supported by clinical or *in vitro* data, previously published studies have suggested a possible role of G-CSF with regard to neuroblastoma proliferation and invasive properties of neuroblastoma cell lines[21]. Also, the tumorigenicity and metastasis formation in human xenograft and murine neuroblastoma tumor models were suggested to be enhanced in some studies[22,23]. Neuroblastoma cells express the G-CSF receptor (**Figure 4A**) and therefore we investigated whether exposure of neuroblastoma cells to G-CSF could alter their phenotype and possibly make them more resistant towards neutrophil-mediated killing. First, we investigated the effect of G-CSF on its cognate receptor signaling. Activation of STAT3 is known to take place downstream of the G-CSF receptor after ligand binding[45] and therefore we measured the level of STAT3 phosphorylation (pSTAT3) by intracellular flow cytometry staining of IMR-32 cells that were exposed to the cytokine for 0, 5, 10 or 20 min. Compared with neutrophils used as positive control, IMR-32 cells did not show any phosphorylation of STAT3, suggesting no STAT3-mediated signaling through the G-CSF receptor (**Suppl. Figure 4A-B**). To verify that no other (*i.e.* long-term) effects due to G-CSF binding to its receptor occurred, we cultured IMR-32 and patient-derived 691B neuroblastoma cells in the absence or presence of G-CSF for up to 3 weeks, as this is the period during which clinical grade pegylated G-CSF stays in circulation[24–27]. No changes in GD2 or G-CSF receptor expression were detected over time and expression remained high at all time points tested (7, 14, and 21 days; **Suppl. Figure 4C-F**). During incubation with G-CSF, the proliferation rates of tumor cell cultures were similar to control, with no proliferative

advantage of the G-CSF exposed cells (**Figure 4B** and **Suppl. Figure 4G-J**). Since IMR-32 and 691B cells have an adrenergic (epithelial) phenotype[34] (**Suppl. Table 1**), we studied whether exposure to G-CSF initiated epithelial-to-mesenchymal transition (EMT) in these cells. The transition from an adrenergic to a mesenchymal phenotype is known to increase invasive properties of tumor cells facilitating metastasis[46–48]. We performed quantitative RT-PCR on RNA samples isolated from IMR-32 and 691B cells cultured with or without G-CSF. We investigated mRNA expression of several adrenergic (*PHOX2B*, *CHRNA3*, *DBH* and *TH*) and mesenchymal (*PRRX1* and *POSTN*) markers that have been described to be specific for neuroblastoma[32–34]. Overall, no differences in mRNA expression were seen for any of the mesenchymal markers *PRRX1* and *POSTN* during G-CSF culture at any of the time points (7, 14 and 21 days) when compared to the control (untreated) condition. Also, no changes of adrenergic markers were detected, implying no signs of EMT (**Figure 4C-D**). Last, the susceptibility of G-CSF exposed neuroblastoma cells towards neutrophil ADCC was studied by co-incubating IMR-32 cells cultured for 0, 7, 14 and 21 days with G-CSF with overnight *in vitro* G-CSF stimulated neutrophils (**Figure 4E**). No differences were found in the extent of neutrophil-mediated cytotoxicity between G-CSF cultured tumor cells and control tumor cells. Altogether, these results show that *in vitro* G-CSF has no detectable effect on neuroblastoma phenotype, nor on the susceptibility of tumor cells towards neutrophil-mediated ADCC.

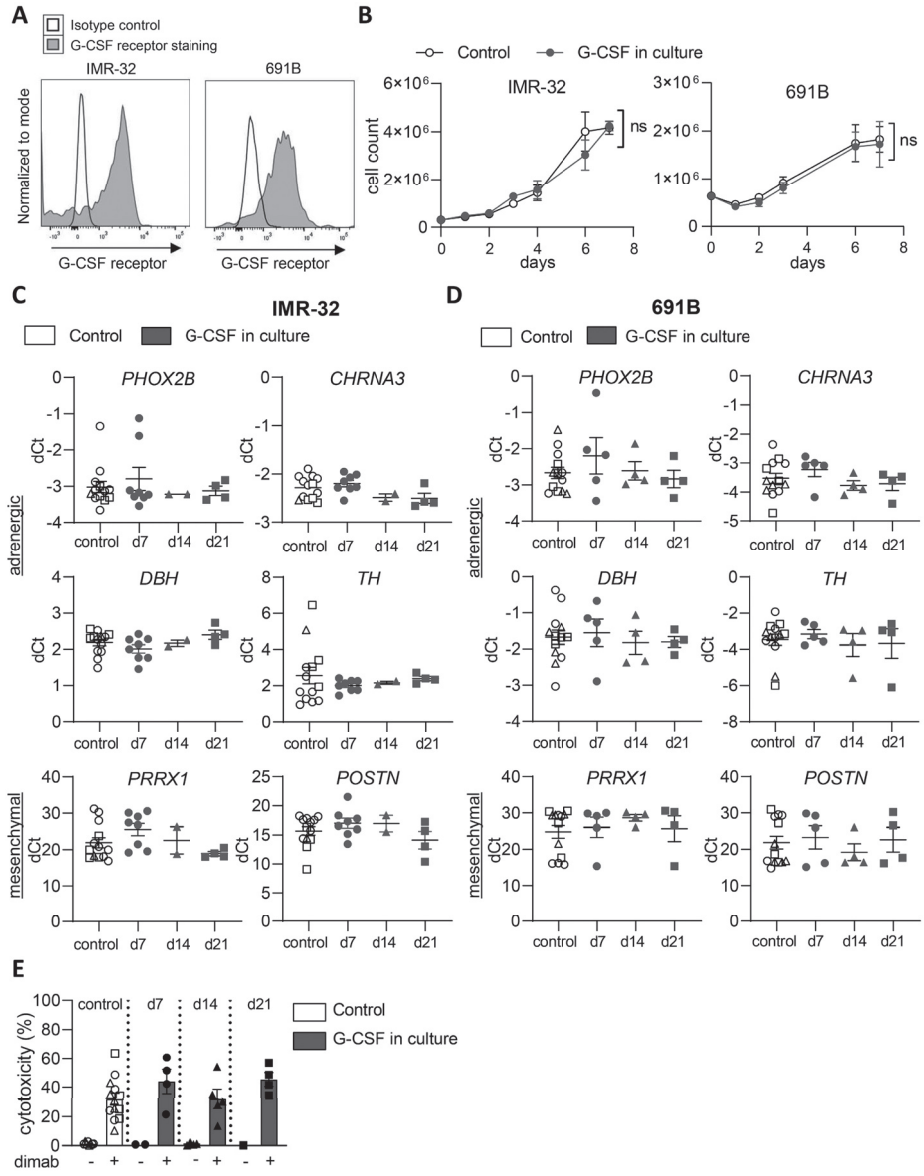


Figure 4. G-CSF treatment does not alter neuroblastoma cell phenotype. (A) *Representative histograms depicting G-CSF receptor expression (gray) on IMR-32 (left panel) and 691B cells (right panel).* **(B)** *Proliferation curves of IMR-32 (left panel) and 691B (right panel) when cultured in the absence (control, white circles) or presence (gray circles) of G-CSF for 7 days. IMR-32 n=4, 691B n=3 of three and four individual experiments, respectively. Statistical significance was assessed with a paired T-test on AUC (***Suppl. Figure 3C-D***).* **(C, D)** *Normalized expression (dCt, delta cycle threshold = Ctmarker−CtGUSB) levels of adrenergic neuroblastoma markers PHOX2B, CHRNA3, DBH and TH, as well as mesenchymal neuroblastoma markers*

PRRX1 and POSTN on IMR-32 cells (C) and patient-derived 691B cells (D) cultured in the absence (control, white symbols) or presence of G-CSF for 7 (gray circles), 14 (gray triangles) or 21 (gray squares) days. IMR-32 n=2–8, 691B n=4–5 of two individual experiments. Statistical differences were tested with ordinary one-way ANOVA with post hoc Sidak test. **(E)** *ADCC of IMR-32 cells cultured in the absence (control, white symbols) or presence of G-CSF (gray bars) for 7 (circles), 14 (triangles) or 21 (squares) days opsonized with (+) or without (–) dinutuximab (dimab) by in vitro G-CSF stimulated neutrophils. N=4–13 of five individual experiments. Statistical significance was tested with ordinary one-way ANOVA with post hoc Sidak test. ADCC, antibody-dependent cellular cytotoxicity; ANOVA, analysis of variance; G-CSF, granulocyte colony-stimulating factor; AUC, areas under curve; ns, not significant.*

Comparable *ex vivo* killing of neuroblastoma cells by *in vivo* GM-CSF or G-CSF stimulated neutrophils

Although *in vitro* GM-CSF and G-CSF stimulated neutrophils show enhanced killing capacity towards neuroblastoma cells (**Figure 1**), whether this also occurs *in vivo* and in patients with neuroblastoma is still unclear. To take this a step closer to the anticipated situation in patients, we investigated the respective abilities of GM-CSF and G-CSF at potentiating neutrophils to kill neuroblastoma cells after *in vivo* stimulation. We obtained blood from patients with high-risk neuroblastoma that were administered GM-CSF subcutaneously for three consecutive days prior to blood sampling (patient characteristics are summarized in **Suppl. Table 2**). In addition, we collected blood from granulocyte transfusion donors ~30 hours after subcutaneous G-CSF injection. We found similar Fcγ receptor expression profiles for both GM-CSF and G-CSF *in vivo* stimulated neutrophils. Compared with unstimulated neutrophils, *in vivo* G-CSF stimulated neutrophils, as well as *in vivo* GM-CSF stimulated neutrophils showed a significant increase of FcγRI expression and a decrease of FcγRIIIb expression, whereas the expression of FcγRIIa remained unaltered (**Figure 5A-D**), similar as observed for the *in vitro* stimulated neutrophils (**Figure 2A-B**). The capacity of *in vivo* stimulated neutrophils to kill dinutuximab-opsonized neuroblastoma cells was investigated *ex vivo*. Neutrophils from GM-CSF injected patients with neuroblastoma induced significantly greater cytotoxicity levels of the GD2-positive neuroblastoma cell lines NMB and IMR-32 as compared with unstimulated neutrophils (**Figure 5E** and **Suppl. Figure 5**). Likewise, the cytotoxic ability of *in vivo* G-CSF stimulated neutrophils was similarly enhanced (**Figure 5F**). Overall, this indicates that both cytokines can stimulate neutrophils *in vivo* to kill neuroblastoma cells *ex vivo*.

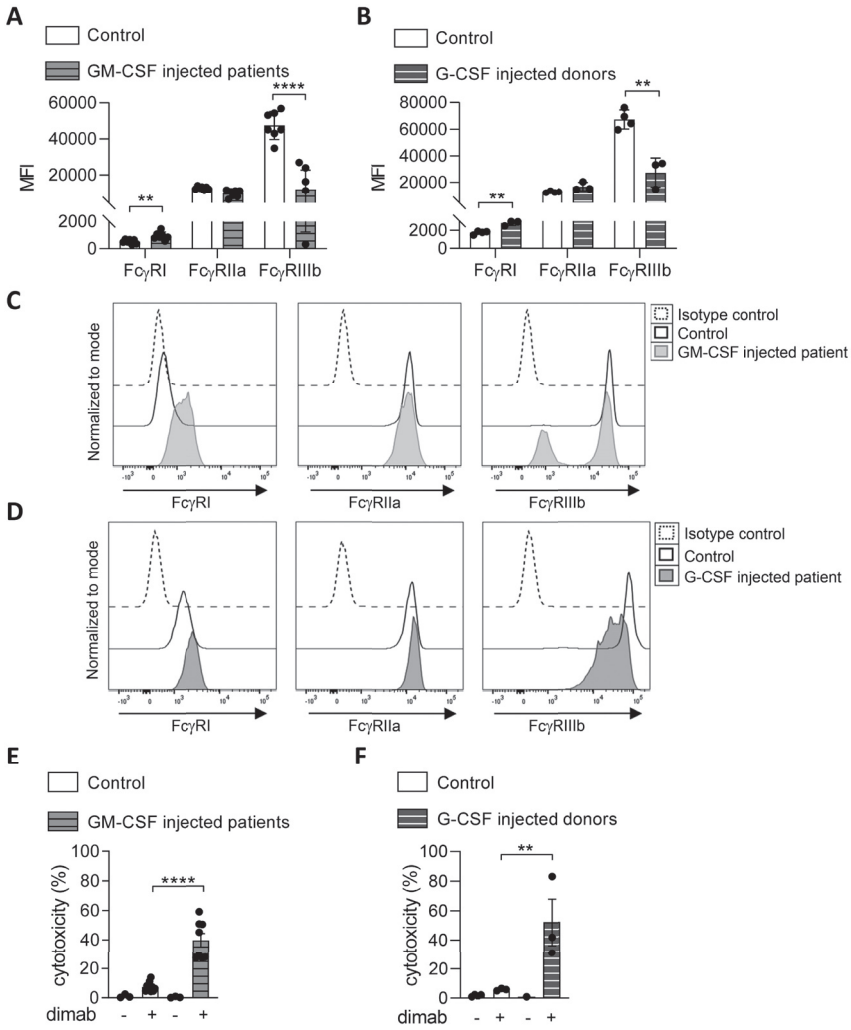


Figure 5. Comparable *ex vivo* killing of neuroblastoma cells by *in vivo* GM-CSF or G-CSF stimulated neutrophils. (A) *Fcγ receptor expression (expressed as MFI) on neutrophils from patients with neuroblastoma that were injected with GM-CSF (striped light gray bars) compared with control neutrophils from healthy donors (white bars). Both for healthy donors and patients, FcγRI n=7, FcγRIIa n=7, FcγRIIIb n=7 of four individual experiments. Statistical analysis was assessed with an unpaired T-test.* **(B)** *Fcγ receptor expression (expressed as MFI) on neutrophils from healthy donors that were injected with G-CSF (striped dark gray bars) compared with control neutrophils from healthy donors (white bars). Both for healthy donors and G-CSF injected donors, FcγRI n=3–4, FcγRIIa n=3–4, FcγRIIIb n=3–4 of two individual experiments. Statistical differences were assessed with an unpaired T-test.* **(C, D)** *Representative histograms of flow cytometry analysis of FcγRI (left panels), FcγRIIa (middle panels) and FcγRIIIb (right panels) expression on control neutrophils from healthy donors (in white) compared with neutrophils from patients with neuro-*

blastoma that were injected with GM-CSF (in light gray) (C), or neutrophils from healthy donors that were administered G-CSF (in dark gray) (D). The dashed line depicts an isotype control. **(E)** *Antibody-dependent cellular cytotoxicity (ADCC) of NMB cells opsonized with (+) or without (–) dinutuximab (dimab) by control neutrophils from healthy donors (white bars) or by neutrophils from patients with neuroblastoma that were administered GM-CSF (striped light gray bars). N=10 healthy donors, n=8 patients of five individual experiments. Statistical differences were assessed with ordinary one-way ANOVA with post hoc Sidak test.* **(F)** *ADCC of NMB cells opsonized with (+) or without (–) dinutuximab (dimab) by control neutrophils from healthy donors (white bars) or by neutrophils from healthy donors that were injected with G-CSF (stripped dark gray bars). N=4 control neutrophils, n=3 G-CSF injected of two individual experiments. Statistical significances was tested with ordinary one-way ANOVA with post hoc Sidak test. ADCC, antibody-dependent cellular cytotoxicity; ANOVA, analysis of variance; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MFI, mean fluorescence intensity. **p<0.01; ****p<0.0001.*

DISCUSSION

High-risk neuroblastoma is an aggressive cancer affecting children mostly before the first year of age. Therapy consists of intense multimodal treatment, including immunotherapy with anti-GD2 antibody dinutuximab. The treatment regimen in the USA encompasses dinutuximab administered in combination with GM-CSF and IL-2 in alternating cycles, as these have been shown to improve therapeutic efficacy[12]. However, this is not the case for other countries, where GM-CSF (sargramostim) is not approved for clinical use. The limited availability of GM-CSF poses a risk of suboptimal treatment of these patients. For this reason, finding a widely available alternative stimulating cytokine that potentiates the killing of neuroblastoma cells is of high clinical relevance in areas where GM-CSF is not available. Enhancing the cytotoxic capacities of effector cells may improve dinutuximab responsiveness, which could further increase the overall survival of patients with high-risk neuroblastoma. As neutrophils are considered the main players in dinutuximab-mediated killing of neuroblastoma cells[8], we tested the capacity of neutrophils stimulated with G-CSF as opposed to GM-CSF in killing dinutuximab-opsonized GD2-positive neuroblastoma cells. For this we used both *in vivo* and *in vitro* GM-CSF or G-CSF stimulated neutrophils from either patients with neuroblastoma or healthy adult donors, and various neuroblastoma cell lines, including primary patient-derived material.

In the present study, we critically compared GM-CSF with G-CSF in the context of neutrophil ADCC of neuroblastoma cells. Previous studies showed that *in vitro* stimulation with GM-CSF increased the magnitude of cytotoxicity of dinutuximab-opsonized neuroblastoma cells specifically for granulocytes, while this effect was not obtained when stimulating peripheral blood mononuclear cells, emphasizing the specificity of GM-CSF on granulocytes[8]. Similarly, G-CSF stimulation of neutrophils has been found to greatly enhance their capacity for ADCC in solid cancers[17–19]. In this report, we found G-CSF to be as effective as GM-CSF in enhancing neutrophil ADCC of neuroblastoma cells,

both after *in vitro* stimulation, as well as after *in vivo* stimulation. For the latter, we were able to use neutrophils from healthy granulocyte transfusion donors injected with G-CSF and neutrophils from patients with neuroblastoma treated with GM-CSF. Both *in vivo* stimulations enhanced neutrophil-mediated ADCC as opposed to unstimulated conditions, demonstrating that *in vivo* stimulated neutrophils can perform ADCC. We did not have access to unstimulated neutrophils of patients with neuroblastoma, but a previous report demonstrated that neutrophils of neuroblastoma patients displayed the same abilities as healthy adult neutrophils in mediating killing of neuroblastoma cells *ex vivo*, supporting comparison between these different cohorts[8].

We found that both GM-CSF and G-CSF stimulated neutrophils induced the same effect on Fcγ receptor and integrin expression on stimulation: shedding of FcγRIIIb, and no apparent changes in expression of FcγRIIa or CD11b/CD18 integrins. We did see an increase in FcγRI expression after *in vivo* stimulation, in line with existing literature, and to a lesser extent also after *in vitro* stimulation. A previous study demonstrated that both FcγRIIa and CD11b/CD18 integrins are indispensable for neutrophil-mediated killing of antibody-opsonized solid tumor cells[36], and this is consistent with our findings in the context of dinutuximab-opsonized neuroblastoma cells; blocking CD11b/CD18 integrin function completely abolished ADCC, with FcγRIIa being a dominant Fcγ receptor contributing to the process. The reason ADCC could not be fully inhibited on FcγRIIa blockade is not completely understood, especially as we found that killing itself seemed to be fully antibody-dependent. It might be that the concentration of FcγRIIa blockade using the $F(ab')_2$ fragments was suboptimal or not complete during the 4 hours ADCC. In addition, the same might hold true for the purified IgG1 Fc tails for the saturation of FcγRI.

Furthermore, neutrophils stimulated overnight with either GM-CSF or G-CSF *in vitro* were able to trogocytose dinutuximab-opsonized neuroblastoma cells and this was followed by tumor cell death. This is in line with previous findings where neutrophils trogocytosed trastuzumab-opsonized breast cancer cells, which led to cell rupture and death[36].

Finally, we investigated the effect of G-CSF on the neuroblastoma cells. Our results showed no unfavorable effects of G-CSF during the 3-week cultures on neuroblastoma cell growth and on the susceptibility towards neutrophil-mediated ADCC *in vitro*, and no signs of EMT were observed. Of interest, a recent phase I/IIa clinical trial in a cohort of patients with neuroblastoma in Japan—where GM-CSF is also unavailable—assessed the tolerability, safety and feasibility of either G-CSF or macrophage colony-stimulating factor (M-CSF) during dinutuximab immunotherapy with IL-2[49]. This study showed that G-CSF was well-tolerated, which complements our preclinical data on the safety of G-CSF.

Although biologically very relevant, validating our findings in an *in vivo* mouse model would technically and methodologically be challenging. Considering syngeneic tumor mouse models, fundamental differences are observed between human and mouse neutrophil biology, reflected in the number of circulating neutrophils, function and their antitumor effects[50,51], which hamper the translatability of preclinical findings in such models. As for a xenogeneic mouse tumor model, the major obstacle is the availability of patient-derived xenograft models in mice with fully functional human immune system, including neutrophils, suitable for studying dinutuximab-based immunotherapies in neuroblastoma[52]. Our *in vitro* preclinical data on the efficacy and safety of G-CSF, together with extensive clinical experience with G-CSF in other (pediatric) indications, support direct evaluation of G-CSF in a clinical setting to improve immunotherapy of patients with neuroblastoma.

AUTHOR CONTRIBUTIONS

PMS and DJvR designed and performed the experiments, analyzed the data and wrote the manuscript. LMJvZ, BK, PB, HO, KS, IK, AS, and KL performed experiments and reviewed the manuscript. IT, MPD, WMK, JJM, and GAMT provided patient material and primary patient-derived cell line 691B and reviewed the manuscript. TWK, RvB, and TKvdB contributed to experimental design and data interpretation and reviewed the manuscript. HLM, GAMT, and KF designed the experiments, interpreted and evaluated the data and wrote the manuscript. All authors approved the submitted version.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

FUNDING

HLM is supported by the Dutch Cancer Society (grant #11537). IT is supported by KiKa (grant #303) and PPOC (grant #19-27).

REFERENCES

1. Whittle SB, Smith V, Doherty E, Zhao S, McCarty S, Zage PE. Overview and recent advances in the treatment of neuroblastoma. *Expert Rev Anticancer Ther.* 2017;17(4):369-386.
2. American Cancer Society. https://www.cancer.org/cancer/neuroblastoma/detection-diagnosis-staging/risk-groups.html
3. Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *The Lancet*. 2007;369(9579):2106-2120.
4. Park JR, Bagatell R, London WB, et al. Children's Oncology Group's 2013 blueprint for research: neuroblastoma. *Pediatr Blood Cancer.* 2013;60(6):985-993.
5. Smith V, Foster J. High-Risk Neuroblastoma Treatment Review. *Children (Basel).* 2018;5(9).
6. Ahmed M, Cheung NK. Engineering anti-GD2 monoclonal antibodies for cancer immunotherapy. *FEBS Lett*. 2014;588(2):288-297.
7. Keyel ME, Reynolds CP. Spotlight on dinutuximab in the treatment of high-risk neuroblastoma: development and place in therapy. *Biologics*. 2019;13:1-12.
8. Barker E, Mueller BM, Handgretinger R, Herter M, Yu AL, Reisfeld RA. Effect of a chimeric anti-ganglioside GD2 antibody on cell-mediated lysis of human neuroblastoma cells. *Cancer Res*. 1991;51(1):144-149.
9. Hank JA, Robinson RR, Surfus J, et al. Augmentation of antibody dependent cell mediated cytotoxicity following in vivo therapy with recombinant interleukin 2. *Cancer Res*. 1990;50(17):5234-5239.
10. Kushner BH, Cheung NK. GM-CSF enhances 3F8 monoclonal antibody-dependent cellular cytotoxicity against human melanoma and neuroblastoma. *Blood.* 1989;73(7):1936-1941.
11. Cheung IY, Hsu K, Cheung NK. Activation of peripheral-blood granulocytes is strongly correlated with patient outcome after immunotherapy with anti-GD2 monoclonal antibody and granulocyte-macrophage colony-stimulating factor. *J Clin Oncol.* 2012;30(4):426-432.
12. Yu AL, Gilman AL, Ozkaynak MF, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N Engl J Med.* 2010;363(14):1324-1334.
13. McGinty L, Kolesar J. Dinutuximab for maintenance therapy in pediatric neuroblastoma. *Am J Health Syst Pharm.* 2017;74(8):563-567.
14. Gilman AL, Ozkaynak MF, Matthay KK, et al. Phase I study of ch14.18 with granulocyte-macrophage colony-stimulating factor and interleukin-2 in children with neuroblastoma after autologous bone marrow transplantation or stem-cell rescue: a report from the Children's Oncology Group. *J Clin Oncol.* 2009;27(1):85-91.
15. Ladenstein R, Pötschger U, Valteau-Couanet D, et al. Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): a multicentre, randomised, phase 3 trial. *Lancet Oncol.* 2018;19(12):1617-1629.
16. European Medicines Agency. https://www.ema.europa.eu/en
17. Michon J, Moutel S, Barbet J, et al. In vitro killing of neuroblastoma cells by neutrophils derived from granulocyte colony-stimulating factor-treated cancer patients using an anti-disialoganglioside/anti-Fc gamma RI bispecific antibody. *Blood.* 1995;86(3):1124-1130.
18. Treffers LW, van Houdt M, Bruggeman CW, et al. FcgammaRIIIb Restricts Antibody-Dependent Destruction of Cancer Cells by Human Neutrophils. *Front Immunol.* 2018;9:3124.

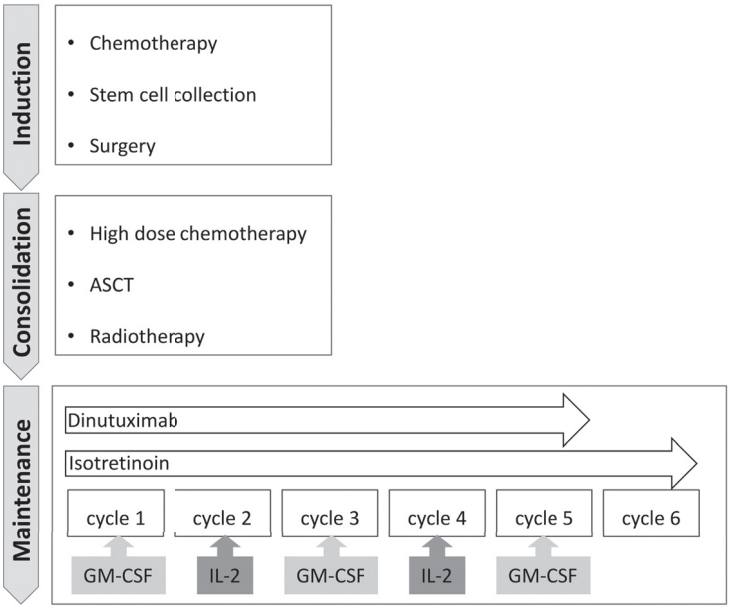
19. Tamamori Y, Sawada T, Nishihara T, et al. Granulocyte-colony stimulating factor enhances chimeric antibody Nd2 dependent cytotoxicity against pancreatic cancer mediated by polymorphonuclear neutrophils. *Int J Oncol*. 2002;21(3):649-654.
20. Carulli G. Effects of recombinant human granulocyte colony-stimulating factor administration on neutrophil phenotype and functions. *Haematologica*. 1997;82(5):606-616.
21. Gay A, Chang S, Rutland L, Yu L, Byeseda S. Granulocyte Colony Stimulating Factor (GCSF) Alters the Phenotype of Neuroblastoma Cells: Implications for disease free survival of high-risk patients. *J Pediatr Surg*. 2008;43(5):837-842.
22. Hsu DM, Agarwal S, Benham A, et al. G-CSF receptor positive neuroblastoma subpopulations are enriched in chemotherapy-resistant or relapsed tumors and are highly tumorigenic. *Cancer Res*. 2013;73(13):4134-4146.
23. Agarwal S, Lakoma A, Chen Z, et al. G-CSF Promotes Neuroblastoma Tumorigenicity and Metastasis via STAT3-Dependent Cancer Stem Cell Activation. *Cancer Res*. 2015;75(12):2566-2579.
24. Green MD, Koelbl H, Baselga J, et al. A randomized double-blind multicenter phase III study of fixed-dose single-administration pegfilgrastim versus daily filgrastim in patients receiving myelosuppressive chemotherapy. *Ann Oncol*. 2003;14(1):29-35.
25. Zamboni WC. Pharmacokinetics of pegfilgrastim. *Pharmacotherapy*. 2003;23(8 Pt 2):9s-14s.
26. Wendelin G, Lackner H, Schwinger W, Sovinz P, Urban C. Once-per-cycle pegfilgrastim versus daily filgrastim in pediatric patients with Ewing sarcoma. *J Pediatr Hematol Oncol*. 2005;27(8):449-451.
27. Borinstein SC, Pollard J, Winter L, Hawkins DS. Pegfilgrastim for prevention of chemotherapy-associated neutropenia in pediatric patients with solid tumors. *Pediatr Blood Cancer*. 2009;53(3):375-378.
28. Kuijpers TW, Tool AT, van der Schoot CE, et al. Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood*. 1991;78(4):1105-1111.
29. Bate-Eya LT, Ebus ME, Koster J, et al. Newly-derived neuroblastoma cell lines propagated in serum-free media recapitulate the genotype and phenotype of primary neuroblastoma tumours. *Eur J Cancer*. 2014;50(3):628-637.
30. Marsman C, Jorritsma T, Ten Brinke A, van Ham SM. Flow Cytometric Methods for the Detection of Intracellular Signaling Proteins and Transcription Factors Reveal Heterogeneity in Differentiating Human B Cell Subsets. *Cells*. 2020;9(12).
31. van Zogchel LMJ, Zappeij-Kannegieter L, Javadi A, et al. Specific and Sensitive Detection of Neuroblastoma mRNA Markers by Multiplex RT-qPCR. *Cancers (Basel)*. 2021;13(1):150.
32. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, et al. PHOX2B is a novel and specific marker for minimal residual disease testing in neuroblastoma. *J Clin Oncol*. 2008;26(33):5443-5449.
33. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, et al. Detecting minimal residual disease in neuroblastoma: the superiority of a panel of real-time quantitative PCR markers. *Clin Chem*. 2009;55(7):1316-1326.
34. van Wezel EM, van Zogchel LMJ, van Wijk J, et al. Mesenchymal Neuroblastoma Cells Are Undetected by Current mRNA Marker Panels: The Development of a Specific Neuroblastoma Mesenchymal Minimal Residual Disease Panel. *JCO Precis Oncol*. 2019;(3):1-11.

35. Metelitsa LS, Gillies SD, Super M, Shimada H, Reynolds CP, Seeger RC. Antidisialoganglioside/granulocyte macrophage-colony-stimulating factor fusion protein facilitates neutrophil antibody-dependent cellular cytotoxicity and depends on FcgammaRII (CD32) and Mac-1 (CD11b/CD18) for enhanced effector cell adhesion and azurophil granule exocytosis. *Blood*. 2002;99(11):4166-4173.
36. Matlung HL, Babes L, Zhao XW, et al. Neutrophils Kill Antibody-Opsonized Cancer Cells by Trogoptosis. *Cell Rep*. 2018;23(13):3946-3959 e6.
37. Repp R, Valerius T, Sendler A, et al. Neutrophils express the high affinity receptor for IgG (Fc gamma RI, CD64) after in vivo application of recombinant human granulocyte colony- stimulating factor. *Blood*. 1991;78(4):885-889.
38. Treffers LW, Hiemstra IH, Kuijpers TW, van den Berg TK, Matlung HL. Neutrophils in cancer. *Immunol Rev*. 2016;273(1):312-328.
39. Peipp M, Lammerts van Bueren JJ, Schneider-Merck T, et al. Antibody fucosylation differentially impacts cytotoxicity mediated by NK and PMN effector cells. *Blood*. 2008;112(6):2390-2399.
40. Bouti P, Zhao XW, Verkuijlen P, et al. Kindlin3-Dependent CD11b/CD18-Integrin Activation Is Required for Potentiation of Neutrophil Cytotoxicity by CD47-SIRPα Checkpoint Disruption. *Cancer Immunol Res*. 2021;9(2):147-155.
41. Tabiasco J, Espinosa E, Hudrisier D, Joly E, Fournie JJ, Vercellone A. Active trans-synaptic capture of membrane fragments by natural killer cells. *Eur J Immunol*. 2002;32(5):1502-1508.
42. Velmurugan R, Challa DK, Ram S, Ober RJ, Ward ES. Macrophage-Mediated Trogocytosis Leads to Death of Antibody-Opsonized Tumor Cells. *Mol Cancer Ther*. 2016;15(8):1879-1889.
43. Ladenstein R, Valteau-Couanet D, Brock P, et al. Randomized Trial of prophylactic granulocyte colony-stimulating factor during rapid COJEC induction in pediatric patients with high-risk neuroblastoma: the European HR-NBL1/SIOPEN study. *J Clin Oncol*. 2010;28(21):3516-3524.
44. Maris JM, Healy J, Park J, Ladenstein R, Potschger U. G-CSF Is a Cancer Stem Cell-Specific Growth Factor-Letter. *Cancer Res*. 2015;75(18):3991.
45. Schneider A, Kruger C, Steigleder T, et al. The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *J Clin Invest*. 2005;115(8):2083-2098.
46. Thiery JP. Epithelial–mesenchymal transitions in tumour progression. *Nat Rev Cancer*. 2002;2(6):442-454.
47. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell*. 2008;14(6):818-829.
48. Van Groningen T, Koster J, Valentijn LJ, et al. Neuroblastoma is composed of two super-enhancer-associated differentiation states. *Nat Genet*. 2017;49(8):1261-1266.
49. Hara J, Nitani C, Kawamoto H, et al. A Phase I/IIa Study of Antidisialoganglioside Antibody Dinutuximab in Japanese Patients With Neuroblastoma. *J Pediatr Hematol Oncol*. 2021;43(3):e358-e364.
50. Eruslanov EB, Singhal S, Albelda SM. Mouse versus Human Neutrophils in Cancer: A Major Knowledge Gap. *Trends Cancer*. 2017;3(2):149-160.
51. Bruhns P, Jonsson F. Mouse and human FcR effector functions. *Immunol Rev*. 2015;268(1):25-51.
52. Braekeveldt N, Bexell D. Patient-derived xenografts as preclinical neuroblastoma models. *Cell Tissue Res*. 2018;372(2):233-243.

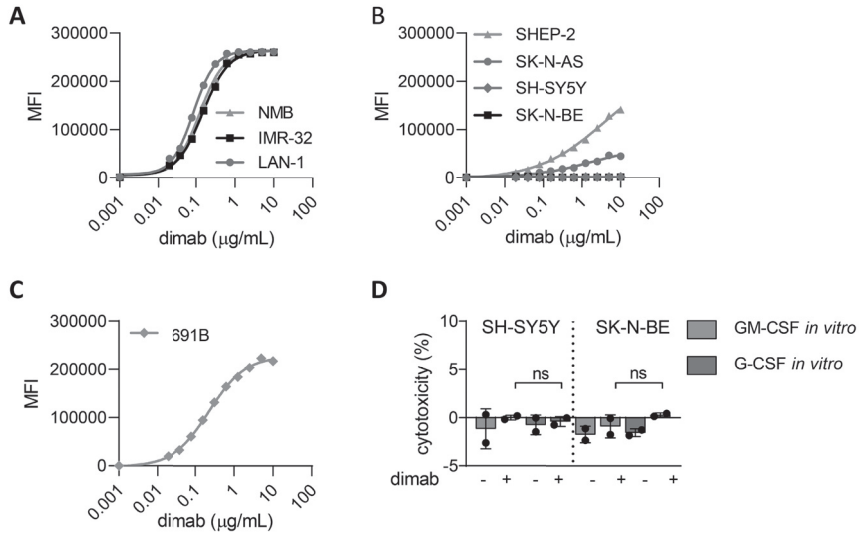
SUPPLEMENTAL DATA

Cell line	Phenotype	GD2 expression	Remarks
NMB	Adrenergic	+++	
IMR-32	Adrenergic	+++	
LAN-1	Adrenergic	+++	
SHEP-2	Mesenchymal	+	Derived from SK-N-SH cells, isogenic pair with SH-SY5Y
SK-N-AS	Mesenchymal	±	
SH-SY5Y	Adrenergic	-	Derived from SK-N-SH cells, isogenic pair with SHEP-2
SK-N-BE	Adrenergic	-	
691B	Adrenergic	+++	Derived from patient 691, bone marrow metastasis (B)

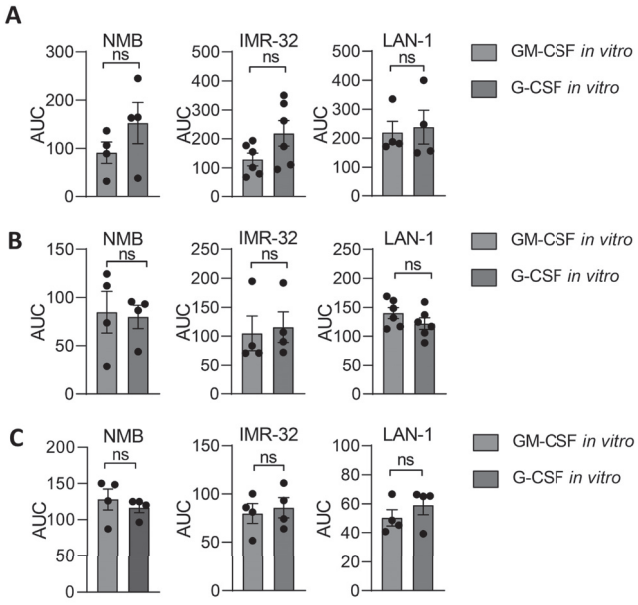
Supplemental Table 1. Characterization of neuroblastoma cell lines. *Phenotypical characterization of the different neuroblastoma cell lines used in this study, including phenotype and GD2 expression levels. GD2 surface expression was established by flow cytometry (***Suppl. Figure 2***).*



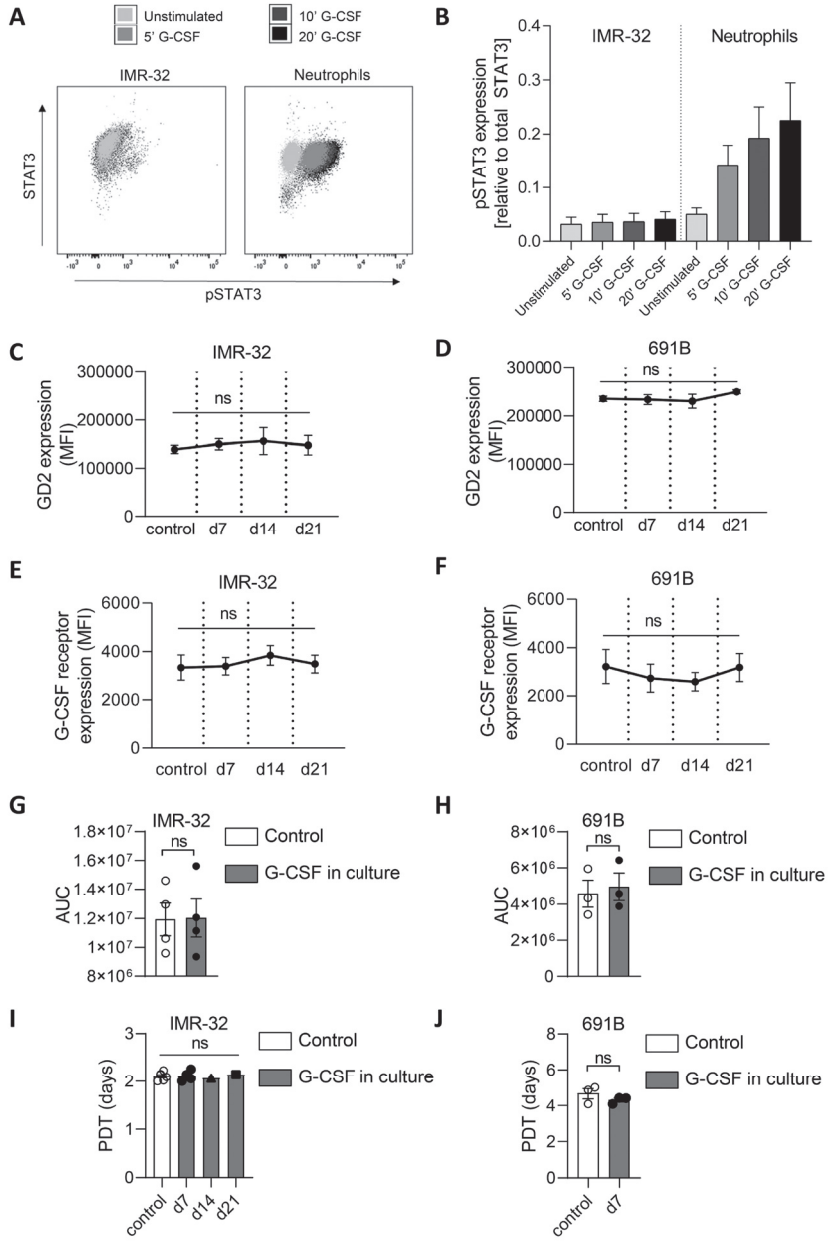
Supplemental Figure 1. Multimodal treatment protocol for high-risk neuroblastoma patients. *Scheme showing the different phases (induction, consolidation and maintenance) of the treatment for high-risk neuroblastoma patients. The immunotherapy regimen, as approved in Northern America, is subdivided in alternating cycles of dinutuximab combined with GM-CSF or IL-2, and isotretinoin (13-cis-retinoic acid). Each cycle consists of 28 days. Figure adapted from Smith, V.; Foster, J. High-Risk Neuroblastoma Treatment Review*[5]*. ASCT, autologous stem cell transplantation.*



Supplemental Figure 2. Binding capacity of dinutuximab to GD2 on neuroblastoma cell lines and ADCC of GD2-negative neuroblastoma cell lines. (A, B, C) *Binding capacity of increasing concentrations of dinutuximab (dimab) to GD2 (expressed as MFI) as measured by flow cytometry on GD2-positive cell lines (A) NMB (light grey triangles), IMR-32 (black squares) and LAN-1 (dark grey circles) and on cell lines expressing lower or none GD2 (B) SHEP-2 (light grey triangles), SK-N-AS (dark grey circles), SH-SY5Y (dark grey diamonds) and SK-N-BE (black squares), and on the GD2-positive primary patient-derived 691B cell line (light grey diamonds) (C). NMB, IMR-32 and LAN-1 n=1, of 2 individual experiments. SHEP-1, SK-N-AS, SH-SY5Y and SK-N-BE n=2, of 2 individual experiments. 691B n=3, of 3 individual experiments.* **(D)** *ADCC of GD2-negative cell lines SH-SY5Y and SK-N-BE opsonized with (+) or without (-) dinutuximab (dimab) by in vitro stimulated neutrophils with GM-CSF (light grey bars) or G-CSF (dark grey bars). SH-SY5Y and SK-N-BE n=2, of 1 individual experiment. Statistical differences were tested with unpaired T-test used to test statistical differences.*



Supplemental Figure 3. Area under the curve for statistics of Figure 1B-D. *Areas under the curve (AUC) of ADCCs of NMB, IMR-32 and LAN-1 cells with dinutuximab titration* **(A)**, *with increasing T:E ratios* **(B)**, *or with cytokines titration* **(C)**, *by neutrophils stimulated in vitro with GM-CSF (light grey bars) or G-CSF (dark grey bars). NMB n=4, IMR-32 n=4-6, LAN-1 n=4-6, of 3 individual experiments. Statistical significance was tested with unpaired T-test to test the difference of the AUC.*

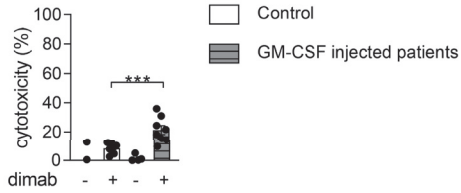

A
Unstimulated
5' G-CSF
10' G-CSF
20' G-CSF
IMR-32
Neutrophils
STAT3
pSTAT3
B
IMR-32
Neutrophils
pSTAT3 expression [relative to total STAT3]
Unstimulated
5' G-CSF
10' G-CSF
20' G-CSF
C
IMR-32
GD2 expression (MFI)
ns
control d7 d14 d21
D
691B
GD2 expression (MFI)
ns
control d7 d14 d21
E
IMR-32
G-CSF receptor expression (MFI)
ns
control d7 d14 d21
F
691B
G-CSF receptor expression (MFI)
ns
control d7 d14 d21
G
IMR-32
AUC
ns
Control
G-CSF in culture
H
691B
AUC
ns
Control
G-CSF in culture
I
IMR-32
PDT (days)
ns
Control
G-CSF in culture
control d7 d14 d21
J
691B
PDT (days)
ns
Control
G-CSF in culture
control d7

Supplemental Figure 4. Effect of G-CSF treatment on proliferation rate and GD2 expression. (A-B) *Representative flow cytometry plots of intracellular total STAT3 plotted against intracellular pSTAT3 (A) and pSTAT3 intracellular expression levels relative to total STAT3 in IMR-32 cell line and neutrophils over the course of G-CSF exposure (0 to 20 minutes). N=2, of 2 individual experiments (B).* **(C)** *GD2 expression (expressed as MFI) measured over time on IMR-32 cells cultured in absence (control) or presence of G-CSF for 7, 14 and 21 days detected with 1 µg/mL of dinutuximab. N=3-13, of 6 individual experiments. Statistical significances were tested with ordinary one-way ANOVA with post-hoc Sidak test.* **(D)** *GD2 expression (expressed as MFI) measured over time on 691B cells cultured in absence (control) or presence of G-CSF for 7, 14 and 21 days detected with 2.5 µg/mL of dinutuximab. N=4-14, of 6 individual experiments. Statistical differences were assessed with ordinary one-way ANOVA with post-hoc Sidak test.* **(E-F)** *G-CSF receptor expression (expressed as MFI) measured over time on IMR-32 and 691B cells cultured in absence (control) or presence of G-CSF for 7, 14 and 21 days. IMR-32 n=2-3, 691B: n= 3-4, of 3 and 4 individual experiments, respectively. Statistical significance was assessed with ordinary one-way ANOVA with post-hoc Sidak test.* **(G-H)** *Area under the curve (AUC) of one-week proliferation rates of IMR-32 cultures (G) and 691B cultures (H) treated without (white bars) or with G-CSF (dark grey bars). IMR-32 n=4, 691B n=3, of 3 and 4 individual experiments, respectively. Statistical significance was tested with unpaired T-test to test the difference of the AUC.* **(I)** *Population doubling time (PDT, expressed in days) of IMR-32 cells upon being cultured in the absence (control, white symbols) or presence of G-CSF for 7 (circles), 14 (triangle) and 21 (squares) days. N=1-6, of 6 individual experiments. Statistical significances were tested with ordinary one-way ANOVA with post-hoc Sidak test.* **(J)** *PDT (expressed in days) of patient-derived 691B cells upon being cultured in the absence (control, white symbols) or presence of G-CSF for 7 (circles) days. N=3, of 3 individual experiments. Statistical significance was assessed with paired T-test.*

Patient	INRG stage*	Gender	Immunotherapy treatment cycle at time of blood sampling**
1	High-risk	Male	1
2	High-risk	Male	3, 5
3	High-risk	Female	5
4	High-risk	Male	1, 3
5	High-risk	Male	1
6	High-risk	Male	1

*INRG= International Neuroblastoma Risk Group
**During cycles 1, 3 and 5 GM-CSF was administered at a dose of 250 µg/m²/day for 3 days prior to blood sampling, and before the start of dinutuximab treatment. No samples were obtained during treatment cycles 2 and 4 where patients receive IL-2.

Supplemental Table 2. GM-CSF injected patient characteristics. *GM-CSF injected patient characteristics, including disease stage as determined by the INRG, age at time of blood sampling (months), gender and treatment cycle 1-5.*



Supplemental Figure 5. *In vivo* GM-CSF stimulated neutrophils show enhanced *ex vivo* killing of IMR-32 neuroblastoma cells. *ADCC of IMR-32 cells opsonized with (+) or without (-) dinutuximab (dimab) by control neutrophils from healthy donors (white bars) or by neutrophils from neuroblastoma patients that were injected with GM-CSF (striped light grey bars). N=10 healthy donors, n=8 patients, of 4 individual experiments. Statistical differences were tested with ordinary one-way ANOVA with post-hoc Sidak test.*

CHAPTER 4

CD47-SIRPα CHECKPOINT INHIBITION ENHANCES NEUTROPHIL-MEDIATED KILLING OF DINUTUXIMAB-OPSONIZED NEUROBLASTOMA CELLS

Paula Martinez-Sanz, Arjan J. Hoogendijk, Paul J. J. H. Verkuijlen, Karin Schornagel, Robin van Bruggen, Timo K. Van den Berg, Godelieve A. M. Tytgat, Katka Franke, Taco W. Kuijpers*, Hanke L. Matlung*

* Authors contributed equally

Cancers 2021 Aug 24; 13(17):4261

SIMPLE SUMMARY

Current immunotherapy for high-risk neuroblastoma patients involves treatment with anti-GD2 antibody dinutuximab, which has significantly improved the survival rate. Still, approximately half of the patients succumb to the tumor; therefore, efforts to improve their prognosis are urgently needed. Since T cell targeting immune checkpoint inhibitors in neuroblastoma are limited due to the low immunogenicity of these tumors, alternative immunotherapeutic approaches should be studied. The therapeutic targeting of the innate immune checkpoint CD47-SIRPα has the ability to enhance antitumor effects of myeloid cells, especially in the presence of cancer-opsonizing antibodies. Given that neutrophil ADCC is a dominant effector mechanism leading to the eradication of dinutuximab-opsonized neuroblastoma cells, we have investigated the therapeutic potential of anti-GD2 antibody in combination with CD47-SIRPα inhibition. We demonstrate here that the capacity of neutrophils to kill dinutuximab-opsonized neuroblastoma cells is controlled by the CD47-SIRPα axis and its disruption promotes their cytotoxic potential even further, significantly improving dinutuximab responsiveness.

ABSTRACT

High-risk neuroblastoma, especially after recurrence, still has a very low survival rate. Immune checkpoint inhibitors targeting T cells have shown remarkable clinical efficacy in adult solid tumors, but their effects in pediatric cancers have been limited so far. On the other hand, targeting myeloid immune checkpoints, such as CD47-SIPRa, provide the opportunity to enhance antitumor effects of myeloid cells, including that of neutrophils, especially in the presence of cancer-opsonizing antibodies. Disialoganglioside (GD2)-expressing neuroblastoma cells targeted with anti-GD2 antibody dinutuximab are in part eradicated by neutrophils, as they recognize and bind the antibody targeted tumor cells through their Fc receptors. Therapeutic targeting of the innate immune checkpoint CD47-SIRPα has been shown to promote the potential of neutrophils as cytotoxic cells in different solid tumor indications using different cancer-targeting antibodies. Here, we demonstrate that the capacity of neutrophils to kill dinutuximab-opsonized neuroblastoma cells is also controlled by the CD47-SIRPα axis and can be further enhanced by antagonizing CD47-SIRPα interactions. In particular, CD47-SIRPα checkpoint inhibition enhanced neutrophil-mediated ADCC of dinutuximab-opsonized adrenergic neuroblastoma cells, whereas mesenchymal neuroblastoma cells may evade immune recognition by a reduction of GD2 expression. These findings provide a rational basis for targeting CD47-SIRPα interactions to potentiate dinutuximab responsiveness in neuroblastomas with adrenergic phenotype.

INTRODUCTION

Immune checkpoint inhibitors of the adaptive immune system have in the last decades revolutionized the treatment landscape of cancer by demonstrating unprecedented success across a wide spectrum of adult advanced cancers[1]. Despite the progress seen with checkpoint blockade in adults, the use of such approaches in pediatric cancers has to date failed to show meaningful clinical efficacy[2,3]. One major factor behind the pediatric tumor resistance to immune checkpoint immunotherapy is considered to be the low mutation rate that these tumors present[4–6]. This results in an important scarcity of neoantigens that can be recognized by T cells, giving rise to a lack of T cell-containing or so-called 'cold tumors'. Unfortunately, patients with such tumors appear unable to benefit from T cell-directed checkpoint blockade therapies. Another consideration for immunotherapy resistance is the highly sophisticated immunosuppressive tumor microenvironment found in most pediatric malignancies[7,8]. The presence of M2 (pro-tumoral) macrophages and a dense stroma packed with fibroblasts is believed to prevent effective adaptive immune responses[2].

Neuroblastoma, an aggressive and bulky cancer affecting very young children, is one example of a pediatric tumor in which the above-mentioned features are inherently present; hence, little has been accomplished regarding the application of adaptive checkpoint blockade immunotherapies in this tumor type. There are, however, effector immune cells other than T cells that can efficiently combat cancer and lead to the eradication of the tumor cells. Neutrophils are the most abundant leukocytes present in human blood and compelling evidence has put them in the spotlight as cells with significant antitumor capacities[9–11]. Among their immune-mediated effects is induction of tumor cell death of antibody opsonized cancer cells, a process known as antibody-dependent cellular cytotoxicity (ADCC)[12,13]. In neuroblastoma, the standard of care for high-risk patients involves antibody therapy with the anti-disialoganglioside (GD2) antibody dinutuximab, which has greatly increased the survival of patients since its implementation in the treatment protocol[14,15]. Among others, neutrophil ADCC has been recognized as an important effector mechanism contributing to the eradication of these dinutuximab-opsonized neuroblastoma cells[16]. Evidence for the relevant role of neutrophils in this cancer type comes from the favorable correlation with clinical outcome found after anti-GD2 immunotherapy with a specific polymorphic variant of *FCGR2A*[17]. This receptor, present exclusively on myeloid cells, represents the predominant activating FcγR present on neutrophils, and it has been demonstrated to be the principal mediator of neutrophil ADCC through recognition of the IgG tail of cancer-targeting therapeutic antibodies directed against other solid cancer cells[18–20]. Furthermore, the overall clinical response of neuroblastoma patients has been found to be further improved by the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF), acting on myeloid cells including neutrophils, to the anti-GD2 treatment regime[14,21–24]. Overall, from the above-mentioned studies and a variety of others, it has

further become apparent that neutrophils can be stimulated by cytokines such as GM-CSF or granulocyte colony-stimulating factor (G-CSF), given either alone or in combination with interferon-gamma (IFNγ), in order to improve their in vitro IgG-mediated cytotoxicity[25–29].

Neutrophils, as well as T cells, are endowed with inhibitory receptors so as to suppress their activity when necessary, which turns them into potential therapeutic targets for checkpoint blockade therapy[30,31]. Signal regulatory protein alpha (SIRPα) is one well-established example of an immunoreceptor expressed on neutrophils that can be successfully targeted for checkpoint-blockade. Its ligand, CD47, a molecule present on normal cells that generally acts as a 'don't eat me signal', is often overexpressed by tumor cells, leading to an evasion of tumor cell recognition and hampered elimination by the immune system[32]. We and others have shown that CD47-SIRPα interactions negatively regulate antibody-mediated cytotoxicity by neutrophils both in vitro and in vivo for a number of cancers (i.e. $Her2^{+}$-breast cancer, $EGFR^{+}$-carcinoma), and that a blockade of the interaction substantially potentiates the cytotoxic capabilities of these effector cells[33–35]. In a clinical setting, several approaches to block CD47-SIRPα axis are already in clinical development for multiple cancer indications[36–38]. The involvement of the CD47-SIRPα checkpoint in neuroblastoma has, however, not been thoroughly investigated yet. Here, we examined whether an inhibition of CD47-SIRPα axis, by either a genetic disruption or by using an antagonistic agent for SIRPα, allows neutrophils to more efficiently kill dinutuximab-opsonized neuroblastoma cells in vitro. By testing cells of either an adrenergic or mesenchymal phenotype, the two divergent cellular phenotypes responsible for a large part of the tumor heterogeneity found in neuroblastoma, we further characterized the involvement of CD47-SIRPα checkpoint in this cancer type. Overall, this provides a rational basis for the targeting of CD47-SIRPα interactions to improve the clinical response to anti-GD2 therapy in children suffering from neuroblastoma.

MATERIALS AND METHODS

Neutrophil gene signature enrichment in neuroblastoma samples

Neuroblastoma stage-specific regulated transcript listings were obtained from Zhang et al.[39]. Regulated transcripts were collapsed to SYMBOL identifiers. We defined a neutrophil gene signature based on all transcripts that were upregulated in a dataset of differentiating primary neutrophils[40]. These neutrophil transcripts were collapsed to SYMBOL identifiers and merged with the neuroblastoma regulated transcript list.

mRNA data analysis for CD47 expression

mRNA sequencing data on expression levels for *CD47* from healthy tissue and neuroblastoma tumors collected from Genotype-Tissue Expression Project (GTEx) and the

Therapeutically Applicable Research to Generate Effective Treatment Program (TARGET) studies, respectively, were downloaded as log2 values from the Xena Functional Genomics Explorer (https://xenabrowser.net/, accessed on 21/02/2021) under the query "TCGA TARGET GTEx". GTEx healthy tissue samples were filtered in for adrenal gland, while TARGET tumor samples were filtered in for neuroblastoma.

Microarray sequencing data regarding *CD47* mRNA expression levels in the different neuroblastoma disease stages were obtained from the Zhang et al. dataset with Gene Expression Omnibus (GEO) accession number GSE49710[39]. Other *CD47* expression profiles used in this study are available from GEO: neuroblastoma cell line panel (GSE28019) and isogenic neuroblastoma cell line pairs of adrenergic or mesenchymal phenotypes (GSE90803). All gene expression analyses were performed in the R2 genomics analysis and visualization platform (http://r2.amc.nl, accessed on 22/03/2021). Where applicable, normalization for expression was based on the expression of *GUSB* and was defined as log2 *CD47*-log2 *GUSB*. Supplemental data on *CD47* expression of other databases analyzed in the present study can be accessed from GEO or R2 browser with the following identifiers: adrenal gland (various: GSE3527, GSE7307, GSE8514) and neuroblastoma (GSE49710, GSE16476, GSE14880, GSE16237, GSE13136).

Neutrophil isolation and stimulation

Neutrophils from heparinized peripheral blood were isolated as previously described by density gradient centrifugation with isotonic Percoll (GE Healthcare, Chicago, IL, USA) and erythrocyte lysis with ice cold hypotonic ammonium chloride solution[41]. Neutrophils were used either directly after isolation (unstimulated) or were stimulated for 30 min or overnight at 37 °C and 5% CO_2 with recombinant human GM-CSF (10 ng/mL; Peprotech, Cranbury, NJ, USA), clinical grade G-CSF (10 ng/mL; Neupogen, Amgen, Thousand Oaks, CA, USA), or a combination of clinical grade G-CSF (10 ng/mL) and recombinant human IFNγ (50 ng/mL; Peprotech). After overnight incubation, the percentage of apoptotic cells was determined using Annexin V staining (BD Biosciences, Franklin Lakes, NJ, USA) to correct for the number of viable neutrophils prior to any experiments. All human blood samples were obtained and used according to the declaration of Helsinki 1964.

Cell culture

The human neuroblastoma cell lines NMB, LAN-1, and IMR-32 were obtained in 2018 from the Leibniz Institute DSMZ, Germany. These cells were routinely cultured at 37 °C and 5% CO_2 and maintained in Iscove's modified Dulbecco's medium (IMDM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% of heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. The human neuroblastoma cell lines SHEP-2 and SK-N-AS (kindly provided by the department of

Oncogenomics, Amsterdam UMC, Amsterdam, the Netherlands) were routinely cultured at 37 °C and 5% CO_2 and maintained in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific) supplemented with 20% of FCS, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin.

The primary patient-derived neuroblastoma spheroid lines AMC691T and AMC691B (hereafter 691T and 691B) were derived from either the primary tumor site (T) or a bone marrow metastasis (B) of patient 691[42]. 691T and 691B cells were cultured and maintained in DMEM (Thermo Fisher Scientific) with low glucose and sodium pyruvate (Thermo Fisher Scientific) supplemented with 25% Ham's F12 nutrient mixture (Thermo Fisher Scientific), B-27 supplement minus vitamin A (50X; Thermo Fisher Scientific), N-2 supplement (100X; Thermo Fisher Scientific), 20 ng/mL animal-free human epidermal growth factor (Peprotech), 40 ng/mL human basic fibroblast growth factor (Peprotech), 200 ng/mL human insulin-like growth factor (Peprotech), 10 ng/mL human platelet-derived growth factor-AA (Peprotech), 10 ng/mL human platelet-derived growth factor-BB (Peprotech), 100 units/mL penicillin, and 100 µg/mL streptomycin.

All cells were kept in culture for up to 3 months and were routinely tested for potential mycoplasma infection using polymerase chain reaction.

Generation of genetically modified cells

CD47 was knocked out from the neuroblastoma cell lines by lentiviral plasmid pLentiCrispRv2 transduction (Addgene, Watertown, MA, USA) containing gRNA against the gene of interest. Knockout cells for CD47 were obtained when using either gRNA 5′ CCAGCAACAGCGCCGCTACC 3′ (hereafter CD47 KO1) or gRNA 5′ CAGCAACAGCGCCGCTACCA 3′ (hereafter CD47 KO2). Tumor cells expressing scrambled gRNA were used as a control for transduction (scrambled: 5′ GCACTACCAGAGCTAACTCA 3′). Lentivirus was grown by transient transfection of HEK293T cells. Virussup was harvested on day 2 and 3 after transfection, filtered through 0.45 µM, and added to the target cells. Transduced cells were selected with 1–2 µg/mL Purocymin (Invivogen, San Diego, CA, USA) and were kept in Puromycin selection until flow cytometry sorting on BD FACSAria™ III Cell Sorter (BD Biosciences). The transduction resulted in 60% to 80% of cells with no CD47 expression, and CD47 KO cells were collected and further expanded in culture. Knockout of CD47 on the different cell lines was routinely verified by flow cytometry.

Flow cytometry staining

For GD2 detection on target cells, the human anti-GD2 antibody dinutuximab (Unituxin, Ch14.18; United Therapeutics, Silver Spring, MD, USA) was previously conjugated to a 633

dye with Lightning-Link™ Atto 633 kit (Innova Biosciences Ltd., Cambridge, UK) according to manufacturers' instructions. After conjugation, 10 µg/mL of the directly labeled dinutuximab was used to quantify GD2 expression by flow cytometry. To detect CD47 on target cells, 10 µg/mL anti-human CD47 (clone B6H12; own hybridoma) and Alexa Fluor 633 $F(ab')_2$ antibody (Thermo Fisher Scientific) were used for primary and secondary staining, respectively. Cell viability of target cells was determined using Hoechst 33,342 solution (Thermo Fisher Scientific). SIRPα expression on neutrophils was detected with 10 µg/mL anti-human SIRPα (clone 12C4; own hybridoma) and a subsequent incubation with Alexa Fluor 488 antibody (Thermo Fisher Scientific) for secondary staining, or with a FITC-labeled antibody on SHEP-2 and SK-N-AS tumor cells. Where needed, isotypes and secondary antibody controls were used to correct for any potential background. Fluorescence was measured on BD FACSCanto™ II flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (version 10.6.1, Becton Dickinson, Ashland, OR, USA).

ADCC

Target cells (1×10^6) were labeled in their culture medium for 90 min at 37 °C with 100 µCi ^{51}Cr (PerkinElmer, Waltham, MA, USA) and finally diluted to 0.1×10^6 cells/mL after several washing steps. Neutrophils were either left untreated or were pre-incubated with 10 µg/mL anti-SIRPα at room temperature for the indicated conditions. Co-incubation of target and effector cells was carried out at a target:effector (T:E) ratio of 1:50 (i.e. 5000:250,000 cells), unless specified otherwise, for 4 h at 37 °C and 5% CO_2 in the absence or presence of 0.5 µg/mL dinutuximab. Spontaneous and maximum ^{51}Cr release were determined by incubating the target cells without effector cells and by treating them with a 0.1% triton X-100 (Sigma Aldrich, St. Louis, MO, USA), respectively. After incubation, supernatant was harvested and analyzed for radioactivity in a Wallac Wizard gamma counter or a MicroBeta^2 plate reader (PerkinElmer). The percentage of cytotoxicity was calculated as: [(experimental counts per minute (CPM)-spontaneous CPM)/(maximum CPM-spontaneous CPM)] × 100%. All conditions were performed in duplo or triplicate.

Statistical analysis

Gene overrepresentation for neutrophil gene signature enrichment in neuroblastoma samples was determined using Fisher exact tests. Where applicable, *p*-values were adjusted using Benjamini–Hochberg multiple test correction. Statistical differences between groups were evaluated by one- or two-way ANOVA, or by student's *t*-test using GraphPad Prism version 8. Where indicated, correction for multiple comparisons using either Sidak's or Tukey's test was performed. Data were considered significant when $p < 0.05$. The results are presented as the mean ± standard error of the mean.

RESULTS

Neuroblastoma tumors contain neutrophil mRNA signatures and upregulate CD47 expression

To further support the idea that neutrophils are relevant players in neuroblastoma tumors, we first investigated the presence of neutrophil mRNA markers in a dataset based on almost 500 biopsies of primary neuroblastomas[39]. From the 55.945 transcripts found in this malignancy, 18,469 were found to be differentially expressed. The differentially expressed neuroblastoma mRNA transcripts were compared with a neutrophil mRNA gene signature extracted from Grassi et al.[40]. This analysis revealed that 1.918 of the 18.469 differentially expressed neuroblastoma transcripts were neutrophil-related, which included highly specific neutrophil markers such as *FCGR3B*, *FPR1*, *S100A8/9*, and *SIGLEC9*, among others (**Figure 1A**). Furthermore, the ratio of upregulated neutrophil mRNA signature versus total mRNA indicated a significant influx of neutrophils in neuroblastoma tumors of either disease stage 1 (very low risk) or 4 (high risk), as established by the International Neuroblastoma Staging System[43] (**Figure 1B**). Altogether, these data insinuate that both early as well as advanced stage neuroblastoma tumors contain neutrophil mRNA signatures.

To assess the relevance of CD47-SIRPα signaling in neuroblastoma, we first examined the gene expression levels of CD47 in this tumor type and compared it to the levels in the respective healthy tissue, being the adrenal gland in our case[44,45]. To do so, mRNA-sequencing data from the publicly available GTEx study was used to extract the data from healthy tissue, which was filtered in for adrenal gland tissue. Meanwhile, the TARGET study, specialized in genomic data of pediatric cancers, was used to obtain the respective mRNA data of neuroblastoma samples. We found that human neuroblastoma tumors expressed significantly higher levels of the immune checkpoint molecule CD47 relative to normal adrenal gland tissue (**Figure 1C**), suggesting a pronounced ~twofold upregulation on mRNA level of the molecule. To check whether this finding was not an isolated phenomenon for this particular database, four other neuroblastoma datasets (GSE49710, GSE16476, GSE14880, GSE16237, GSE13136) were examined in which a significant CD47 overexpression was found in three out of the four studies when compared to the adrenal gland values of a different dataset (GSE3527, GSE7307, GSE8514; **Suppl. Figure 1**). In addition, we investigated CD47 mRNA expression levels in neuroblastoma stages 1, 2, 3, 4, and 4S in the cohort of Zhang et al.[39]. After disease stage stratification we found that CD47 expression remained high and was unaltered over all stages (**Figure 1D**).

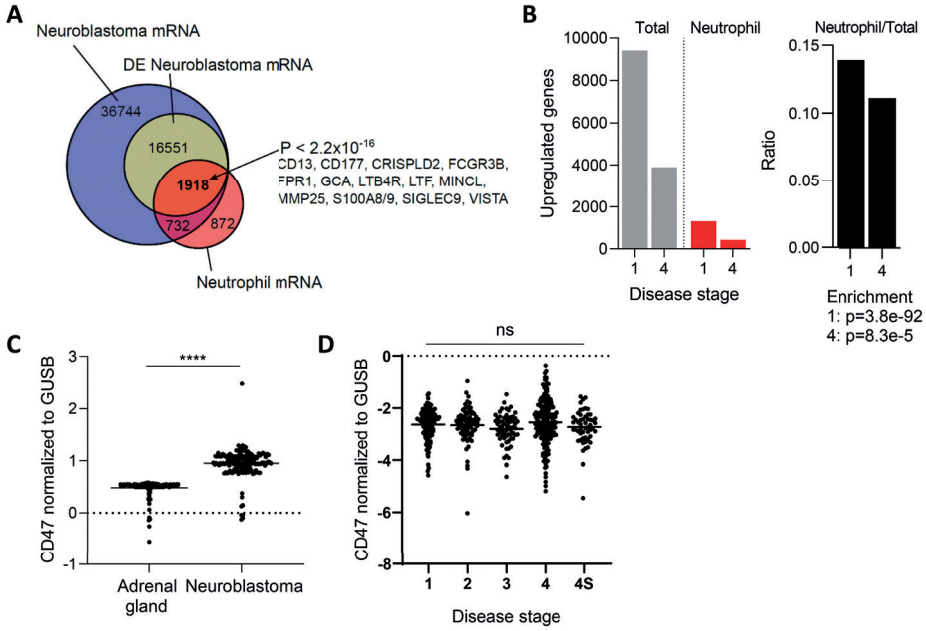


Figure 1. (A) *Venn diagram showing overlap between neuroblastoma (Zhang et al.[39]) and neutrophil (Grassi et al.[40]) regulated transcripts. Overrepresentation was determined with a Fisher exact test. DE, differentially expressed.* **(B)** *Proportion of neutrophil associated genes in neuroblastoma stratified according to disease stages 1 and 4 (left panel: absolute numbers, right panel: ratios). Enrichment determined with Fisher exact tests.* **(C)** *Normalized CD47 mRNA expression levels in healthy adrenal gland and neuroblastoma tumors. Adrenal gland: n = 127, neuroblastoma: n = 162. Statistical significance was tested with unpaired t-test; *** p < 0.001.* **(D)** *Normalized CD47 mRNA expression levels in neuroblastoma patients stratified by disease stage. Stage 1: n = 121, stage 2: n = 78, stage 3: n = 63, stage 4: n = 183, stage 4S: n = 53. Statistics were performed by one-way ANOVA; ns, not significant.*

CD47-SIRPα disruption potentiates neutrophil-dependent antitumor activity towards neuroblastoma cells

To investigate the role of CD47-SIRPα as checkpoint in neutrophil-mediated ADCC in neuroblastoma, we verified CD47 expression in the GD2-positive neuroblastoma cell lines NMB, LAN-1, and IMR-32 (**Figure 2A** and **Suppl. Figure 2A**). To genetically disrupt CD47-SIRPα interaction, CD47 expression was deleted on all cell lines by CrispR/Cas9 with two different guide RNAs using lentiviral transduction. This resulted in full CD47 knockouts (KO; **Figure 2A**) that did not interfere with GD2 expression on any of the cell lines (**Figure Suppl. Figure 2A**). Co-cultured dinutuximab-opsonized CD47 KO cells with neutrophils expressing high levels of SIRPα (**Suppl. Figure 3A–B**) were more readily killed as compared with wild-type or scrambled cells (used as control for transduction). This

resulted in significantly higher levels of neutrophil-mediated cytotoxicity regardless of the stimulus used (**Figure 2B–D**). Importantly, the most compelling enhancing effect was seen for unstimulated neutrophils, for which unmodified neuroblastoma cells as targets barely resulted in 5–20% of killing, whereas for the CD47 KO cell lines, the neutrophil-mediated cytotoxicity levels were enhanced up to 50–80%. This enhancement in tumor cell killing of CD47 KO cell lines happened similarly upon a longer stimulation overnight of the neutrophils with either GM-CSF or G-CSF cytokines (**Suppl. Figure 2B–D**). Of note, as for the genetically unmodified target cells, no killing of the CD47 deleted cells occurred in the absence of the therapeutic antibody dinutuximab, demonstrating that CD47–SIRPα interactions only control antibody-dependent mechanisms of neuroblastoma killing by neutrophils.

Since neutrophils are considered promising effector cells for anti-SIRPα antibody therapy as these cells express high levels of this inhibitory receptor both at a basal state (unstimulated) and upon stimulation with growth factors and cytokines (**Suppl. Figure 3A–B**), we next evaluated the effect of directly blocking SIRPα in vitro. The therapeutic activity of the antagonistic antibody against SIRPα was first tested either alone or in combination with the tumor-opsonizing antibody dinutuximab on the above-mentioned GD2-positive neuroblastoma cell lines NMB, LAN-1, and IMR-32 (wild types). Blockade of SIRPα resulted in a significantly augmented neutrophil-mediated ADCC of all three cell lines when co-cultured with either unstimulated or stimulated neutrophils with GM-CSF alone, G-CSF alone, or G-CSF in combination with IFNγ (**Figure 2E–G** and **Suppl. Figure 2E–G**). In line with our data on CD47 KO neuroblastoma cells, we found the SIRPα blocking agent alone did not induce neutrophil-mediated cytotoxicity of tumor cells unopsonized with dinituximab. To test the effect of decreasing numbers of neutrophils available, we investigated the cytotoxic capabilities of anti-SIRPα treated neutrophils by reducing their numbers. We found that even at relatively low T:E ratios, i.e. 1:12.5 or 1:25, the therapeutic activity of the SIRPα blocking antibody was still detectable, which can be especially appreciated for LAN-1 and IMR-32 target cells (**Figure 2H–J** and **Suppl. Figure 2H–J**). Particularly, the condition of unstimulated neutrophils following anti-SIRPα treatment resulted in cytotoxicity levels as high as those induced by GM-CSF stimulated neutrophils in the absence of SIRPα blocking antibody.

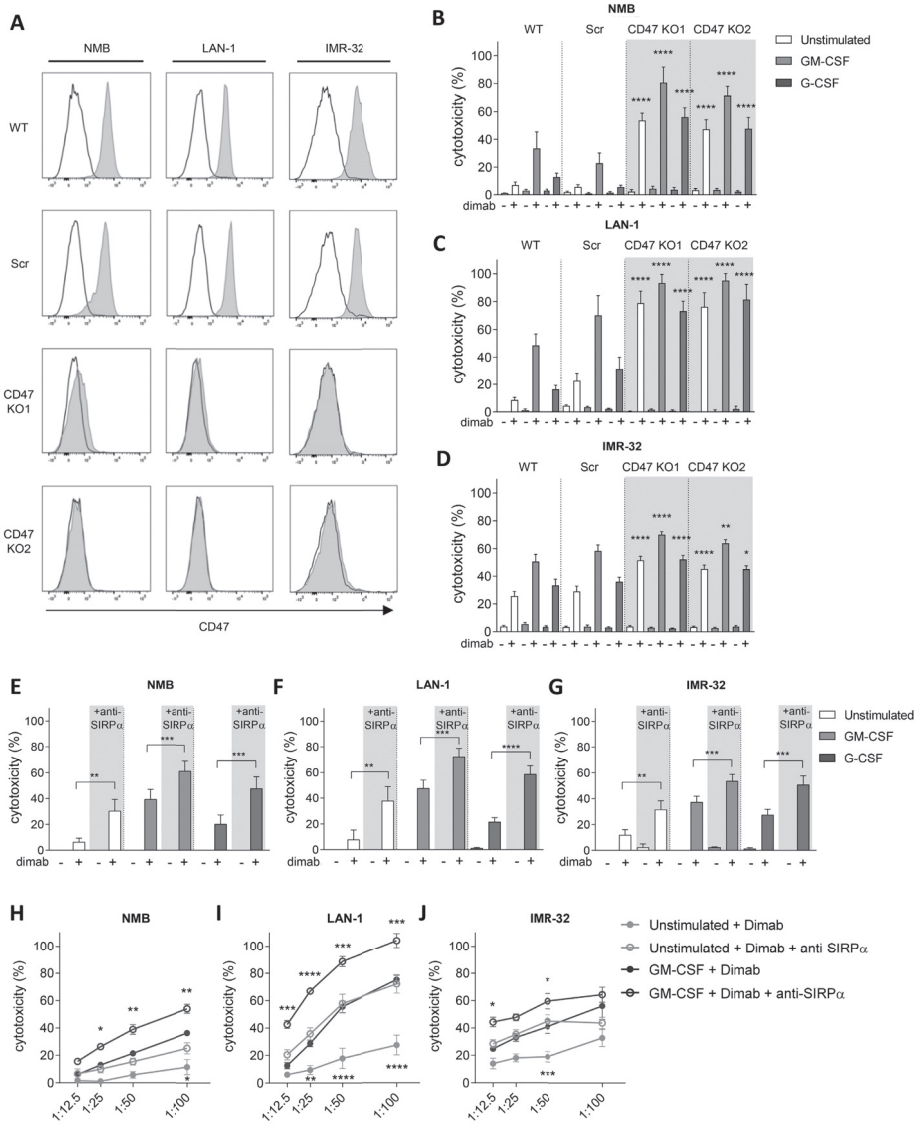


Figure 2. (A) *Representative histograms depicting CD47 surface expression (n = 3) as analyzed by flow cytometry on (from left to right) NMB, LAN-1, and IMR-32 control cells (top two rows) and their respective CD47 KO variants (bottom two rows). Secondary antibody controls are represented in white.* **(B–D)** *ADCC of control (WT and Scr, no background) and CD47 KO (CD47 KO1 and CD47 KO2, grey background) NMB (***B***), LAN-1 (***C***), and IMR-32 (***D***) cells opsonized with (+) or without (-) dinutuximab (dimab) by unstimulated neutrophils (white bars) or stimulated with GM-CSF (light grey bars) or G-CSF (dark grey bars). n = 6, of 3 individual experiments. Statistics were performed by one-way ANOVA with Sidak correction for multiple comparisons.* **(E–G)** *ADCC of NMB (***E***), LAN-1 (***F***), and IMR-32 (***G***) cells opsonized with (+) or without*

(-) dinutuximab (dimab) by unstimulated neutrophils (white bars) or stimulated with GM-CSF (light grey bars) or G-CSF (dark grey bars) in the absence (no background) or presence (grey background) of SIRPα blocking agent. n = 6–14, of 7 independent experiments. Statistical analysis was assessed with by a paired t-test. **(H–J)** *ADCC of dinutuximab-opsonized NMB (***H***), LAN-1 (***I***), and IMR-32 (***J***) cells by unstimulated neutrophils (light grey circles) or stimulated with GM-CSF (dark grey circles) in the absence (filled circles) or presence (empty circles) of SIRPα blocking agent at different T:E ratios ranging from 1:12.5 to 1:100. n = 5, of 4 individual experiments. Statistical differences were tested with two-way ANOVA with Tukey's post hoc test; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001. ADCC, antibody-dependent cellular cytotoxicity. WT, wildtype. Scr, scrambled.*

Tumor cell opsonization determines anti-SIRPα treatment efficacy

Another level of complexity in neuroblastoma tumors being responsible for the intratumoral heterogeneity among patients is the existence of two divergent cellular phenotypes with distinct gene expression profiles: the adrenergic and mesenchymal phenotypes[46,47]. It is now widely established that committed adrenergic neuroblastoma cells can switch their fate and interconvert into undifferentiated mesenchymal cells, which are known to be enriched in post-therapy and relapsing tumors as a result of epithelial-to-mesenchymal transition processes[46]. Therefore, we sought to investigate the involvement of CD47-SIRPα axis in neuroblastoma cells of mesenchymal phenotype as well. First, we examined CD47 mRNA expression on a panel of 24 neuroblastoma cell lines from tissue banks which included cells of both phenotypes (**Figure 3A**). We found a significantly higher expression of CD47 on cell lines with a mesenchymal phenotype compared to the adrenergic cells (**Figure 3B**). From these, we focused on two of the mesenchymal cell lines: SHEP-2 and SK-N-AS, which were characterized for GD2 and CD47 expression by flow cytometry. SHEP-2 and SK-N-AS cells express relatively low levels of GD2 antigen compared to one of the earlier mentioned adrenergic cell lines, LAN-1 (**Figure 3C** and **Suppl. Figure 4A**). Both cell lines were confirmed to express CD47 at high levels also at protein level (**Figure 3C**), and, in line with the mRNA expression dataset (**Figure 3A**), were found to express higher CD47 levels as compared to LAN-1 (**Suppl. Figure 4B**). Despite the high expression of the SIRPα ligand on the surface of SHEP-2 and SK-N-AS cell lines, a SIRPα block on either unstimulated or stimulated neutrophils in an ADCC assay had no effect on the cytotoxicity of these cells (**Figure 3D–E**). This suggests it is not the inhibitory signal provided by SIRPα binding to CD47 on these tumor cells that hampers neutrophil-mediated ADCC, but rather an insufficient tumor cell opsonization due to low GD2 expression is limiting the full cytotoxic capacity of neutrophils towards dinutuximab-opsonized SHEP-2 and SK-N-AS cells. Although SHEP-2 and SK-N-AS cells were shown to express relatively low levels of SIRPα on their surface (**Suppl. Figure 3C–D**), no neutrophil-cytotoxicity effect was detected in the conditions with anti-SIRPα treatment only, suggesting that the level of opsonization that the anti-SIRPα antibodies could possibly cause on the tumor cells is not sufficient to trigger neutrophil killing by itself.

To better evaluate the involvement of CD47-SIRPα axis in the two divergent phenotypes that can be found in neuroblastoma, we examined CD47 expression in four isogenic neuroblastoma cell line pairs with opposite phenotypes that were isolated from individual patients[48]. Again, a trend for higher CD47 mRNA expression was found for the cells of mesenchymal phenotype, as compared to their respective adrenergic pair (**Figure 3F**). Of these, we further characterized the isogenic pair from patient 691 — 691T cells were derived from the primary tumor site of a neuroblastoma patient while 691B cells were isolated from the bone marrow metastasis of the same patient[42] — for the markers of interest by flow cytometry. CD47 expression could be detected on the surface of both counterparts (**Figure 3G**), and, despite not showing statistical significance, its expression seemed higher for the mesenchymal 691T cells as compared to the adrenergic 691B cells (**Suppl. Figure 4B**), correlating with the findings at mRNA level (**Figure 3F**). The 691B cell line of adrenergic phenotype expressed GD2 in the same order of magnitude compared to LAN-1, while the mesenchymal 691T cells lost most of the expression of the ganglioside on the surface membrane, similar to SK-N-AS (**Figure 3G** and **Suppl. Figure 4A**). Next, we assessed the ability of neutrophils to kill the two primary patient-derived tumor cell lines 691B and 691T. Anti-SIRPα treatment of neutrophils further enhanced the killing of GD2-positive adrenergic 691B cells, reaching cytotoxicity levels up to 100%, even by unstimulated neutrophils (**Figure 3H** and **Suppl. Figure 4C**). Conversely, SIRPα blockade did not induce any neutrophil-mediated killing of GD2-low expressing mesenchymal 691T cells (**Figure 3I** and **Suppl. Figure 4D**). Altogether, these results suggest that SIRPα blockade therapy may only be applicable and of benefit when the tumor antigen GD2 is present on the surface of neuroblastoma cells in sufficient amounts and hence ADCC can be triggered upon antibody therapy with dinutuximab.

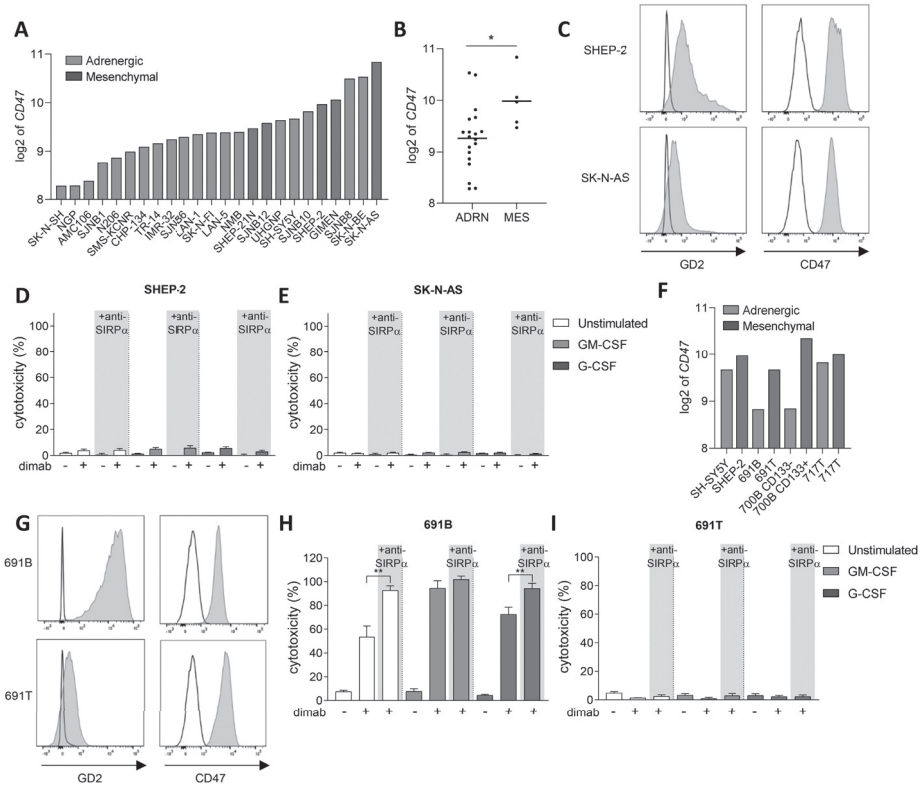


Figure 3. (A) *CD47 mRNA expression levels (expressed as log2) on a panel of 24 neuroblastoma cell lines. Depicted in light grey are the cell lines of adrenergic phenotype while dark grey bars represent mesenchymal cell lines.* **(B)** *Scatterplot showing the pooled CD47 mRNA expression (expressed as log2) of the panel of 24 neuroblastoma cell lines divided by phenotype: adrenergic (n = 19) and mesenchymal (n = 5). Statistical significance was assessed with unpaired t-test; * p < 0.05.* **(C)** *Representative histograms depicting GD2 (left) and CD47 (right) surface expression (n = 3) as analyzed by flow cytometry on SHEP-2 (top) and SK-N-AS (bottom) cells. Isotype and secondary antibody controls are represented in white.* **(D–E)** *ADCC of SHEP-2 (***D***) and SK-N-AS (***E***) cells opsonized with (+) or without (-) dinutuximab (dimab) by unstimulated neutrophils (white bars) or stimulated with GM-CSF (light grey bars) or G-CSF (dark grey bars). n = 6, of 3 individual experiments. Statistical significance was tested with a paired t-test.* **(F)** *CD47 mRNA expression (expressed as log2) on a panel of four isogenic neuroblastoma cell line pairs with opposite phenotype: adrenergic (light grey) and mesenchymal (dark grey).* **(G)** *Representative histograms showing GD2 (left) and CD47 (right) surface expression (n = 2) as analyzed by flow cytometry on 691B (top) and 691T (bottom) cells. Isotype and secondary antibody controls are represented in white.* **(H–I)** *ADCC of primary patient-derived 691B (***H***) and 691T (***I***) cells opsonized with (+) or without (-) dinutuximab (dimab) by unstimulated neutrophils (white bars) or stimulated with GM-CSF (light grey bars) or G-CSF (dark grey bars) n = 4–6, of 3 individual experiments. Statistical significance was tested with a paired t-test; ** p < 0.01. ADRN, adrenergic. MES, mesenchymal.*

DISCUSSION

In recent years, multiple immunotherapeutic approaches have demonstrated promise in the field of pediatric oncology. One undeniable example of this is the use of antibody therapy targeting GD2 with dinutuximab in neuroblastoma. The implementation of dinutuximab into the standard of care for neuroblastoma has significantly increased the 5-year survival rate of high-risk patients from roughly 20% to 50%[49]. Despite the encouraging results of dinutuximab treatment, the prognosis of high-risk neuroblastoma patients remains poor; therefore, intense attempts are currently being made to identify novel immunotherapeutic approaches for the treatment of neuroblastoma. The reduced infiltration and activity of lymphocytes in this low immunogenic tumor, as well as in other pediatric tumors[2,8,50], limits, for now, the application of T cell targeting immune checkpoint inhibitors; therefore, the efforts of researchers are directed at the exploitation of other powerful immune modalities involving, for instance, the innate immune system.

In the present study, we have investigated the role of the CD47-SIRPα innate immune checkpoint in the context of antibody therapy with dinutuximab in neuroblastoma. First, we provided evidence of neutrophil infiltration in neuroblastoma tumors, as well as an upregulation of CD47 molecule throughout all disease stages relative to the levels found in the adrenal gland. These findings were used as a basis to study the involvement of CD47-SIRPα interactions in the neutrophil-mediated cytotoxicity of neuroblastoma cells. We found CD47-SIRPα interactions between neutrophil and tumor cells to limit the neutrophil's capability of inducing antibody-mediated cytotoxicity in vitro. This was shown by either genetically deleting CD47 molecules from the surface of several neuroblastoma cells or by using a blocking antibody for SIRPα. Just as found for other cancer types[18,33,34], we demonstrated how a disruption of the interaction potentiated the killing capabilities of neutrophils resulting in higher cytotoxicity towards the dinutuximab-opsonized target cells. From a therapeutic point of view, it seems beneficial to focus on the targeting of SIRPα with a blocking agent, given its more restricted expression on myeloid cells as compared to the ubiquitous expression of CD47[36]. Nonetheless, clinical trials that are currently being explored with antibodies targeting the CD47-SIRPα axis from the CD47 side in combination with tumor-specific monoclonal antibody therapy have shown minimal to moderate toxicity effects[36–38]. More importantly, the success rates of these clinical trials for adult cancers, together with the pre-clinical findings described in the present study, clearly support the clinical application of such a therapeutic approach for neuroblastoma patients in the near future. Furthermore, the experiments where lower neutrophil T:E ratios were used demonstrate the strength of anti-SIRPα treatment as it still significantly enhanced the cytotoxicity levels in the presence of low neutrophil counts.

In the absence of opsonizing dinituximab, we found no enhancing effects of CD47-SIRPα blockade, suggesting that CD47-SIRPα blockers may primarily be useful in combination with dinutuximab, and obviously when GD2 is present. This could be a potential drawback for patients with GD2-negative/low neuroblastoma variants that will not benefit from anti-GD2 immunotherapy. Despite the fact that the loss of GD2 antigen following monoclonal antibody therapy has been described as a rare phenomenon[51], it has been detected in a number of cases[52]. The prevalence of this event could also be appreciated in the present study, wherein we found neuroblastoma cells of mesenchymal phenotype to have lost some or all of the expression of the ganglioside on their surface membrane. To date, the exact mechanism behind GD2 loss is not fully understood, but the results from a recent study by Terzic et al. suggest that resistance to anti-GD2 immunotherapy may be due to selection, i.e. the presence of GD2-negative/low cells in primary tumors that may preferentially grow out during therapy[53].

The combination of GD2 loss (antigen-negative/low clones), perhaps together with the overexpression of CD47, may constitute an immune escape mechanism that tumors use in their favor. This can ultimately lead to clinical resistance or recurrence, a mechanism suggested by our findings, and which is in particular present in the mesenchymal phenotype. Therefore, alternative immunotherapeutic targets for antibody therapy are highly needed for neuroblastoma patients with GD2-negative/low variants. One example of an additional immunotherapeutic target currently under investigation that may be of interest in neuroblastoma is the B7-H3 molecule. This member of the B7 family of immunomodulatory regulators is homogeneously expressed in both primary and metastatic neuroblastomas, as well as in a large variety of solid cancers, while it shows low or null protein surface expression in most normal tissues[54,55]. More specifically, a recent study found neuroblastoma patients with GD2-negative/low variants to still express B7-H3 molecule in high levels, suggesting that B7-H3 might represent an optimal alternative targetable molecule for these patients in particular[56]. At least one anti-B7-H3 monoclonal antibody has already been developed, enoblituzumab, which showed potent antitumor activity by peripheral blood mononuclear cells towards B7-H3-expressing tumors[55–57] and has been recently clinically tested in a phase I trial for solid pediatric tumors, including neuroblastoma (www.clinicaltrials.gov: NCT02982941, accessed on 21/04/2021). In combination with CD47-SIRPα checkpoint blockade, this could be a feasible alternative to dinutuximab for patients with GD2-negative/low neuroblastoma variants. Nevertheless, CD47-SIRPα may not be the only mechanism by which tumor cells can evade neutrophil-mediated immune destruction as neutrophils are endowed with other potent inhibitory receptors[31].

CONCLUSIONS

Collectively, our findings provide a rational basis for the combination of the therapeutic antibody dinutuximab with CD47-SIRPα checkpoint blockade to potentiate the antitumor efficacy of neutrophils towards neuroblastomas, at least of adrenergic phenotype, which is expected to significantly improve the dinutuximab responsiveness and patients' prognosis.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://xenabrowser.net/ (accessed on 21/02/2021), http://r2.amc.nl (accessed on 22/03/2021) and https://www.ncbi.nlm.nih.gov/geo/ (accessed on 21/04/2021).

ACKNOWLEDGEMENTS

We wish to thank I. Timmerman for her input on the mesenchymal and adrenergic phenotype part of the study and for critically reading the manuscript.

AUTHOR CONTRIBUTIONS

Conceptualization, P.M.-S., G.A.M.T., K.F., and H.L.M.; methodology, P.M.-S., T.W.K., and H.L.M.; validation, P.M.-S., A.J.H., P.J.J.H.V., and K.S.; formal analysis, P.M.-S., A.J.H., and H.L.M.; writing—original draft preparation, P.M.-S., A.J.H., and H.L.M.; writing—review and editing, P.M.-S., A.J.H., P.J.J.H.V., K.S., R.v.B., T.K.v.d.B., G.A.M.T, K.F., T.W.K., and H.L.M.; visualization, P.M.-S and A.J.H.; supervision, K.F., T.W.K., and H.L.M.; funding acquisition, H.L.M. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST DISCLOSURE

T.K.v.d. B. is the inventor of patent EP2282772, owned by Stichting Sanquin Bloedvoorziening, entitled “Compositions and Methods to Enhance the Immune System”, which describes targeting CD47-SIRPα interactions during antibody therapy in cancer. Other co-authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

FUNDING

This research was funded by the Dutch Cancer Society, grant #11537.

REFERENCES

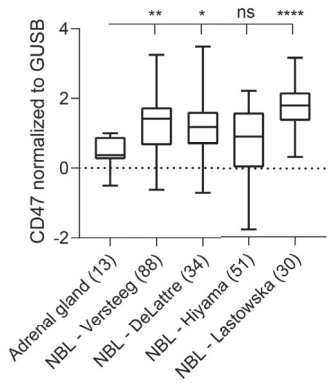
1. Darvin P, Toor SM, Sasidharan Nair V, Elkord E. Immune checkpoint inhibitors: recent progress and potential biomarkers. *Exp Mol Med*. 2018;50(12):1-11.
2. Park JA, Cheung N V. Limitations and opportunities for immune checkpoint inhibitors in pediatric malignancies. *Cancer Treat Rev*. 2017;58:22-33.
3. Majzner RG, Heitzeneder S, Mackall CL. Harnessing the Immunotherapy Revolution for the Treatment of Childhood Cancers. *Cancer Cell*. 2017;31(4):476-485.
4. Lawrence MS, Stojanov P, Polak P, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature*. 2013;499(7457):214-218.
5. Wedekind MF, Denton NL, Chen CY, Cripe TP. Pediatric Cancer Immunotherapy: Opportunities and Challenges. *Paediatr Drugs*. 2018;20(5):395-408.
6. Grobner SN, Worst BC, Weischenfeldt J, et al. The landscape of genomic alterations across childhood cancers. *Nature*. 2018;555(7696):321-327.
7. Raffaghello L, Prigione I, Airoldi I, et al. Mechanisms of immune evasion of human neuroblastoma. *Cancer Lett*. 2005;228(1-2):155-161.
8. Wienke J, Dierselhuis MP, Tytgat GAM, Kunkele A, Nierkens S, Molenaar JJ. The immune landscape of neuroblastoma: Challenges and opportunities for novel therapeutic strategies in pediatric oncology. *Eur J Cancer*. 2020;144:123-150.
9. van Egmond M, Bakema JE. Neutrophils as effector cells for antibody-based immunotherapy of cancer. *Semin Cancer Biol*. 2013;23(3):190-199.
10. Treffers LW, Hiemstra IH, Kuijpers TW, van den Berg TK, Matlung HL. Neutrophils in cancer. *Immunol Rev*. 2016;273(1):312-328.
11. Furumaya C, Martinez-Sanz P, Bouti P, Kuijpers TW, Matlung HL. Plasticity in Pro- and Anti-tumor Activity of Neutrophils: Shifting the Balance. *Front Immunol*. 2020;11.
12. Gale R, Zighelbloim J. Polymorphonuclear leukocytes in antibody-dependent cellular cytotoxicity. *J Immunol*. 1975;114(3):1047-1050.
13. Albanesi M, Mancardi DA, Jonsson F, et al. Neutrophils mediate antibody-induced antitumor effects in mice. *Blood*. 2013;122(18):3160-3164.
14. Yu AL, Gilman AL, Ozkaynak MF, et al. Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N Engl J Med*. 2010;363(14):1324-1334.
15. McGinty L, Kolesar J. Dinutuximab for maintenance therapy in pediatric neuroblastoma. *Am J Health Syst Pharm*. 2017;74(8):563-567.
16. Cheung NK, Dyer MA. Neuroblastoma: developmental biology, cancer genomics and immunotherapy. *Nat Rev Cancer*. 2013;13(6):397-411.
17. Cheung NK V., Sowers R, Vickers AJ, Cheung IY, Kushner BH, Gorlick R. FCGR2A polymorphism is correlated with clinical outcome after immunotherapy of neuroblastoma with anti-GD2 antibody and granulocyte macrophage colony-stimulating factor. *J Clin Oncol*. 2006;24(18):2885-2890.
18. Matlung HL, Babes L, Zhao XW, et al. Neutrophils Kill Antibody-Opsonized Cancer Cells by Trogoptosis. *Cell Rep*. 2018;23(13):3946-3959 e6.
19. Treffers LW, Zhao XW, van der Heijden J, et al. Genetic variation of human neutrophil Fcgamma receptors and SIRPalpha in antibody-dependent cellular cytotoxicity towards cancer cells. *Eur J Immunol*. 2018;48(2):344-354.

20. Derer S, Glorius P, Schlaeth M, et al. Increasing FcgammaRIIa affinity of an FcgammaRIII-optimized anti-EGFR antibody restores neutrophil-mediated cytotoxicity. *MAbs*. 2014;6(2):409-421.
21. Barker E, Mueller BM, Handgretinger R, Herter M, Yu AL, Reisfeld RA. Effect of a chimeric anti-ganglioside GD2 antibody on cell-mediated lysis of human neuroblastoma cells. *Cancer Res*. 1991;51(1):144-149.
22. Kushner BH, Cheung NK. Clinically effective monoclonal antibody 3F8 mediates nonoxidative lysis of human neuroectodermal tumor cells by polymorphonuclear leukocytes. *Cancer Res*. 1991;51(18):4865-4870.
23. Michon J, Moutel S, Barbet J, et al. In vitro killing of neuroblastoma cells by neutrophils derived from granulocyte colony-stimulating factor-treated cancer patients using an anti-disialoganglioside/anti-Fc gamma RI bispecific antibody. *Blood*. 1995;86(3):1124-1130.
24. Chen RL, Reynolds CP, Seeger RC. Neutrophils are cytotoxic and growth-inhibiting for neuroblastoma cells with an anti-GD2 antibody but, without cytotoxicity, can be growth-stimulating. *Cancer Immunol Immunother*. 2000;48(11):603-612.
25. Treffers LW, van Houdt M, Bruggeman CW, et al. FcgammaRIIIb Restricts Antibody-Dependent Destruction of Cancer Cells by Human Neutrophils. *Front Immunol*. 2018;9:3124.
26. Kushner BH, Cheung NK. GM-CSF enhances 3F8 monoclonal antibody-dependent cellular cytotoxicity against human melanoma and neuroblastoma. *Blood*. 1989;73(7):1936-1941.
27. van der Kolk LE, de Haas M, Grillo-López AJ, Baars JW, van Oers MH. Analysis of CD20-dependent cellular cytotoxicity by G-CSF-stimulated neutrophils. *Leukemia*. 2002;16(4):693-699.
28. Ragnhammar P, Frödin JE, Trotta PP, Mellstedt H. Cytotoxicity of white blood cells activated by granulocyte-colony-stimulating factor, granulocyte/macrophage-colony-stimulating factor and macrophage-colony-stimulating factor against tumor cells in the presence of various monoclonal antibodies. *Cancer Immunol Immunother*. 1994;39(4):254-262.
29. Martinez Sanz P, van Rees DJ, van Zogchel LMJ, et al. G-CSF as a suitable alternative to GM-CSF to boost dinutuximab-mediated neutrophil cytotoxicity in neuroblastoma treatment. *J Immunother Cancer*. 2021;9(5).
30. Favier B. Regulation of neutrophil functions through inhibitory receptors: an emerging paradigm in health and disease. *Immunol Rev*. 2016;273(1):140-155.
31. van Rees DJ, Szilagyi K, Kuijpers TW, Matlung HL, van den Berg TK. Immunoreceptors on neutrophils. *Semin Immunol*. 2016;28(2):94-108.
32. Willingham SB, Volkmer JP, Gentles AJ, et al. The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Proc Natl Acad Sci U S A*. 2012;109(17):6662-6667.
33. Zhao XW, van Beek EM, Schornagel K, et al. CD47-signal regulatory protein-α (SIRPα) interactions form a barrier for antibody-mediated tumor cell destruction. *Proc Natl Acad Sci U S A*. 2011;108(45):18342-18347.
34. Ring NG, Herndler-Brandstetter D, Weiskopf K, et al. Anti-SIRPα antibody immunotherapy enhances neutrophil and macrophage antitumor activity. *Proc Natl Acad Sci U S A*. 2017;114(49):E10578-e10585.
35. Horrigan SK, Reproducibility Project: Cancer Biology. Replication Study: The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Elife*. 2017;6:e18173.
36. Weiskopf K. Cancer immunotherapy targeting the CD47/SIRPα axis. *Eur J Cancer*. 2017;76:100-109.

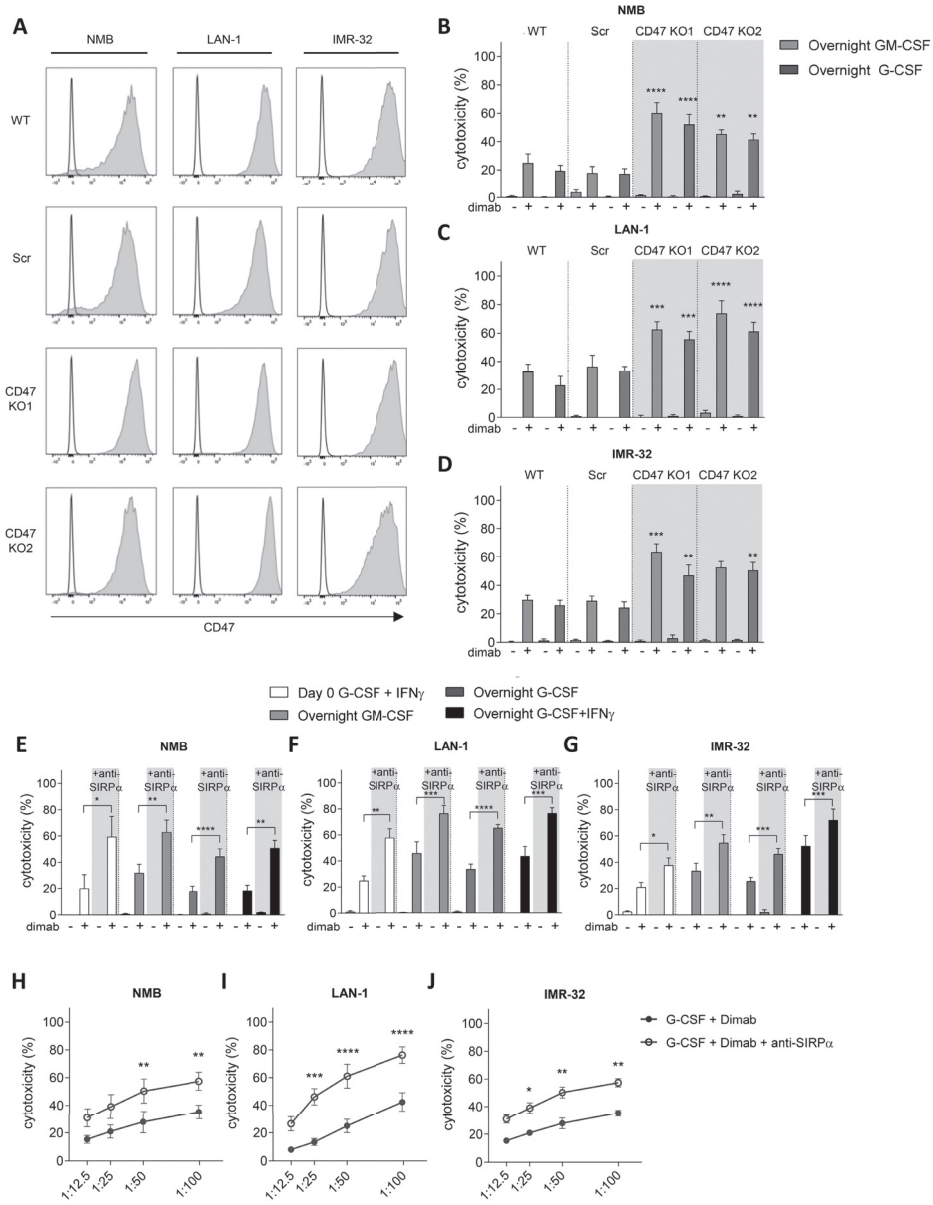
37. Advani R, Flinn I, Popplewell L, et al. CD47 Blockade by Hu5F9-G4 and Rituximab in Non-Hodgkin's Lymphoma. *N Engl J Med*. 2018;379(18):1711-1721.
38. Sikic BI, Lakhani N, Patnaik A, et al. First-in-Human, First-in-Class Phase I Trial of the Anti-CD47 Antibody Hu5F9-G4 in Patients With Advanced Cancers. *J Clin Oncol*. 2019;37(12):946-953.
39. Zhang W, Yu Y, Hertwig F, et al. Comparison of RNA-seq and microarray-based models for clinical endpoint prediction. *Genome Biol*. 2015;16:133.
40. Grassi L, Pourfarzad F, Ullrich S, et al. Dynamics of Transcription Regulation in Human Bone Marrow Myeloid Differentiation to Mature Blood Neutrophils. *Cell Rep*. 2018;24(10):2784-2794.
41. Kuijpers TW, Tool AT, van der Schoot CE, et al. Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood*. 1991;78(4):1105-1111.
42. Bate-Eya LT, Ebus ME, Koster J, et al. Newly-derived neuroblastoma cell lines propagated in serum-free media recapitulate the genotype and phenotype of primary neuroblastoma tumours. *Eur J Cancer*. 2014;50(3):628-637.
43. Neuroblastoma Stages and Prognostic Markers. Am. Cancer Soc. Published 2021. Accessed January 10, 2021. https://www.cancer.org/cancer/neuroblastoma/detection-diagnosis-staging/staging.html
44. Brisse HJ, McCarville MB, Granata C, et al. Guidelines for imaging and staging of neuroblastic tumors: consensus report from the International Neuroblastoma Risk Group Project. *Radiology*. 2011;261(1):243-257.
45. Maris JM. Recent advances in neuroblastoma. *N Engl J Med*. 2010;362(23):2202-2211.
46. Van Groningen T, Koster J, Valentijn LJ, et al. Neuroblastoma is composed of two super-enhancer-associated differentiation states. *Nat Genet*. 2017;49(8):1261-1266.
47. Boeva V, Louis-Brennetot C, Peltier A, et al. Heterogeneity of neuroblastoma cell identity defined by transcriptional circuitries. *Nat Genet*. 2017;49(9):1408-1413.
48. van Wezel EM, van Zogchel LMJ, van Wijk J, et al. Mesenchymal Neuroblastoma Cells Are Undetected by Current mRNA Marker Panels: The Development of a Specific Neuroblastoma Mesenchymal Minimal Residual Disease Panel. *JCO Precis Oncol*. 2019;(3):1-11.
49. Smith V, Foster J. High-Risk Neuroblastoma Treatment Review. *Children (Basel)*. 2018;5(9).
50. Vakkila J, Jaffe R, Michelow M, Lotze MT. Pediatric cancers are infiltrated predominantly by macrophages and contain a paucity of dendritic cells: a major nosologic difference with adult tumors. *Clin Cancer Res*. 2006;12(7 Pt 1):2049-2054.
51. Kramer K, Gerald WL, Kushner BH, Larson SM, Hameed M, Cheung NK. Disaloganglioside GD2 loss following monoclonal antibody therapy is rare in neuroblastoma. *Med Pediatr Oncol*. 2001;36(1):194-196.
52. Schumacher-Kuckelkorn R, Volland R, Gradehandt A, Hero B, Simon T, Berthold F. Lack of immunocytological GD2 expression on neuroblastoma cells in bone marrow at diagnosis, during treatment, and at recurrence. *Pediatr Blood Cancer*. 2017;64(1):46-56.
53. Terzic T, Cordeau M, Herblot S, et al. Expression of Disialoganglioside (GD2) in Neuroblastic Tumors: A Prognostic Value for Patients Treated With Anti-GD2 Immunotherapy. *Pediatr Dev Pathol*. 2018;21(4):355-362.
54. Seeger RC, Rosenblatt HM, Imai K, Ferrone S. Common antigenic determinants on human melanoma, glioma, neuroblastoma, and sarcoma cells defined with monoclonal antibodies. *Cancer Res*. 1981;41(7):2714-2717.

55. Loo D, Alderson RF, Chen FZ, et al. Development of an Fc-enhanced anti-B7-H3 monoclonal antibody with potent antitumor activity. *Clin Cancer Res*. 2012;18(14):3834-3845.
56. Dondero A, Morini M, Cangelosi D, et al. Multiparametric flow cytometry highlights B7-H3 as a novel diagnostic/therapeutic target in GD2neg/low neuroblastoma variants. *J Immunother Cancer*. 2021;9(4).
57. Castriconi R, Dondero A, Augugliaro R, et al. Identification of 4Ig-B7-H3 as a neuroblastoma-associated molecule that exerts a protective role from an NK cell-mediated lysis. *Proc Natl Acad Sci U S A*. 2004;101(34):12640-12645.

SUPPLEMENTAL DATA

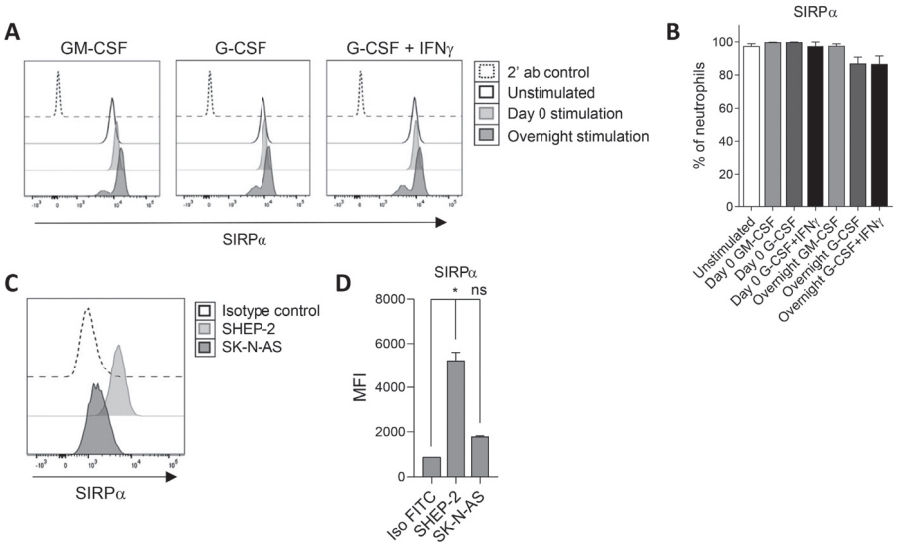


Supplemental Figure 1. CD47 expression in neuroblastoma. *Normalized CD47 mRNA expression levels in healthy adrenal gland and different neuroblastoma tumor databases. Number of individuals per dataset indicated in between brackets. Statistical significance was tested with one-way ANOVA with Sidak correction for multiple comparisons; ns, not significant; * p < 0.05; ** p < 0.01; **** p < 0.0001.*

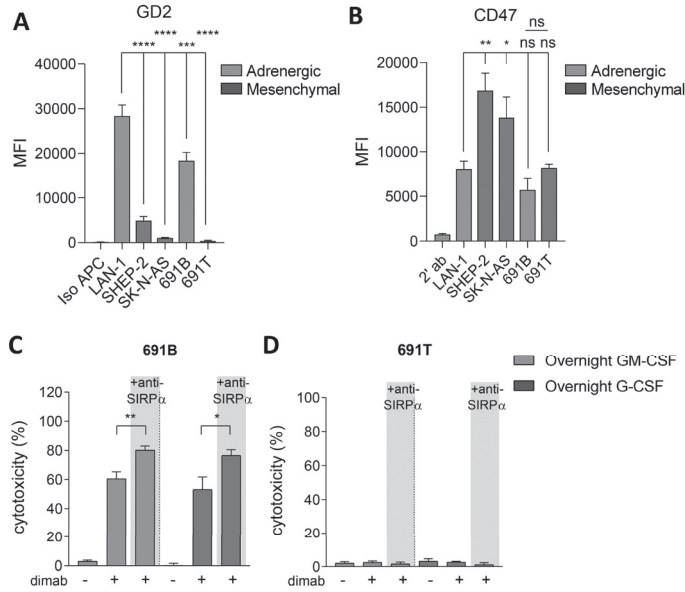


Supplemental Figure 2. GD2 expression and ADCCs of standard neuroblastoma cell lines upon overnight stimulation of neutrophils. (A) *Representative histograms depicting GD2 surface expression (n = 3) as analyzed by flow cytometry on (from left to right) NMB, LAN-1 and IMR-32 control cells (top two rows) and their respective CD47 KO variants (bottom two rows). Isotype controls are represented in white.* **(B–D)** *ADCC of control (WT and Scr, no background) and CD47 KO (CD47 KO1 and CD47 KO2, grey background) NMB (***B***), LAN-1 (***C***) and IMR-32 (***D***) cells opsonized with (+) or without (-) dinutuximab (dimab)*

by neutrophils stimulated overnight with GM-CSF (light grey bars) or G-CSF (dark grey bars). n = 6–8, of 4 individual experiments. Statistics were performed by one-way ANOVA with Sidak correction for multiple comparisons. **(E–G)** *ADCC of NMB (***E***), LAN-1 (***F***) and IMR-32 (***G***) cells opsonized with (+) or without (-) dinutuximab (dimab) by neutrophils stimulated with G-CSF in combination with IFNγ on day of isolation (white bars) or overnight stimulated neutrophils with GM-CSF (light grey bars), G-CSF (dark grey bars) or G-CSF in combination with IFNγ (black bars) in the absence (no background) or presence (grey background) of SIRPα blocking agent. n = 6–14, of 7 independent experiments. Statistical analysis was assessed with by a paired t-test.* **(H–J)** *ADCC of dinutuximab-opsonized NMB (H), LAN-1 (***I***) and IMR-32 (***J***) cells by neutrophils stimulated with G-CSF in the absence (filled circles) or presence (empty circles) of SIRPα blocking agent at different T:E ratios ranging from 1:12.5 to 1:100. n = 5, of 4 individual experiments. Statistical differences were tested with two-way ANOVA with Tukey's post hoc test; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001. WT, wildtype. Scr, scrambled.*



Supplemental Figure 3. SIRPα expression on neutrophils upon different stimulation conditions and on tumor cells. (A) *Representative histograms depicting SIRPα surface expression as analyzed by flow cytometry on unstimulated neutrophils (white histograms), or neutrophils stimulated with either (from left to right) GM-CSF, G-CSF or G-CSF in combination with IFNγ on the day of isolation (day 0 stimulation, light grey histograms) or after an overnight stimulation (dark grey histograms). Secondary antibody controls are represented with a dashed line.* **(B)** *SIRPα expression expressed as % for the different stimulation conditions. n = 4–12, from 5 independent experiments. Statistical differences were tested with ordinary one-way ANOVA with post hoc Sidak test.* **(C)** *Representative histogram showing SIRPα expression as analyzed by flow cytometry on SHEP-2 (light grey histogram) and SK-N-AS (dark grey histogram) neuroblastoma cells. Isotype antibody control is represented with a dashed line.* **(D)** *SIRPα expression on SHEP-2 and SK-N-AS cells expressed as MFI. n = 2, from 2 independent experiments. Statistical differences were tested with ordinary one-way ANOVA with post hoc Sidak test; * p < 0.05. MFI, mean fluorescence intensity.*



Supplemental Figure 4. Surface marker's comparison between a panel of neuroblastoma cell lines and ADCC of 691B and 691T with overnight stimulated neutrophils. (A–B) *GD2 (***A***) and CD47 (***B***) surface expression as analyzed by flow cytometry (MFI) on LAN-1, SHEP-2, SK-N-AS, 691B and 691T cell lines. n = 2–3, of 3 independent experiments. Statistical differences were tested with ordinary one-way ANOVA with post hoc Sidak test.* **(C–D)** *ADCC of primary patient-derived 691B (***C***) and 691T (***D***) cells opsonized with (+) or without (-) dinutuximab (dimab) by neutrophils stimulated overnight with GM-CSF (light grey bars) or G-CSF (dark grey bars). n = 4–6, of 3 individual experiments. Statistical significance was tested with a paired t-test; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001. MFI, mean fluorescence intensity.*

CHAPTER 5

FcαR-BEARING CAR NB4 NEUTROPHILS DISPLAY BUILT-IN ANTIGEN-SPECIFIC CYTOTOXIC ACTIVITY TOWARDS SOLID TUMOR TARGETS

Paula Martinez-Sanz, Bart Klein, Karin Schornagel, Paul J. J. H. Verkuijlen, Iris Langerak, Robin van Bruggen, Taco W. Kuijpers, Hanke L. Matlung

ABSTRACT

Chimeric antigen receptor (CAR)-T cells are used to enhance killing by T cells, however, this treatment shows limited efficacy in solid tumors. In this study, the cytotoxicity toward specific antigen-expressing tumor cells by differentiated neutrophil-like NB4 cells expressing an innovative CAR is examined. These differentiated NB4 CAR cells express a single chain variable fragment (scFv) of a tumor-specific antibody linked to the transmembrane and intracellular domains of the Fcα-receptor (FcαR). CARs directed to three different solid tumor antigens of different cancer origins (GD2, EGFR and HER2/neu) were transduced and showed direct cytotoxicity towards their respective targets. In contrast to the NB4 CAR neutrophils, control cells required target cell opsonization with tumor-specific antibody to achieve the same level of tumor cell killing. We further demonstrate that the killing induced by the CAR-transduced NB4 neutrophils was fully dependent on the downstream signaling by the intracellular domain of the activating FcαR, as demonstrated by pharmacological inhibition of Syk tyrosine kinase downstream of the receptor or the lack of activity with truncated CARs without the intracellular signaling domain. It is worth mentioning that CAR receptor expression did neither affect cell differentiation, nor the canonical anti-microbial functions of NB4 neutrophils. Overall, we provide evidence of a functional antigen-targeting CAR bearing a FcαR signaling domain for NB4 neutrophils as a proof of concept for further research on the generation of innate CAR approaches to provide long-lasting tumor control of solid tumors.

INTRODUCTION

Adoptive Cell Therapy (ACT) is a promising cancer immunotherapy consisting of the transfer of *in vitro* modified and expanded immune cells to a patient to help the body fight a particular tumor.[1,2] Currently, ACT mainly involves methods that use T cells as a basis and can be classified into three different subtypes, including ACT with tumor-infiltrating lymphocytes, ACT with T cells for which the T cell receptor has been genetically modified, and ACT with a Chimeric Antigen Receptor (CAR).[1,2] The latter, which are receptors initially designed to specifically redirect T cell cytotoxic activity towards a specific tumor antigen, have been used successfully in targeting hematological cancers, but unfortunately they have been less successful for its application in solid tumors.[3,4] Three unique challenges are posed to CAR T cell therapy by solid tumors. These include in first place the difficulty of finding a sufficiently safe tumor-associated antigen to target that will not increase the risks for on-target off-tumor toxicities.[4] Secondly, CAR T cells must be able to reach the tumor site, an arduous task due to the multiple physical and biological barriers that these T lymphocytes have to overcome, which result in the downregulation of their anti-tumor potential.[4] Last, the tumor microenvironment of solid tumors has been extensively characterized as hostile for T cells, attributable to immune resistance mechanisms of all kinds, which negatively influences T cell survival and induces exhaustion.[4] It is therefore of clinical relevance to find immunotherapeutic strategies that can be especially suitable and broadly applicable in tumor types where T cell-based therapies show limited success.

Innate cells, including neutrophils and macrophages, among others, are essential effectors of the immune system, and harnessing these against tumor cells is of longstanding interest. Myeloid cells infiltrate solid tumors very well,[5,6] yet, most studies show that they often support tumor progression.[7] In optimizing CAR therapy, specifically directing innate effector functions of these tumor-infiltrating cells against tumors by equipping them with target-specific CARs might provide them with the means to tip the balance towards a more anti-tumor environment, opening up new possibilities for long-lasting tumor control in solid tumors. At the same time, given that such innate cells also contribute to the generation of adaptive immune responses by inducing tumor antigen release,[8–11] these tumor-infiltrating cells are placed in the spotlight for targeted cancer therapy for non-haematological tumors in view to the believe that immunotherapeutic approaches that integrate both the adaptive and innate immune compartments result in more successful therapeutic outcomes.[12,13]

Here, we provide proof of concept of the cytotoxic potential towards solid tumor targets of a CAR technology specifically designed for its insertion in the maturation-inducible neutrophil-like NB4 cell line as a model system for neutrophils. The endodomain of our CAR constructs is composed of the transmembrane and intracellular part of the Fcα-receptor (FcαR), hypothesized to induce stronger anti-tumor effects when compared to Fcγ-receptor

(FcγR) signaling.[14–16] On the other end, the extracellular antigen recognition domain of our FcαR-based CARs consisted of the single chain variable fragment (scFv) derived from monoclonal antibodies recognizing relevant solid tumor antigens. To broaden the potential therapeutic applicability of our CAR, we focused on three well-known antigens including GD2 (overexpressed on tumors of neuroectodermal origin), EGFR (overexpressed on carcinomas), and HER2/neu (overexpressed on a subset of breast cancers). We showed that genetically modified NB4 CAR neutrophils were equipped with the intrinsic ability to recognize and effectively trogocytose and kill their respective unopsonized tumor cells, in contrast to the control cells. In addition, we found that the downstream signaling of the activating FcαR comprising the intracellular part of our CAR was indispensable for the generation of potent anti-tumor responses by the NB4 CAR neutrophils. By combining the specificity of antigen recognition with the lethality of these potent cytotoxic innate cells, our data provide sufficient preclinical evidence to support the translation of our findings to primary human innate cells. Ultimately, this would help to overcome the challenges that therapies such as CAR T cells face for the treatment of solid tumors.

MATERIALS AND METHODS

In vitro culture and genetic modifications

LAN-1, NMB, IMR-32, TC-71 (GD2-expressing neuroblastoma and Ewing's sarcoma cell lines), A431 (EGFR-expressing epidermoid carcinoma cell line), and SKBR3 (HER2/neu-expressing breast cancer cell line) were obtained from either ATCC or from Leibniz Institute and were routinely cultured at 37°C and 5% CO_2 and maintained in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 20% of heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (further referred to as IMDM complete medium) for up to 3 months. NB4 cells, a maturation-inducible cell line derived from a patient with acute promyelocytic leukemia[17] obtained from ATCC and routinely cultured and maintained in IMDM complete medium, were differentiated towards neutrophil-like cells by 7 days stimulation with 5 µmol/L All-Trans Retinoic Acid (ATRA; Sigma Aldrich) before their use in functional assays. All cell lines tested negative for Mycoplasma using PCR.

For antigen-targeting CAR expression in NB4 neutrophils, the coding sequences of the heavy and light chain variable fragment (scFv) of either GD2-targeting antibody dinutuximab, EGFR-targeting antibody cetuximab, or HER2/neu-targeting antibody trastuzumab were connected via a linker and coupled to the transmembrane domain and intracellular tail of an FcαR followed by an IRES GFP tracer, ensuring the cells transduced properly were GFP positive. For specific experiments, a truncated version of the CAR lacking the intracellular tail of the FcαR was also generated. The DNA sequences encoding the CAR, linker, spacer

and FcαR were ordered at Thermo Fisher Scientific and cloned into pENTR1A (Invitrogen), in which an IRES GFP was cloned previously. The resulting pENTR1A – CAR-FcαR IRES GFP constructs were recombined with lentiviral vector pRRL PPT SFFV prester SIN – Gateway Cassette B, using LR Clonase II (Thermo Fisher Scientific). To produce lentiviral particles, HEK293T cells were co-transfected with lentiviral vector, pMDLg/pRRE, pRSV-rev and pCMV-VSVg in IMDM complete medium. After 2 and 3 days of transfection, the lentivirus containing supernatant was filtered through a 0.45 μM filter and added to NB4 cells, which were then routinely passed. The cells were sorted on scFv of anti-GD2, anti-EGFR, or anti-HER2/neu antibody expression, as well as on GFP positivity by flow cytometry (explained below). As control for transfection, wildtype NB4 neutrophils transduced with an IRES GFP tracer only were taken along (further referred to as control NB4).

To knock out GD2 ganglioside from LAN-1 cells, gRNAs directed against B4GALNT1 were selected using the CRISPOR online tool. These gRNAs were ordered at Integrated DNA Technologies and cloned into lentiviral plasmid pLentiCrispR-v2, after which they were sequence verified. Production of lentiviral particles and transduction of LAN-1 cells were performed as described above for the CAR-expressing NB4 cells. Transduced LAN-1 cells were selected on 1 μg/ml puromycin (Invivogen) and GD2KO cells were sorted on the lack of GD2 expression by flow cytometry. A successful knockout was found using gRNA 5' cgtcccgggtgctcgcgtac 3'.

Flow cytometry for surface markers

The expression of the scFv of the anti-GD2 and anti-EGFR antibodies, or the anti-HER2/neu antibody on the CAR transduced NB4 cells was detected with the primary antibody Biotin-SP AffiniPure F(ab')2 goat anti-mouse IgG or Biotin-SP AffiniPure F(ab')2 goat anti-human IgG (both at 1 μg/mL; Jackson Immunoresearch), respectively. Streptavidin Alexa Fluor 647 (10 μg/mL; Life Technologies) was used for secondary staining. For detection of maturation markers, NB4 neutrophils were labeled with APC-labeled mAbs against FcγRI (CD64, clone 10.1; Bio-Rad), FcγRII (CD32, clone AT10; Bio-Rad), and CD11b (clone CLB-mon-gran/1-B2; Sanquin Pharmaceuticals), and PE-Cy7-labeled mAb against FcγRIII (CD16, clone 3G8; BD Pharmingen). SIRPα was detected with a mAb against human SIRPα (clone 4G5, produced in house) and goat anti-mouse F(ab')2 Alexa Fluor 633 (Thermo Fisher Scientific) was used for the secondary staining. For target cell characterization, the used cell lines and their derivatives were authenticated by flow cytometry using the following antibodies: dinutuximab (anti-GD2, Unituxin, Ch14.19, 1 μg/mL; United Therapeutics) for LAN-1, NMB, IMR-32 and TC-71 cells; cetuximab (anti-EGFR, 10 μg/ml; Merck KGaA) for A431 cells, and trastuzumab (Herceptin, anti-HER2/neu, 10 μg/ml; Roche) for SKBR3 cells. Each incubation lasted 20 minutes and was performed on ice. Flow cytometry data were

acquired using BD FACS CantoII™ flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 10.8; Becton Dickinson).

Western blotting

5×10^6 NB4 neutrophils were washed in phosphate-buffered saline (PBS) and incubated with 1 µL of serine protease inhibitor diisopropyl fluorophosphate (DFP; Sigma Aldrich) for 10 minutes on ice. After incubation, cells were centrifuged at high speed (14.000 rpm) and pellets were resuspended in 50 µL of complete Protease Inhibitor Cocktail (Roche diagnostics)/ ethylenediaminetetraacetic acid (EDTA, 0.45 mol/L) solution and 50 µL of 2x sample buffer (25 mL Tris B, Invitrogen; 20 mL 100% glycerol, Sigma Aldrich; 5 g sodium dodecyl sulphate (SDS), Serva; 1.54 g dithiothreitol (DTT), Sigma Aldrich; 20 mg bromophenol blue, Sigma Aldrich; 1.7 mL β-mercaptoethanol, Bio-Rad; and H_2O to 50 mL, Gibco) at 95˚C for 30 minutes while vortexing every 10 minutes. For electrophoresis, 1×10^6 cells were loaded onto a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel and ran at 80 to 120 V. Proteins were transferred to a nitrocellulose membrane (GE Healthcare Life Science) while running at 0.33 A for 1.5 hours. The membrane was blocked with 5% Bovine serum albumin (BSA; Sigma)/ tris-buffered saline with Tween 20 (TBST) for 1 hour at room temperature and further stained with antibodies diluted in 2.5% BSA/TBST. The Biotin-SP AffiniPure F(ab')2 goat anti-mouse antibody (0.1 µg/mL, incubated overnight at 4˚C) was used for detection of the scFv region of the GD2-CAR constructs, and IRDye 680 Streptavidin (0.4 µg/mL, 1 hour at room temperature; LICOR) was used for analysis with Odyssey (LI-COR Biosciences). Loading control was assessed by using rabbit anti-GAPDH (1.5 mg diluted in 3 mL of 2.5% BSA/TBST; Millipore) and donkey anti-rabbit IgG IRDye 800 (LI-COR) suitable for analysis using Odyssey (LI-COR Biosciences).

NADPH oxidase activity assay

The nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity was assessed by measuring the release of hydrogen peroxide (H_2O_2) with an Amplex Red kit (Molecular Probes). NB4 neutrophils (0.25×10^6) were left unstimulated in 4-(2-hydroxyethyl) –1-piperazineethanesulfonic acid supplemented with 5 g/L human albumin (Albuman, Sanquin Plasma Products), 1 mM CaCl and 5.5 mM glucose (further referred to as HEPES buffer) or were stimulated for 30 minutes at 37°C with unopsonized zymosan (1 mg/mL; MP Biomedicals), serum-treated zymosan (STZ, 1 mg/mL), phorbol 12-myrisatate 13-acetate (PMA, 100 ng/mL; Sigma Aldrich), *N*-formylmethionine-leucyl-phenylalanine (fMLP, 1 µmol/L; Sigma Aldrich) or platelet-activation factor (PAF, 1 µmol/L; Sigma Aldrich)/ fMLP, in the presence of Amplex Red (0.5 µmol/L) and horseradish peroxidase (1 U/mL). Fluorescence derived from Amplex Red conversion into Resorufin was measured at 30-second intervals for 30 minutes with an Infinite F200 PRO plate reader (Tecan). Results are shown as nmol H_2O_2/min $\times 10^6$ cells.

Adhesion assay

NB4 neutrophils (5 × 10^6/mL) were fluorescently labeled with 1 μM calcein-AM (Molecular Probes) for 30 minutes at 37°C and brought to a concentration of 2 × 10^6/mL in HEPES buffer. Calcein-labeled cells were stimulated with Pam3Cys (20 mg/mL; EMC Microcollections), C5a (10 nmol/L; Sigma Aldrich), tumor necrosis factor α (TNFα, 10 ng/mL; Peprotech), PAF (100 ng/mL), fMLP (30 nmol/L), DTT (10 mmol/L; Sigma Aldrich) or PMA (100 ng/mL) in an uncoated 96-well MaxiSorp plate (Nunc) for 30 minutes at 37°C and 5% CO_2. Cells in HEPES buffer were used to determine spontaneous adhesion. After stimulation, plates were washed with PBS and adhesiveness of the cells was determined by a Tecan Infinite F200 PRO plate reader after lysis with 0.5% Triton X-100 solution (Sigma Aldrich) for 10 minutes at room temperature. Results are shown as percentage of total input of calcein-labeled cells.

Chromium-based ADCC assay

The cytotoxic activity of NB4 neutrophils was assessed in a standard 6-hour chromium-release assay. In brief, target cells were labeled with 100 μCi ^{51}Cr (PerkinElmer) for 90 minutes at 37°C and subsequently co-incubated with NB4 cells over a range of target:effector (T:E) ratio from 1:25 to 1:200 in a 96-well U-bottom plate (Corning) at 37°C and 5% CO_2 in their culture medium. Where indicated, target cells were opsonized with the respective opsonizing antibody: LAN-1, NMB, IMR-32 and TC-71 with dinutuximab (1 μg/mL), A431 with cetuximab (10 μg/ml), and SKBR3 with trastuzumab (10 μg/ml). For the indicated experiments, effector cells were pre-incubate with Syk block BAY61-3606 (10 μg/mL; Sigma Aldrich). Spontaneous and maximum ^{51}Cr release were determined by incubating the target cells without effector cells and by treating the target cells with a 0.1% Triton X-100 in culture medium, respectively. After the 6-hour incubation, the quantification of ^{51}Cr release into the supernatant was analyzed in a MicroBeta^2 plate reader (PerkinElmer) and percentage of cytotoxicity was determined using the following formula: [(experimental release−spontaneous release)/(maximum release−spontaneous release)]×100%. All conditions were performed in duplicate.

FACS-based trogocytosis

Tumor cells were stained with either 1 μM (for LAN-1) or 5 μM (for A431 and SKBR3) lipophilic membrane dye 1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine (DiD; Invitrogen). After labeling, cells were washed twice with PBS. Cells were co-incubated at a T:E ratio of 1:5 with NB4 neutrophils in the absence or presence of appropriate opsonizing antibodies (described above) in a U-bottom 96-well plate (Greiner Bio-One) for 90 minutes at 37°C and 5% CO_2 in their culture medium. After incubation, cells were fixed with STOPbuffer (PBS containing 20 mM sodium fluoride, 0.5% paraformaldehyde (PFA) and 1% BSA) and analyzed using flow cytometer Canto II (BD Biosciences). The NB4

neutrophil population was assessed for the uptake of tumor cell membrane dye DiD with FlowJo software (V.10.6.1; Becton Dickinson).

Live cell trogocytosis imaging

LAN-1 cells labeled with DiD and 2.5 nM cytoplasmic dye Calcein Red-Orange-AM (ThermoFisher) were co-incubated with unstained NB4 neutrophils at a T:E ratio of 1:5 in glass chambered coverslips (Ibidi) of 9.4×10.7×6.8 mm^3 well dimensions in IMDM complete medium at 37°C. Where indicated, 1 μg/mL dinutuximab was added. Imaging started 5 minutes after start of co-culture and lasted up to 117 minutes using a Leica TCM SP8 confocal microscope (Leica).

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 9 (GraphPad Software). Data were evaluated by paired one-way or two-way ANOVA, or two-tailed student's t-test. Where indicated, correction for multiple comparisons using either Sidak's test was performed. The results are presented as the mean ± SEM. Data were considered significant when $p < 0.05$ for all comparisons.

RESULTS

FcαR-bearing CAR NB4 neutrophils differentiate normally after transduction

To model the potential for CAR-mediated redirection of neutrophil cytotoxicity towards tumors, we transduced the human maturation-inducible neutrophil-like NB4 cell line with an anti-GD2 CAR (further referred to as GD2-FcαR CAR or GD2-CAR, unless otherwise specified), composed of the V_H and V_L (scFv) of dinutuximab antibody, bearing the FcαR intracellular domain (**Figure 1A**). After lentiviral transduction and enrichment by flow cytometry sorting, we demonstrated that transduced NB4 neutrophils expressed the GD2-CAR with high efficiency as detected by cell surface staining for the scFv fragment of the CAR (>80% expression) as well as by western blot of NB4 lysates (84.3 kDa, **Figure 1B-C**).

To assess whether the lentiviral transduction did not hamper the differentiation process of these cells towards neutrophil-like cells upon stimulation with All-Trans Retinoic Acid (ATRA), we examined the surface expression levels of specific NB4 maturation markers (FcγRI, FcγRII, FcγRIII, CD11b and SIRPα) by flow cytometry. We detected no differences in MFI for any of the cell surface markers when comparing control NB4 neutrophils with those expressing the GD2-CAR (**Figure 1D-E**). In addition, two anti-microbial effector functions of neutrophils were evaluated: the capability to produce reactive oxygen species (ROS), and CD11b/CD18-mediated adhesion. Both ROS production and their ability to adhere did not significantly differ between control NB4 and CAR transduced NB4 neutrophils in response

to specific stimuli (**Figure 1F-G**). Together, these data indicate that CAR transduction on NB4 neutrophils did not alter cell maturation nor the two main anti-microbial properties of these cells, showing a similar phenotype as their respective control cells.

FcαR-bearing CAR NB4 neutrophils acquire direct antigen-specific cytotoxic activity towards unopsonized targets

Just as primary neutrophils, NB4 differentiated neutrophils are endowed with the ability to induce antibody-dependent cellular cytotoxicity (ADCC) against antibody-opsonized tumor cells via the engagement of Fc receptors on their surface.[18–22] Here, we aimed at evaluating whether the retargeted FcαR-based CAR-expressing NB4 neutrophils displayed cytotoxic ability towards their respective solid tumor targets in the absence of an opsonizing antibody. GD2-CAR transduced NB4 neutrophils showed efficient killing of $GD2^+$ neuroblastoma cells LAN-1, NMB and IMR-32, as well as the $GD2^+$ Ewing's sarcoma TC-71 cell line, without the need for anti-GD2 opsonization with dinutuximab (**Figure 2A-D**). This seemed particularly efficient for T:E ratios from 1:50 to 1:200 for the majority of the cell lines tested. The level of cytotoxicity by the retargeted CAR-transduced cells in unopsonized conditions was as strong as that induced by control NB4 neutrophils against the same dinutuximab-opsonized targets, showing no significant differences, while the unopsonized control cells turned out resistant in the absence of antibody (**Figure 2A-D**).

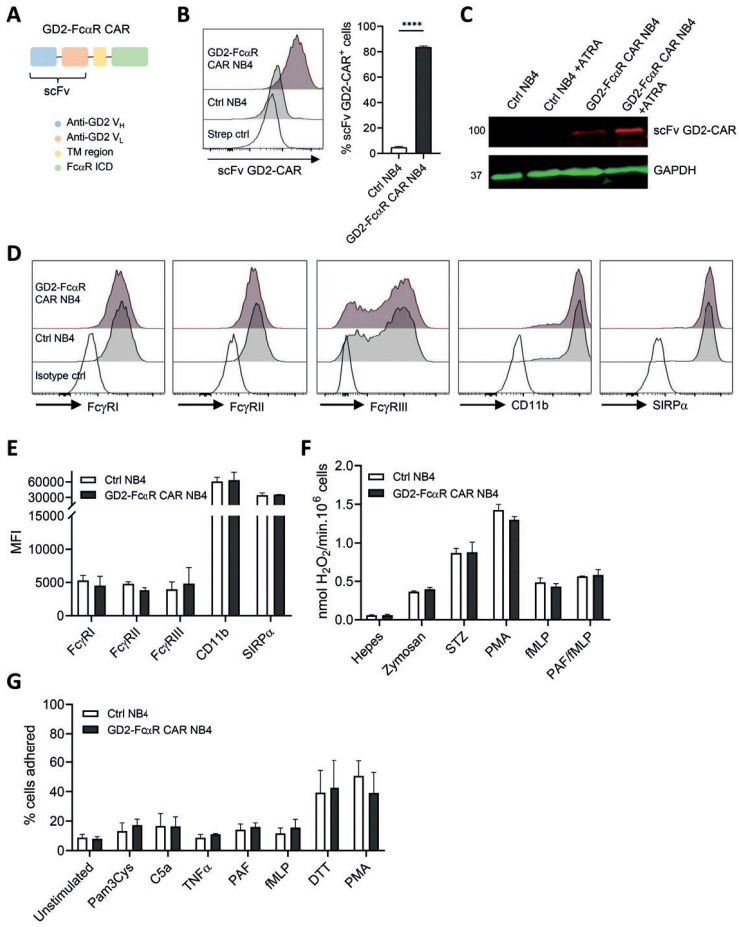


Figure 1. Anti-GD2-FcαR CAR NB4 cells differentiate normally after transduction (A) *Schematic representation of the construct used in lentiviral vectors to express anti-GD2-FcαR CAR in NB4 cells.* **(B)** *Representative flow cytometry histogram (left) and quantification of GD2-CAR expression expressed as % of positive cells (right) after sorting of transduced and untransduced NB4 cells. N=3.* **(C)** *Representative example of western blot stained for detection of scFv GD2-CAR (84.3 kDa) of undifferentiated or ATRA-differentiated control or CAR transduced NB4 cells. Staining for GAPDH was used as a loading control.* **(D-E)** *Representative flow cytometry histograms (D) and quantification (E) of surface expression levels of NB4 maturation markers expressed as MFI of control and CAR transduced cells. N=2-4.* **(F)** *NADPH-oxidase activity of control or CAR transduced cells in the presence of the indicated stimuli expressed as nmol H_2O_2/min per 10^6 cells. N=2-4.* **(G)** *Percentage of adhered control or CAR transduced cells in response to the indicated stimuli. N=4. V_H and V_L, V domains of the heavy and light chains. TM, transmembrane. ICD, intracellular domain. scFv, single chain variable fragment. ATRA, All-Trans Retinoic Acid.*

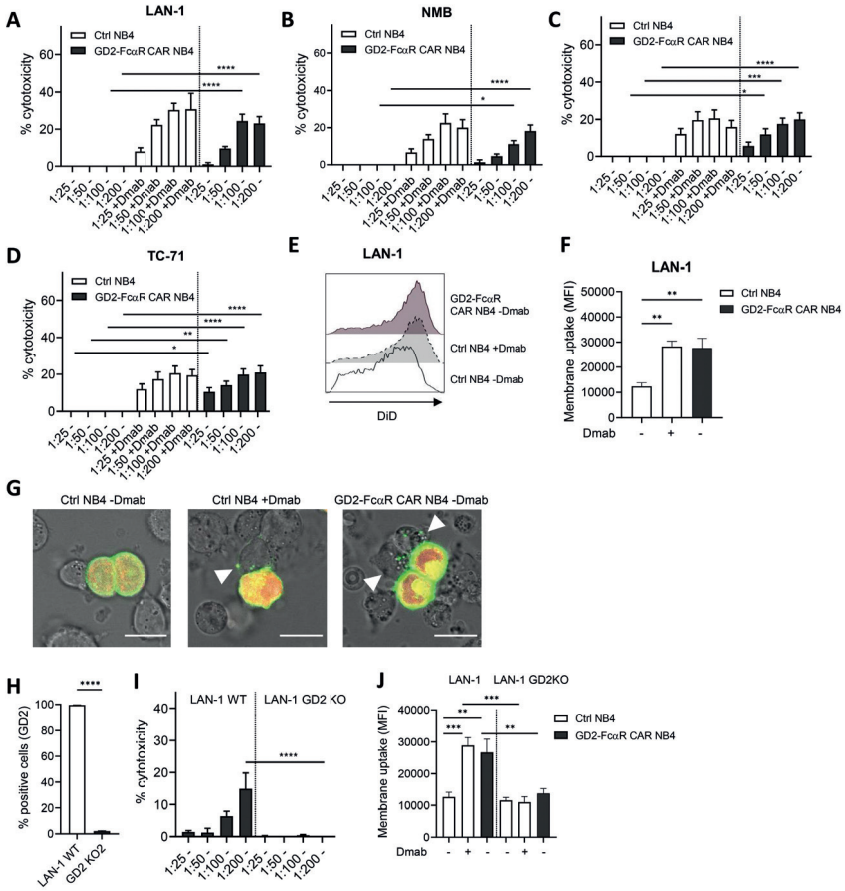


Figure 2. Anti-GD2-FcαR CAR-expressing NB4 cells acquire intrinsic antigen-specific cytotoxic activity (A-D) *Assessment of control and CAR transduced NB4-mediated cytotoxicity against GD2⁺ LAN-1, NMB, IMR-32 and TC-71 target cells in the absence or presence of dinutuximab (Dmab) at different T:E ratios in a ^{51}Cr-release assay. N=8-12.* **(E-F)** *Representative flow cytometry histogram (E) and quantification (F) of trogocytosis against GD2⁺ LAN-1 target cells in the presence or absence of dinutuximab (Dmab) assessed by the DiD positivity within the effector cells as analyzed by flow cytometry. A T:E ratio of 1:5 was used. N=4.* **(G)** *Live cell confocal imaging stills showing NB4 cells taking up pieces (white arrows) of DiD (green) and Calcein Red-Orange-AM- (orange) labeled LAN-1 cells. Note the uptake of the membrane dye only and no uptake of the orange cytoplasmic dye by the effector cells. Imaging started 5 minutes after start of co-culture and took place within 117 minutes from the start of the recording. Scale bar represents 20 µm.* **(H)** *Surface expression levels of GD2 antigen on the membrane of LAN-1 WT or the GD2 KO variant expressed as percentage of positive cells. N=2.* **(I)** *Assessment of NB4-mediated cytotoxicity by GD2-FcαR CAR transduced cells against LAN-1 WT or LAN-1 GD2KO targets at different T:E ratios in a ^{51}Cr-release assay. N=4.* **(J)** *Quantification of trogocytosis by control and CAR transduced NB4 cells towards LAN-1 WT or LAN-1 GD2KO targets as analyzed in a FACS-based trogocytosis assay. N=4. Dmab, dinutuximab. MFI, mean fluorescence intensity.*

We further evaluated the ability of the GD2-CAR expressing NB4 neutrophils to perform trogocytosis, an active mechanism involving the uptake of plasma membrane from a donor cell[23] that has been claimed to be a cytotoxic mechanism in the context of neutrophil-mediated antibody-dependent tumor cell killing.[24,25] The amount of plasma membrane uptake by the effector cells can be measured by a FACS-based trogocytosis assay upon co-culture with tumor cells that have been labeled with a membrane dye. Here, LAN-1 cells were labeled with the membrane dye DiD. In contrast to control NB4 neutrophils, GD2-CAR expressing NB4 neutrophils became positive for the membrane dye DiD in conditions where no opsonizing antibody was present, indicative of trogocytosis (**Figure 2E-F**). Just as seen for the killing assays, the trogocytosis levels by CAR expressing cells in unopsonized conditions were as potent as those found for the control cells in co-culture with dinutuximab-opsonized cells, suggesting that the CAR expressing cells are able to effectively trogocytose the target cells. In an attempt to visualize trogocytic events, we performed live cell confocal imaging for the same conditions. To distinguish trogocytosis from phagocytosis we additionally labeled the target cells with the cytoplasmic dye Calcein Red-Orange-AM. Indeed, the retargeted GD2-CAR NB4 neutrophils had the ability to take up pieces of membrane dye when put in co-culture with their unopsonized targets, as observed by the effector cells becoming positive for the tumor membrane dye, but not the cytoplasmic dye (which would indicate phagocytosis, **Figure 2G**).

To confirm that the GD2-CAR-induced cytotoxicity of NB4 neutrophils was antigen-dependent, we knocked out GD2 from the cell membrane of LAN-1 cells using CRISPR-Cas9. By interfering with B4GALNT1 (GD2 synthase), one of the enzymes directly responsible for GD2 synthesis,[26] we generated LAN-1 GD2KO cells with a KO efficiency of more than 90% (**Figure 2H**). We demonstrated that the cytolytic activity of the GD2-CAR NB4 neutrophils was highly ligand-specific as shown by the absence of detectable tumor cell killing and trogocytosis towards the LAN-1 GD2KO targets (**Figure I-J**).

In parallel, we also assessed the effector functions of two other FcαR-based CAR constructs directed towards EGFR and HER2/neu, two major solid tumor antigens abundantly expressed in carcinomas and a subset of breast cancers, respectively.[27–31] **Figures 3A-B** and **F-G** show the schematic representation and the specific expression of the constructs after transduction in NB4 neutrophils. Functionally, we obtained similar results as shown for the GD2-CAR differentiated neutrophils: both the killing and trogocytic capacities by the EGFR- or HER2/neu-CAR-transduced NB4 cells towards their unopsonized targets ($EGFR^+$ epidermoid carcinoma A431 cells, and $HER2/neu^+$ breast cancer SKBR3 cells, respectively) were at least as potent as those exerted by the control NB4 neutrophils when in presence of the respective opsonizing antibody (**Figures 3C-E** and **H-J**). Exceptionally, although not significant, we found a trend for even higher killing and trogocytosis by the HER2/neu-CAR NB4 neutrophils in unopsonized conditions, when compared to the control cells in the presence of trastuzumab (**Figure 3H-J**).

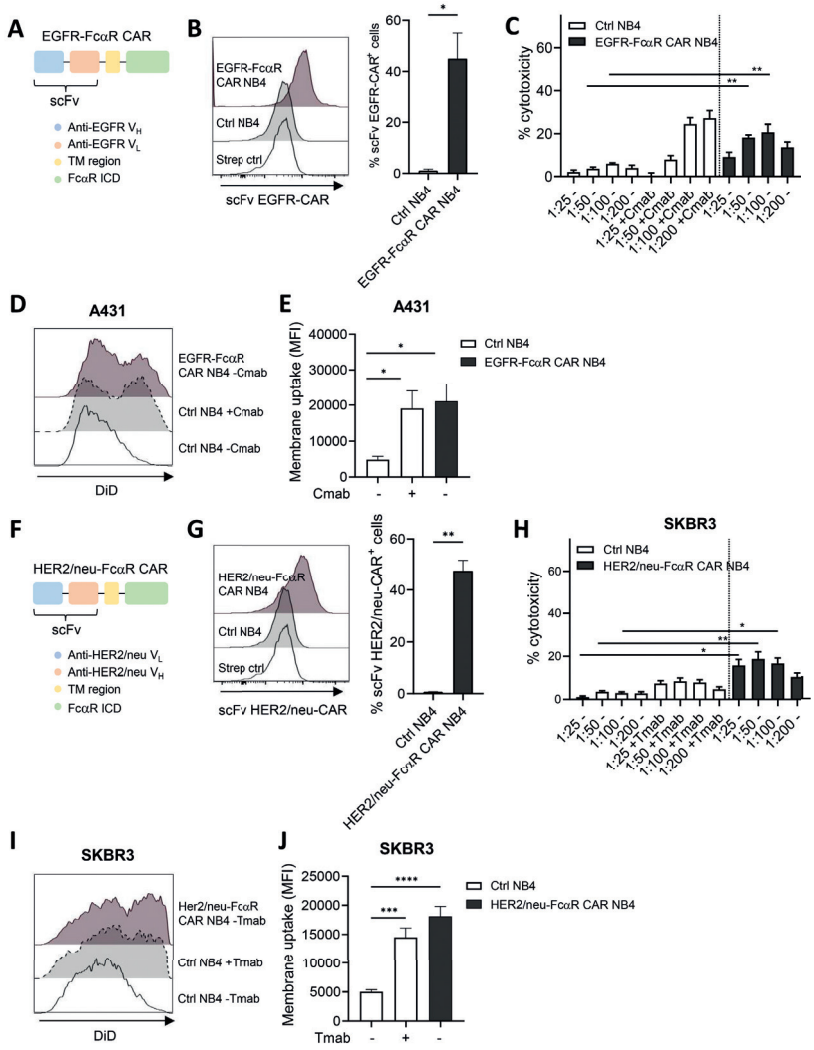


Figure 3. Cytotoxic activity of anti-EGFR- and anti-HER2/neu-FcαR CAR-expressing NB4 cells is redirected towards their respective unopsonized targets (A) *Schematic representation of the construct used in lentiviral vectors to express anti-EGFR-FcαR CAR in NB4 cells.* **(B)** *Representative flow cytometry histogram (left) and quantification of EGFR-CAR expression expressed as % of positive cells (right) after sorting of transduced and untransduced NB4 cells. N=2.* **(C)** *Assessment of control and CAR transduced NB4-mediated cytotoxicity against EGFR⁺ A431 target cells in the absence or presence of cetuximab (Cmab) at different T:E ratios in a ^{51}Cr-release assay. N=5.* **(D-E)** *Representative flow cytometry histogram (D) and quantification (E) of trogocytosis against EGFR⁺ A431 target cells in the presence or absence of cetuximab (Cmab) assessed by the DiD positivity within the effector cells as analyzed by flow cytometry. A T:E ratio of 1:5 was used. N=8.* **(F)** *Schematic representation of the construct used in lentiviral vectors to express anti-HER2/neu-FcαR CAR in NB4 cells.* **(G)** *Representative flow cytometry histogram (left) and quantification of HER2/neu-CAR expres-*

sion expressed as % of positive cells (right) after sorting of transduced and untransduced NB4 cells. N=2. **(H)** *Assessment of control and CAR transduced NB4-mediated cytotoxicity against HER2/neu⁺ SKBR3 target cells in the absence or presence of trastuzumab (Tmab) at different T:E ratios in a ^{51}Cr-release assay. N=12.* **(I-J)** *Representative flow cytometry histogram (I) and quantification (J) of trogocytosis against HER2/neu⁺ SKBR3 target cells in the presence or absence of trastuzumab (Tmab) assessed by the DiD positivity within the effector cells as analyzed by flow cytometry. A T:E ratio of 1:5 was used. N=8. V_H and V_L, V domains of the heavy and light chains. TM, transmembrane. ICD, intracellular domain. scFv, single chain variable fragment. Cmab, cetuximab. Tmab, trastuzumab. MFI, mean fluorescence intensity.*

Functionality of NB4 CAR depends on the signaling downstream FcαR domain

As described by a large body of literature, the Fc receptor crosslinking of activating Fc receptors, including the FcαR, initiates a signal transduction cascade which begins with activation of Syk tyrosine kinases that phosphorylate ITAM motifs culminating in cellular activation.[14,32,33] Hence, we tested an inhibitor approach to study the involvement of the intracellular domain of our Fcα-based CAR by pharmacologically interfering with the downstream signaling. As expected, the Syk tyrosine kinase inhibitor BAY61-3606 essentially abolished the killing ability by the CAR transduced NB4 neutrophils (**Figure 4A**), indicating that an activation of this kinase is indispensable for CAR-mediated cytotoxicity, just as also detected by the antibody-mediated killing induced by control NB4 neutrophils (**Suppl. Figure 1**).

Additionally, to provide further evidence that the induction of CAR-mediated cytotoxicity is an active process requiring signaling downstream of FcαR to which our CAR has been fused to, we sought to compare the cytotoxic potential of NB4 neutrophils expressing either the intact (GD2-FcαR) or the truncated (GD2-Δcyt) version of the CAR construct lacking the FcαR intracellular domain (**Figure 4B**). The transduction efficiency of NB4 neutrophils bearing the truncated construct was equal to that of the cells transduced with the intact construct (>80%, **Figure 4C**). Western blot analysis confirmed that the GD2-Δcyt CAR construct resulted in a shorter protein (79.8 kDa) compared to the full-length construct (84.3 kDa, **Figure 4D**). We further validated efficient cell differentiation of the GD2-Δcyt CAR transduced cells by evaluating the expression of the various NB4 maturation markers, as well as their ability to produce ROS and their adhesion capacities, which were comparable to that of the intact CAR-transduced cells (**Figure 4E-H**). Importantly, we found that the cytoplasmic tail of our CAR encoding for the intracellular signaling domain of a FcαR was absolutely required for the effector cells to be able to kill (**Figure 4I**). Moreover, the trogocytosis levels induced by the GD2-Δcyt truncated CAR cells were also significantly reduced in comparison to the intact CAR transduced NB4 neutrophils in unopsonized conditions, yet a (not significant) trend of leftover trogocytosis was detected both in the FACS-based assay (**Figure 4J**) as well as by live cell imaging (**Figure 4K**), probably explained by the fact that such cells are able to attach to the tumor antigen by the extracellular domain of the CAR.

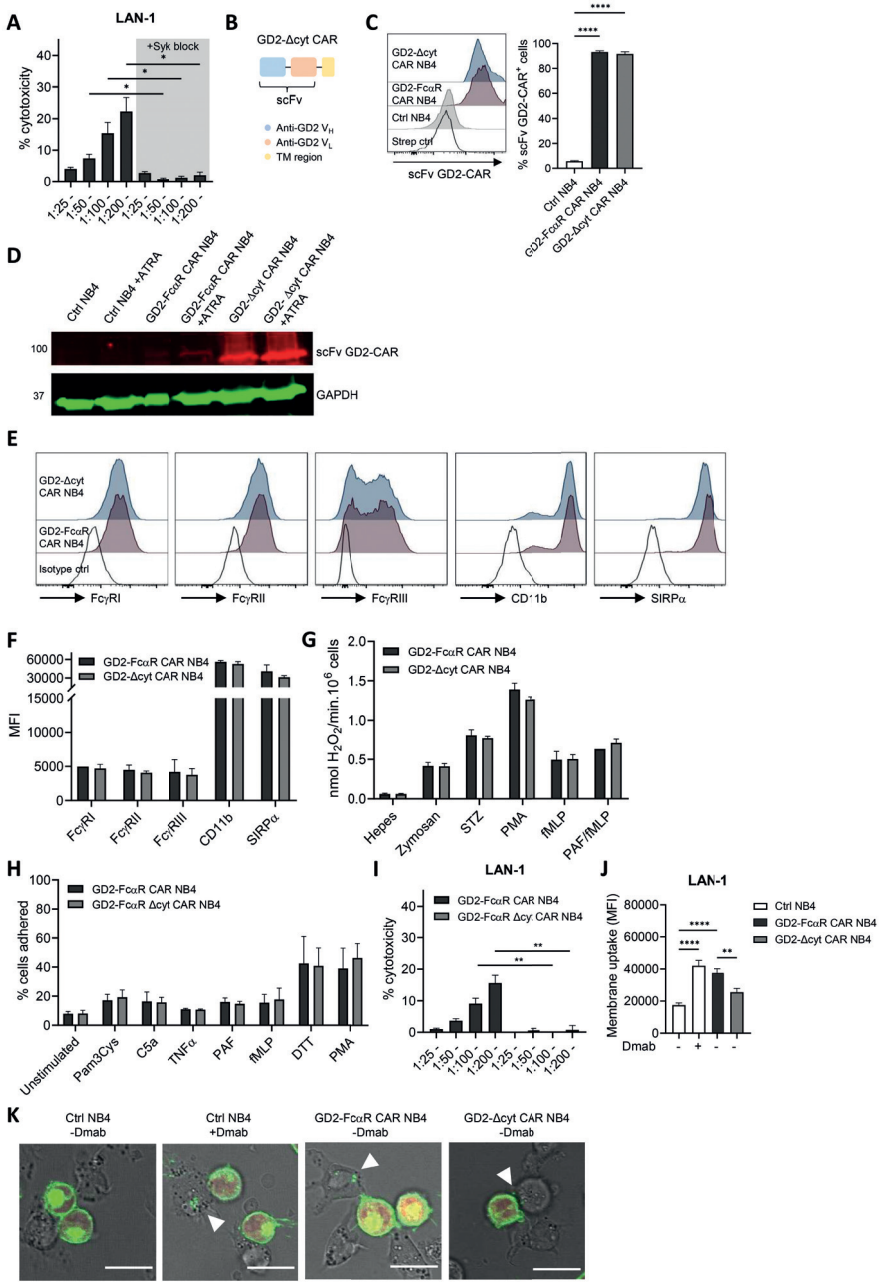


Figure 4. Functionality of anti-GD2-FcαR CAR depends on the signaling downstream FcαR domain

(A) *Assessment of CAR transduced NB4-mediated cytotoxicity against GD2⁺ LAN-1 target cells in the absence of dinutuximab (-) and the absence of presence of Syk tyrosine kinase inhibitor at different T:E ratios in a ^{51}Cr-release assay. N=6. V_H and V_L, V domains of the heavy and light chains.* **(B)** *Schematic represen-*

tation of the construct used in lentiviral vectors to express the truncated version of anti-GD2 CAR in NB4 cells, GD2-Δcyt CAR. **(C)** *Representative flow cytometry histogram (left) and quantification of GD2-CAR expression expressed as % of positive cells (right) after sorting on GD2-FcαR CAR, GD2-Δcyt CAR and control NB4 cells. N=7.* **(D)** *Representative example of western blot stained for detection of scFv GD2-CAR of undifferentiated or ATRA-differentiated control, GD2-FcαR CAR (84.3 kDa) and GD2-Δcyt CAR (79.8 kDa) transduced NB4 cells. Staining for GAPDH was used as a loading control.* **(E-F)** *Representative flow cytometry histograms (E) and quantification (F) of surface expression levels of NB4 maturation markers expressed as MFI of intact or truncated CAR transduced cells. N=2-4.* **(G)** *NADPH-oxidase activity of intact or truncated CAR transduced cells in the presence of the indicated stimuli expressed as nmol H_2O_2/min per 10^6 cells. N=2-4.* **(H)** *Percentage of adhered intact or truncated CAR transduced cells in response to the indicated stimuli. N=4.* **(I)** *Assessment of CAR transduced NB4-mediated cytotoxicity of GD2-FcαR CAR and GD2-Δcyt CAR transduced cells against $GD2^+$ LAN-1 target cells in the absence of dinutuximab (-) at different T:E ratios in a ^{51}Cr-release assay. N=9.* **(J)** *Quantification of trogocytosis against $GD2^+$ LAN-1 target cells in the presence or absence of dinutuximab (Dmab) assessed by the DiD positivity within the effector cells as analyzed by flow cytometry. A T:E ratio of 1:5 was used. N=12.* **(K)** *Live cell confocal imaging stills showing NB4 cells taking up pieces (white arrows) of DiD (green) and Calcein Red-Orange-AM- (orange) labeled LAN-1 cells. Note the uptake of the membrane dye only and no uptake of the orange cytoplasmic dye by the effector cells. Imaging started 5 minutes after start of co-culture and took place within 117 minutes from the start of the recording. Scale bar represents 20 µm. TM, transmembrane. ICD, intracellular domain. scFv, single chain variable fragment. Dmab, dinutuximab. MFI, mean fluorescence intensity.*

DISCUSSION

One of the major advancements in cellular cancer immunotherapy has been the development of CAR T cells, which has led to unprecedented cures in patients suffering from hematological malignancies.[1,2] These remarkable outcomes, which resulted in the FDA approval of CD19-CAR T cells for the treatment of a number of B cell lymphomas,[34,35] have as yet not been obtained in the fight against solid tumors,[3,4,36] highlighting a clear unmet clinical need for additional therapies that are designed to overcome the CAR T cell therapy-associated challenges found in the latter. Exploring immune cells other than the traditional T cells as alternative CAR-vehicles and exploiting their unique advantages when submerged in the highly immunosuppressive tumor microenvironment of solid tumors has in the last years gained a lot of attention. For this matter, NK cells and myeloid cells are especially attractive as promising tools for ACT purposes, owing to their ability to efficiently infiltrate non-hematological tumors. There, their effector functions could be specifically harnessed to redirect the tumor microenvironment towards tumor suppressing by means of genetic engineering. In this study, we have genetically modified human NB4 neutrophils – a commonly used maturation-inducible neutrophil-like cell line displaying potent ADCC activity –, to express anti-GD2-, anti-EGFR-, or anti-HER2/neu-specific CARs coupled to the FcαR signaling moiety to trigger cytolytic activity upon target cell recognition. In fact, we showed that CAR engineering provided the effector cells with

built-in ADCC-like activity against relevant targets without need for opsonization, while antigen-negative targets remained unaffected. In addition, receptor expression did not alter cell differentiation nor the canonical NB4 neutrophil functions (*i.e.* respiratory burst and CD11b/CD18-mediated adhesion).

Our data especially emphasize the potential of using a FcαR signaling moiety as intracellular domain to directly couple the antigen-specific recognition part of the CAR with the execution of cytotoxicity by the engineered cell. Similar to circulating neutrophils, NB4 neutrophils express several activating Fc receptors on their cell surface: FcγRI (CD64), FcγRII (CD32) and FcαR (CD89), which upon ligand binding to an antibody Fc domain, such as in the context of antibody therapy in cancer, signal through their ITAM domains. Despite the higher surface expression of FcγRs, the engagement of FcαRs by IgA antibodies have been shown to induce markedly more robust neutrophil activation.[37] Nonetheless, the clinical use of IgA antibodies targeting FcαRs is limited due to the very short serum half-life of about 4 days, compared to the 21 days of IgG.[38–40] Our CAR approach bypasses the need for a therapeutic IgA antibody circumventing its limitations, and again confirmed that the use of FcαR domains in our CAR model outperformed the cytotoxic capacities of an FcγRIIa-based CAR directed towards the same tumor antigen (data not shown).

Due to the neutrophil's ability to induce potent ADCC against tumor cells, alternative strategies to the immortal neutrophil-like cell models are currently being developed to engage their cytotoxic abilities. These include the genetic modification of hematopoietic stem cell (HSC) progenitors with antigen-specific CAR followed by differentiation into neutrophils. Such HSC cultures already yield sufficient numbers of functional neutrophils within 2 weeks,[41] in contrast to the longer differentiation periods (up to months) needed for NK or T cell differentiation. The introduction of an antigen-specific CAR into $CD34^+$ HSCs that were differentiated into mature neutrophils indicated antigen-specific cytolysis by CD34-derived neutrophils expressing an anti-CD4 CAR bearing either a ζ or a Fcγ signaling domain[42]. Proof of the ability to generate CAR-neutrophil cells from human induced pluripotent stem cells (iPSCs) aimed for targeted immunotherapy also displayed potent anti-tumor activities towards relevant targets as an alternative to further explore.[43] Such novel approaches demonstrate the feasibility to reprogram HSC progenitors or iPSCs to generate off-the-shelf antigen-specific CAR neutrophil cells with enhanced graft-versus-malignancy activity while ensuring persistent production. Although repeated infusions of CAR-neutrophils may be required because of their limited survival *in vivo*, it may actually aid by contributing to the recruitment and maturation of antigen-presenting cells to the tumor site by the increased local inflammatory response, thereby stimulating adaptive immune responses to improve tumor control.[8,12,44]

In conclusion, in the present study we preclinically evaluated the cytotoxic potential of a FcαR-bearing CAR moiety directed towards solid tumor targets for its insertion in neutrophil-like cells as an attractive innovative approach in the ACT field. Our data demonstrate the feasibility of relying on the neutrophil's unique advantages to fight solid tumors to allow the rational design of novel immunotherapeutic strategies. Further studies on CAR-transfected primary or CD34-derived neutrophils are needed to make more educated assessments about their clinical applicability as future CAR therapy. Altogether, this paves the way for myeloid cell-based therapeutic strategies that could take over in situations where T cell-based therapies show limited efficacy.

AUTHOR CONTRIBUTIONS

PMS, BK, KS, PJJHV and IL designed and performed the experiments, analyzed the data and wrote the manuscript. BK, KS, PJJHV and IL performed experiments and reviewed the manuscript. TWK and RvB contributed to data interpretation and reviewed the manuscript. HLM designed the experiments, interpreted and evaluated the data and reviewed the manuscript. All authors approved the submitted version.

CONFLICT OF INTEREST DISCLOSURE

HLM is the inventor of patent PCT/NL2022/050225, owned by Sanquin IP B.V., entitled "Chimeric Fc-alpha receptors and uses thereof".

FUNDING

This project is funded by Sanquin Innovatie B.V.

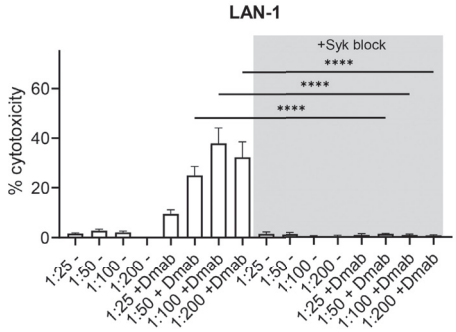
REFERENCES

1. Hayes C. Cellular immunotherapies for cancer. *Ir J Med Sci*. 2021;190(1):41-57.
2. Rohaan MW, Wilgenhof S, Haanen JBAG. Adoptive cellular therapies: the current landscape. *Virchows Arch*. 2019;474(4):449-461.
3. Beatty GL, O'Hara M. Chimeric antigen receptor-modified T cells for the treatment of solid tumors: Defining the challenges and next steps. *Pharmacol Ther*. 2016;166:30-39.
4. Martinez M, Moon EK. CAR T Cells for Solid Tumors: New Strategies for Finding, Infiltrating, and Surviving in the Tumor Microenvironment. *Front Immunol*. 2019;10:128.
5. Balakrishnan A, Vig M, Dubey S. Role of myeloid cells in the tumor microenvironment. *J Cancer Metastasis Treat*. 2022;8(27).
6. Elliott LA, Doherty GA, Sheahan K, Ryan EJ. Human Tumor-Infiltrating Myeloid Cells: Phenotypic and Functional Diversity. *Front Immunol*. 2017;8:86.
7. Awad RM, de Vlaeminck Y, Maebe J, Goyvaerts C, Breckpot K. Turn Back the TIMe: Targeting Tumor Infiltrating Myeloid Cells to Revert Cancer Progression. *Front Immunol*. 2018;9:1977.
8. Megiovanni AM, Sanchez F, Robledo-Sarmiento M, Morel C, Gluckman JC, Boudaly S. Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *J Leukoc Biol*. 2006;79(5):977-988.
9. Stopforth RJ, Ward ES. The Role of Antigen Presentation in Tumor-Associated Macrophages. *Crit Rev Immunol*. 2020;40(3):205-224.
10. Vono M, Lin A, Norrby-Teglund A, Koup RA, Liang F, Loré K. Neutrophils acquire the capacity for antigen presentation to memory CD4(+) T cells in vitro and ex vivo. *Blood*. 2017;129(14):1991-2001.
11. Muntjewerff EM, Meesters LD, van den Bogaart G. Antigen Cross-Presentation by Macrophages. *Front Immunol*. 2020;11:1276.
12. Zhu EF, Gai SA, Opel CF, et al. Synergistic innate and adaptive immune response to combination immunotherapy with anti-tumor antigen antibodies and extended serum half-life IL-2. *Cancer Cell*. 2015;27(4):489-501.
13. Moynihan KD, Opel CF, Szeto GL, et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. *Nat Med*. 2016;22(12):1402-1410.
14. Brandsma AM, Bondza S, Evers M, et al. Potent Fc Receptor Signaling by IgA Leads to Superior Killing of Cancer Cells by Neutrophils Compared to IgG. *Front Immunol*. 2019;10:704.
15. Treffers LW, Ten Broeke T, Rosner T, et al. IgA-Mediated Killing of Tumor Cells by Neutrophils Is Enhanced by CD47-SIRPalpha Checkpoint Inhibition. *Cancer Immunol Res*. 2020;8(1):120-130.
16. Chan C, Lustig M, Baumann N, Valerius T, van Tetering G, Leusen JHW. Targeting Myeloid Checkpoint Molecules in Combination With Antibody Therapy: A Novel Anti-Cancer Strategy With IgA Antibodies? *Front Immunol*. 2022;13:932155.
17. Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. NB4, a Maturation Inducible Cell Line With t(15;17) Marker Isolated From a Human Acute Promyelocytic Leukemia (M3). *Blood*. 1991;77(5):1080-1086.
18. Gale R, Zighelbloim J. Polymorphonuclear leukocytes in antibody-dependent cellular cytotoxicity. *J Immunol*. 1975;114(3):1047-1050.
19. Treffers LW, Hiemstra IH, Kuijpers TW, van den Berg TK, Matlung HL. Neutrophils in cancer. *Immunol Rev*. 2016;273(1):312-328.

20. Barker E, Mueller BM, Handgretinger R, Herter M, Yu AL, Reisfeld RA. Effect of a Chimeric Anti-Ganglioside G D2 Antibody on Cell-mediated Lysis of Human Neuroblastoma Cells. *Cancer Res*. 1991;51:144-149.
21. Siders WM, Shields J, Garron C, et al. Involvement of neutrophils and natural killer cells in the anti-tumor activity of alemtuzumab in xenograft tumor models. *Leuk Lymphoma*. 2010;51(7):1293-1304.
22. Albanesi M, Mancardi DA, Jonsson F, et al. Neutrophils mediate antibody-induced antitumor effects in mice. *Blood*. 2013;122(18):3160-3164.
23. Tabiasco J, Espinosa E, Hudrisier D, Joly E, Fournie JJ, Vercellone A. Active trans-synaptic capture of membrane fragments by natural killer cells. *Eur J Immunol*. 2002;32(5):1502-1508.
24. Matlung HL, Babes L, Zhao XW, et al. Neutrophils Kill Antibody-Opsonized Cancer Cells by Trogoptosis. *Cell Rep*. 2018;23(13):3946-3959 e6.
25. Martinez Sanz P, van Rees DJ, van Zogchel LMJ, et al. G-CSF as a suitable alternative to GM-CSF to boost dinutuximab-mediated neutrophil cytotoxicity in neuroblastoma treatment. *J Immunother Cancer*. 2021;9(5).
26. Cavdarli S, Dewald JH, Yamakawa N, et al. Identification of 9-O-acetyl-N-acetylneuraminic acid (Neu5,9Ac(2)) as main O-acetylated sialic acid species of GD2 in breast cancer cells. *Glycoconj J*. 2019;36(1):79-90.
27. London M, Gallo E. Epidermal growth factor receptor (EGFR) involvement in epithelial-derived cancers and its current antibody-based immunotherapies. *Cell Biol Int*. 2020;44(6):1267-1282.
28. Jiang N, Lin JJ, Wang J, et al. Novel treatment strategies for patients with HER2-positive breast cancer who do not benefit from current targeted therapy drugs. *Exp Ther Med*. 2018;16(3):2183-2192.
29. Ménard S, Casalini P, Campiglio M, Pupa SM, Tagliabue E. Role of HER2/neu in tumor progression and therapy. *Cell Mol Life Sci*. 2004;61(23):2965-2978.
30. Nicholson RI, Gee JMW, Harper ME. EGFR and cancer prognosis. *Eur J Cancer*. 2001;37:9-15.
31. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/neu Oncogene. *Science (1979)*. 1987;235(4785):177-182.
32. Mócsai A, Ruland J, Tybulewicz VLJ. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat Rev Immunol*. 2010;10(6):387-402.
33. Daëron M. Fc RECEPTOR BIOLOGY. *Annu Rev Immunol*. 1997;15(1):203-234.
34. NIH. CAR T-Cell Therapy Approved for Some Children and Young Adults with Leukemia. Published September 11, 2017. Accessed January 16, 2023. https://www.cancer.gov/news-events/cancer-currents-blog/2017/tisagenlecleucel-fda-childhood-leukemia
35. FDA. FDA approves CAR-T cell therapy to treat adults with certain types of large B-cell lymphoma. Published October 18, 2018. Accessed January 23, 2023. https://www.fda.gov/news-events/press-announcements/fda-approves-car-t-cell-therapy-treat-adults-certain-types-large-b-cell-lymphoma
36. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J*. 2021;11(4):69.
37. ben Mkaddem S, Benhamou M, Monteiro R. Understanding Fc Receptor Involvement in Inflammatory Diseases: From Mechanisms to New Therapeutic Tools. *Front Immunol*. 2019;12:10-811.

38. Boross P, Lohse S, Nederend M, et al. IgA EGFR antibodies mediate tumour killing in vivo. *EMBO Mol Med*. 2013;5(8):1213-1226.
39. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol*. 2007;7(9):715-725.
40. van Tetering G, Evers M, Chan C, Stip M, Leusen J. Fc Engineering Strategies to Advance IgA Antibodies as Therapeutic Agents. *Antibodies (Basel)*. 2020;9(4):70.
41. Payuhakrit W, Panichakul T, Charoenphon N, et al. In vitro production of functional immune cells derived from human hematopoietic stem cells. *EXCLI J*. 2015;14:1031-1039.
42. Roberts MR, Cooke KS, Tran AC, et al. Antigen-specific cytolysis by neutrophils and NK cells expressing chimeric immune receptors bearing zeta or gamma signaling domains. *J Immunol*. 1998;161(1):375-384.
43. Chang Y, Syahirah R, Wang X, et al. Engineering chimeric antigen receptor neutrophils from human pluripotent stem cells for targeted cancer immunotherapy. *Cell Rep*. 2022;40(3):111128.
44. van Gisbergen KP, Sanchez-Hernandez M, Geijtenbeek TB, van Kooyk Y. Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *J Exp Med*. 2005;201(8):1281-1292.

SUPPLEMENTAL DATA



Supplemental Figure 1. *Assessment of NB4-mediated cytotoxicity by control NB4 cells against GD2⁺ LAN-1 target cells in the absence or presence of dinutuximab (Dmab) and the absence of presence of Syk tyrosine kinase inhibitor at different T:E ratios in a ^{51}Cr-release assay. N=6. Dmab, dinutuximab.*

CHAPTER 6

HUMANIZED MISTRG AS A PRECLINICAL *IN VIVO* MODEL TO STUDY HUMAN NEUTROPHIL-MEDIATED IMMUNE PROCESSES

Paula Martinez-Sanz, Adrien R. G. Laurent, Edith Slot, Mark Hoogenboezem, Nikolina Bąbała, Robin van Bruggen, Anthony Rongvaux, Richard A. Flavell, Godelieve A. M. Tytgat, Katka Franke, Hanke L. Matlung, Taco W. Kuijpers, Derk Amsen*, Julien J. Karrich*

* Authors contributed equally

Frontiers in Immunology 2023 March 8; 14:1105103

ABSTRACT

Introduction: MISTRG mice have been genetically modified to allow development of a human myeloid compartment from engrafted human $CD34^+$ haemopoietic stem cells, making them particularly suited to study the human innate immune system *in vivo*. Here, we characterized the human neutrophil population in these mice to establish a model that can be used to study the biology and contribution in immune processes of these cells *in vivo*.

Methods and results: We could isolate human bone marrow neutrophils from humanized MISTRG mice and confirmed that all neutrophil maturation stages from promyelocytes ($CD11b^-CD16^-$) to end-stage segmented cells ($CD11b^+CD16^+$) were present. We documented that these cells possessed normal functional properties, including degranulation, reactive oxygen species production, adhesion, and antibody-dependent cellular cytotoxicity towards antibody-opsonized tumor cells *ex vivo*. The acquisition of functional capacities positively correlated with the maturation state of the cell. We found that human neutrophils were retained in the bone marrow of humanized MISTRG mice during steady state. However, the mature segmented $CD11b^+CD16^+$ human neutrophils were released from the bone marrow in response to two well-established neutrophil-mobilizing agents (*i.e.* G-CSF and/or CXCR4 antagonist Plerixafor). Moreover, the neutrophil population in the humanized MISTRG mice actively reacted to thioglycolate-induced peritonitis and could infiltrate implanted human tumors, as shown by flow cytometry and fluorescent microscopy.

Discussion: These results show that functional human neutrophils are generated and can be studied *in vivo* using the humanized MISTRG mice, providing a model to study the various functions of neutrophils in inflammation and in tumors.

INTRODUCTION

Pre-clinical mouse models are essential for the understanding of human physiology, and immunity. There are, however, many differences between mice and humans[1], and findings derived from laboratory animals cannot always be directly translated to humans[2,3]. The use of immunodeficient mice grafted with human hematopoietic stem cells (generally referred to as 'humanized mice') is, among others, a promising approach for studying human immune development and function *in vivo*[2,4,5]. Traditional humanized mouse models have been unable to reliably establish a human myeloid compartment, limiting study of especially innate human immune functions[2,6,7]. Immunocompromised *NOD/scid/IL2Rγ*$^{-/-}$ (NSG) mice exhibit largely defective development of human monocytes/macrophages and NK cells after human immune reconstitution with CD34^{+} hematopoietic progenitor cells (HPC)[6,8,9], likely due to the limited cross-reactivity between specific mouse and human cytokine receptors[2,7].

The delivery of human cytokines to humanized mice via the knock in of cytokine-encoding genes can circumvent this challenge[10–13]. Building on the highly immunodeficient *Rag2*$^{-/-}$*/IL2Rγ*$^{-/-}$background, Rongvaux et *al.* generated an improved model in which the human genes encoding macrophage colony-stimulating factor (M-CSF), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and thrombopoietin (TPO) were knocked in to replace their mouse counterparts[6]. These cytokines were strategically chosen: they have critical roles in early hemato- and myelopoiesis, but the human receptors for these cytokines do not respond to the corresponding murine cytokines. An elegant consequence of the improved early human hematopoiesis is that the generated human cell types produce additional human cytokines (such as IL-15), which further bolsters human hematopoiesis[6]. Moreover, a Bac transgene was introduced to express the human signal-regulatory protein alpha (SIRPα), which promotes acceptance of human xenografts in mice by inhibiting phagocytosis by mouse macrophages[14]. This mouse strain was named 'MISTRG', and was shown to allow successful development of not only lymphoid T and B cell compartments, but also of diverse and functional human myeloid and NK cells, resembling those seen in human blood. Human NK cells from humanized MISTRG (huMISTRG) mice were fully functional and exhibited cytotoxic activity towards human tumor cells[6,15], and human macrophages were shown to infiltrate human tumor xenografts in a pattern resembling that observed in tumors from human patients[6]. huMISTRG mice also supported the development of human neutrophils, among other granulocytes[6,16].

Neutrophilic granulocytes play pivotal roles in host immune defense. They constitute an important early barrier to invasion by infectious agents through mechanisms including phagocytosis, the release of reactive oxygen species (ROS), antimicrobial peptides and proteases, as well as through antibody-mediated mechanisms such as antibody-dependent cellular cytotoxicity (ADCC)[17–19], among others. In addition, recent evidence has made clear

that neutrophils can also act as danger sensors and, by interacting with other immune cells, contribute to the establishment of adaptive immune responses[20]. Hence, dysregulation of neutrophils can lead to a variety of pathologies[21]. Neutrophils can both have beneficial as well as adverse functions. An example of this is in cancer, where neutrophils eliminate cancer cells, but also oppose immune control of tumors by differentiating into myeloid-derived suppressor cells[18,19]. It is therefore important to establish pre-clinical models where the biology of human neutrophils and their involvement in health and disease can be studied.

Despite being present in the bone marrow of huMISTRG mice, the frequency of neutrophils in peripheral blood of such mice is negligible. It has been suggested that either the terminal differentiation, the egress from the bone marrow or the peripheral survival of human neutrophils are still suboptimal in this mouse environment[6]. Still, a subset of human neutrophils in huMISTRG mice was found to possess a mature phenotype as described by a $CD33^{+}CD66b^{+}CD16^{+}$ surface phenotype and the presence of segmented nuclei[16]. Here, we aimed to further characterize the human neutrophil population to validate huMISTRG mice as an experimental model to study neutrophil biology and their contribution in various immune processes *in vivo*. We successfully isolated both immature and end-stage human neutrophils from huMISTRG mice and assessed their functionality in a number of neutrophil-specific assays. Moreover, we showed that they could be mobilized into blood in response to two well-established neutrophil-mobilizing agents (*i.e.* granulocyte colony-stimulating factor and/or CXCR4 antagonist Plerixafor), and were actively recruited to inflammation sites induced by thioglycolate as well as by human tumors engrafted *in vivo*.

MATERIALS AND METHODS

Human immune reconstitution of immunodeficient mice

Highly immunodeficient MITRG (M-CSF$^{h/h}$ IL-3/GM-CSF$^{h/h}$ TPO$^{h/h}$ Rag2$^{-/-}$ IL2Rγ$^{-/-}$) and MISTRG (M-CSF$^{h/h}$ IL-3/GM-CSF$^{h/h}$ hSIRPAtg TPO$^{h/h}$ Rag2$^{-/-}$ IL2Rγ$^{-/-}$) mice were generated as described before (Regeneron,[6]), and maintained under specific pathogen free conditions with continuous Enrofloxacin antibiotic treatment in drinking water (Baytril, 0,27 mg/mL; Bayer).

Newborn MISTRG mice (within first 3 days after birth) were sublethally irradiated (X-ray irradiation with Faxitron MultiRad 225, 10 cGy), and were subsequently injected intrahepatically with 1×10^5 cord blood (CB)-derived $CD34^{+}$ cells (CB was collected according to the guidelines of Eurocord Nederland), unless otherwise specified. Level of human immune reconstitution was measured from week 4 after $CD34^{+}$ cell engraftment using flow cytometry on blood samples (percentage of human $CD45^{+}$ cells, as compared

to percentage of murine CD45$^+$ cells within total CD45 immune cells; BV421-labeled anti-human CD45 (clone HI30; BioLegend), PE-Cy7-labeled anti-mouse CD45 (clone 30-F11; BioLegend) (**Figure 1A**). Mice with at least 20% huCD45$^+$ cells in the blood were selected for further experiments. Of note, each experimental replicate presented in this study was performed with a cohort of huMISTRG generated with a different human CB donor.

Tissue sampling

Prior to tissue sampling, mice were sacrificed by carbon dioxide asphyxiation. Peripheral blood samples were harvested by heart puncture with syringe and needle. Contaminating erythrocytes were removed using red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). For the harvesting of bone marrow cell suspension, femur and tibia from both legs were harvested and bones were crushed in PBS supplemented with 0.5% (v/v) fetal calf serum (FCS) with a pestle and mortar. For the removal of fibrous tissue, cell suspension was passed through a 100 µm mesh. Single cell suspensions from spleen were prepared by mechanical disruption via passing of the tissues over a 70 µm cell strainer. Tumor tissue was cut into pieces of 1 mm^2 and enzymatically digested for 30 min at 37°C with 750 U ml-1 Collagenase Type I (Worthington) and 0.31 mg ml-1 DNase I (Roche, from bovine pancreas, grade II) in RPMI 1,640 supplemented with 10% (v/v) FCS. Single cell suspensions were generated by filtering over a 70 µm cell strainer. Whole tumors for histology were embedded in Tissue-Tek O.C.T. Compound (Sakura) and snap frozen in liquid nitrogen and stored at -80°C until further used. For the harvesting of the peritoneal exudate cells (PEC) the abdominal cavities were flushed with 5 mL PBS with a needle and syringe and the suspension containing PEC was extracted with the same syringe. As control, heparinized peripheral blood from healthy human donors (available through the Sanquin Blood bank or from healthy volunteers) was obtained and used according to the Declaration of Helsinki 1964.

Human neutrophil cell isolation and immune compartment characterization

Human neutrophils from healthy human donors or from bone marrow of engrafted mice were enriched either by Percoll fractionation, as previously described[22], or by magnetic-activated cell sorting (MACS) using anti-human CD15 microbeads (Miltenyi Biotec), according to manufacturer's instructions, respectively. The isolated neutrophils were kept in 4-(2- hydroxyethyl) -1- piperazineethanesulfonic acid supplemented with 5 g/L human albumin (Albuman; Sanquin Plasma Products), 1 mM CaCl and 5.5 mM glucose (further referred to as HEPES buffer) and were used for functional experiments.

The following directly conjugated antibodies were used for flow cytometry analysis of human cell populations in whole blood, bone marrow, spleen and tumor samples: CD45-BUV805 (clone HI30; BD Biosciences), CD19-BUV737 (clone SJ25C1; BD Biosciences),

CD3-BUV661 (clone UCHT1; BD Biosciences), CD4-BUV496 (clone SK3; BD Biosciences), CD16-BUV496 (clone 3G8; BD Biosciences), Gr-1-BUV395 (clone RB6-8C5; BD Biosciences), CD15-BV605 (clone W6D3; eBioscience), CD8-BV605 (clone RPA-T8; BD BioLegend), CD25-BV421 (clone 2A3; BD Biosciences), CD11b-BV421 (clone ICRF44; BioLegend), CD11c-PerCP-Cy5.5 (clone 3.9; BioLegend), HLA-DR-FITC (clone C243; BioLegend), FoxP3-PerCP-Cy5.5 (clone 235A/E7; BD Biosciences), CD32-FITC (clone AT10, Bio-Rad), CD64-FITC (clone 10.1, Bio-Rad), CD66b-FITC (clone 80H3; Bio-Rad), CD14-PE-Cy7 (clone 61D3; eBioscience), CD56-PE (clone B159; BD Pharmingen), Siglec-8-PE (clone 7C9; BioLegend), Siglec-9-PE (clone K8; BioLegend), EMR3-APC (clone 3D7; Bio-Rad) and CD62L-APC (DREG-56; BD Pharmingen), CD33-A700 (clone WM53; BD Biosciences). When specified, a human lineage cocktail of biotinylated antibodies (further referred as 'Lineage') followed by PerCP-Cy5.5-conjugated streptavidin (BD Biosciences) was used for exclusion of CD3, CD19 and CD56 populations from the analysis (Biotin-labeled anti-human CD3, clone OKT3; Biotin-labeled anti-human CD19, clone HIB9; Biotin-labeled anti-human CD56, clone CMSSB, all from eBioscience). A LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells.

Flow cytometry data were acquired using FACS Symphony™ or Fortessa™ flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 10.8; Becton Dickinson). Cell quantification was achieved by using Precision Count beads™, according to manufacturer protocol (BioLegend).

For further isolation of the neutrophil progenitors huMISTRG samples from total bone marrow of steady state animals were first enriched for $CD15^+$ via magnetic sorting (see below) and later separated by FACS sorting based on FSC/SSC and the expression of CD11b-BV421 and CD16-PE-Cy7. FACS sorting was performed using BD FACS Aria III™ cell sorter (BD biosciences).

Cytospin preparation and staining

0.5 or 1×10^5 neutrophils were cytospun (Shandon CytoSpin II Cytocentrifuge) for 10 minutes onto 76 × 26 mm glass microscope slides (Menzel-Gläser). The cytospin slides were first air-dried and subsequently stained for 5 minutes in May-Grünwald followed by a 30 minutes staining with Giemsa. Slides were rinsed in deionized water, air-dried and analyzed with Zeiss Axio Scope A1 microscope.

Phagocytosis assay

Phagocytic activity of FITC-labeled zymosan was assessed via flow cytometry. Zymosan particles (10 mg/mL; MP Biomedicals) were labeled with 0.2 mg/mL fluorescein isothiocyanate (FITC; Sigma Aldrich) for 30 minutes at 37°C in a shaker (650 rpm). The

FITC-labeled particles were then opsonized with pooled serum (obtained via plasmapheresis from five healthy donors) for another 30 minutes to which neutrophils (0.5×10^6) were added in HEPES buffer. At the desired time points, samples were added to STOPbuffer (PBS containing 20 mM sodium fluoride, 0.5% PFA and 1% BSA) and the amount of FITC fluorescence within the neutrophil gate was measured on a FACS Fortessa™ flow cytometer (BD Biosciences). Data were analyzed with FlowJo software and expressed as percentage of $FITC^+$ neutrophils.

NADPH oxidase activity assay

Nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity was assessed by measuring the release of hydrogen peroxide (H_2O_2) with an Amplex Red kit (Molecular Probes). Neutrophils (0.25×10^6) were left unstimulated in HEPES buffer or were stimulated for 30 minutes at 37°C with *E. coli* (OD625=0.2, strain ML-35), unopsonized zymosan (1 mg/mL), serum-treated zymosan (STZ, 1 mg/mL), phorbol 12-myrisatate 13-acetate (PMA, 100 ng/mL; Sigma Aldrich) or platelet-activation factor (PAF; 1 µmol/L; Sigma Aldrich)/N-formylmethionine-leucyl-phenylalanine (fMLP, 1 µmol/L; Sigma Aldrich) in the presence of Amplex Red (0.5 µmol/L) and horseradish peroxidase (1 U/mL). Fluorescence derived from Amplex Red conversion into Resorufin was measured at 30-second intervals for 30 minutes with an Infinite F200 PRO plate reader (Tecan). The activity of the NADPH oxidase of neutrophils was determined as nmol H_2O_2/min $\times 10^6$ cells.

Dihydrorhodamine (DHR)-1,2,3 flow cytometry assay

Production of intracellular ROS was analyzed via a flow cytometry-based DHR assay. For discrimination of neutrophil subpopulations, cells (1×10^6/mL) were first pre-incubated with CD11b-BV421 and CD16-PE-Cy7 (clone 3G8, BD Pharmingen) antibodies for 20 minutes on ice in the dark. After washing, cells were mixed with 0.5 µM of DMSO-dissolved DHR (Invitrogen) for 5 minutes at 37°C in a shaker and cells were subsequently stimulated with PMA (100 ng/mL). At the desired time points, samples were added to STOPbuffer and the amount of fluorescent rhodamine-1,2,3 resulting from DHR oxidation by H_2O_2 was measured on a FACS Fortessa™ flow cytometer (BD Biosciences). Data were analyzed with FlowJo software and were expressed as MFI.

Protease release measurement with DQ-BSA

Protease release after degranulation was measured with DQ-BSA (Invitrogen), which becomes fluorescent upon cleavage by proteases. Neutrophils (0.25×10^6) were preincubated with cytochalasin B (CytoB, 5 µg/mL; Sigma Aldrich) for 5 minutes at 37°C in the presence of DQ-BSA (10 µg/mL) and were then stimulated with fMLP (1 µmol/L) or PMA (1 µg/mL). A 100% content value with 0.5% Triton X-100 in water was determined.

Fluorescence was measured with an Infinite F200 PRO plate reader. Data were expressed as relative fluorescence units (RFU)/minute.

Degranulation flow cytometry assay

Neutrophil degranulation was examined by preincubating the isolated cells (5×10^6/mL) with the (priming) agents PAF (1 μmol/L) or CytoB (5 μg/mL) for 5 minutes and by subsequently stimulating with fMLP (1 μmol/L) for 10 minutes. Thereafter, cells were stained with directly labeled antibodies against neutrophil granule markers: CD63-APC (clone MX-49.129.5; Santa Cruz Biotechnology) or CD66b-FITC. CD11b-BV421 and CD16-PE-Cy7 antibodies were also added to the mix for the discrimination of neutrophil subpopulations. Fluorescence was measured on a FACS Fortessa™ flow cytometer (BD Biosciences) and data were analyzed with FlowJo software. Data were expressed as MFI.

Immunohistochemistry of granule markers

Briefly, 4×10^4 neutrophils from each of the flow cytometry-sorted neutrophil bone marrow progenitor were seeded on a 5 mm well of a 18 well μ-slide (Ibidi) and incubated for 30 minutes at 37°C to allow cells to attach. Cells were subsequently fixed with 4% PFA for 10 minutes and permeabilized with 0.1% Triton X-100 solution for 3 minutes. After washing, non-specific staining was reduced during blocking with PBS containing 5% BSA for 30 minutes. Cells were stained for degranulation markers with the following antibodies: unconjugated anti-human neutrophil Elastase (rabbit polyclonal; Sanquin Reagents) followed by secondary donkey anti-rabbit Alexa Fluor-555 conjugated antibody (Thermo Fisher Scientific), and biotinylated anti-human lactoferrin (goat polyclonal; Bethyl) followed by Streptavidin Alexa Fluor-647 (Invitrogen). Hoechst 33342 Solution (Thermo Fisher Scientific) was used for nuclear staining. Incubations were performed in the dark at room temperature for 45 minutes while washing with PBS in between incubations. Imaging was performed with the LSM 980 Airyscan 2 microscope (Zeiss).

Adhesion assay

Neutrophils (5×10^6/mL) were labeled with 1 μM calcein-AM (Molecular Probes) for 30 minutes at 37°C and brought to a concentration of 2×10^6/mL in HEPES buffer. Calcein-labeled cells were stimulated with either PMA (100 ng/mL) or dithiothreitol (DTT, 10 mmol/L; Sigma Aldrich) in an uncoated 96-well MaxiSorp plate (Nunc) for 30 minutes at 37°C and 5% CO_2. Cells in HEPES buffer were used to determine spontaneous adhesion. After stimulation, plates were washed with PBS to remove non-adherent cells. Adherent cells were subsequently lysed for 10 minutes at room temperature using 0.5% Triton X-100 solution in water and fluorescence was measured with a Tecan Infinite F200 PRO plate reader. Adhesion was determined as percentage of total input of calcein-labeled cells.

Antibody-dependent cellular cytotoxicity (ADCC) assay

Target cells IMR-32 and NMB (1×10^6), which were obtained and cultured as described previously[22], were labeled with 100 µCi 51Cr (PerkinElmer) for 90 minutes at 37°C. Chromium-labeled target cells (5×10^3) were co-incubated with either unstimulated or granulocyte-macrophage colony-stimulated factor (GM-CSF, 10 ng/mL; Peptrotech) stimulated neutrophils in a 96-well U-bottom plate (Corning) in the absence or presence of dinutuximab (1 µg/mL, Unituxin, Ch14.18; United Therapeutics) in culture medium for 4 hours at 37°C and 5% CO_2. A target:effector (T:E) ratio of 1:50 (*i.e.* 5.000:250.000 cells) was used. Spontaneous and maximum ^{51}Cr release were determined by incubating the target cells without effector cells and by treating the target cells with a 0.1% Triton X-100 solution in culture medium, respectively. After incubation, 30 µL of supernatant was transferred to Lumaplates (PerkinElmer). The plates were dried overnight at room temperature and analyzed in a MicroBeta2 plate reader (PerkinElmer). The percentage of cytotoxicity was calculated as: [(experimental counts per minute ((CPM)−spontaneous CPM)/(maximum CPM−spontaneous CPM)]×100%. All conditions were performed in duplicate.

Neutrophil mobilization

Mice were injected subcutaneously (S.C.) with 250 µg/kg of recombinant human granulocyte colony-stimulating factor (G-CSF, Neupogen, clinical grade, unused remains from patient treatment regimen; Amgen), 5 mg/kg CXCR4 antagonist Plerixafor (Mobizil, clinical grade, unused remains from patient treatment regimen; Sanofi/Genzyme, kindly provided by Pharmacy of Princess Maxima Center, Utrecht) or a combination of the two agents on the two consecutive days prior to sampling. Control mice were injected S.C. with sterile PBS (Gibco). On day of experiment, mice were sacrificed by carbon dioxide asphyxiation for the harvesting of peripheral blood and bone marrow samples.

Thioglycolate-induced peritonitis model

Peritonitis was induced by a single intraperitoneal (I.P.) injection of 1 mL sterile 4% thioglycolate (Sigma Aldrich). Control mice were injected I.P. with 1 mL sterile PBS. At 16 hours after injection the mice were sacrificed and PECs were harvested from the abdominal cavities.

Tumor model

Mel526 and NKIRTIL006 human melanoma lines were established from patient material obtained following informed consent and in accordance with local guidelines (kind gift from T. Schumacher, NKI, Amsterdam). Tumor cells were cultured in RPMI1640 supplemented with 10% FCS and penicillin (100 IU/mL) and streptomycin (100 mg/mL). 5×10^6 cells in 200 mL PBS were injected S.C. in the flank of huMISTRG (level of chimerism > 20%

huCD45^{+}). Tumor-bearing huMISTRG animals were analyzed from 3 weeks following tumor cell engraftment.

Immunohistochemistry of tumor samples

Frozen tumors were cut with a Leica CM1850 UV cryostat (Leica) into 10 µm thickness serial sections and subsequently collected onto SuperFrost Plus glass slides (Avantor). Prior to staining, tumor sections were fixed with 4% PFA for 10 minutes and blocked with PBS containing 0.5% BSA for 30 minutes to reduce non-specific staining. Sections were then stained for human cell populations with the following monoclonal antibodies: CD66b-BB515 (clone G10F5; BD Biosciences), purified CD3 (clone HIT3a; BioLegend) followed by secondary PE-conjugated anti-mouse antibody (Invitrogen), and Biotin-labeled CD19 (clone HIB9; eBioscience) followed by APC-conjugated streptavidin (Invitrogen). Hoechst 33342 Solution (Thermo Fisher Scientific) was used for nuclear staining. Incubations were performed in the dark at room temperature for 45 minutes, using Tris-Buffered Saline with 0.1% Tween-20 detergent for washes between incubation steps. Sections were subsequently mounted with 10% Mowiol supplemented with 2.5% DABCO and analyzed with the Nikon Ti2e microscope (Leica Microsystems). A Tilescan of the entire tumor was taken with Kinetix sCMOS camera (objective 10x; Photometrics). Files were first Denoised using the Algorithm provided by Nikon and subsequently processed with a rolling ball filter (14.86 µm). Crops were taken from the Tilescans.

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 9 (GraphPad Software). Data were evaluated by one-way or two-way ANOVA, and, where indicated, correction for multiple comparisons using either Sidak's or Tukey's test was performed, or paired two-tailed student's t-test. The results are presented as the mean ± SEM. Data were considered significant when $p < 0.05$.

RESULTS

General characterization of human immune compartments, including human neutrophils, after reconstitution of huMISTRG mice

To study the level of human immune reconstitution in huMISTRG mice (generated as shown in **Figure 1A**), we studied the kinetics of the humanization procedure by determining the percentage of human CD45$^+$ cells in peripheral blood of the mice at different timepoints after CD34$^+$ cell engraftment (**Figure 1B**). As early as 8 weeks post-injection, >50% human CD45$^+$ cells were detected in blood, and these levels steadily increased over time, reaching up to ~70-90% of human CD45$^+$ cells 10 to 14 weeks from transplantation. We characterized adult huMISTRG for multilineage immune cell differentiation and, consistent with other literature in huMISTRG animals[6], we found all major immune compartments represented in spleen, peripheral blood and bone marrow (**Figure 1C**). This included T (CD3$^+$) and B lymphocytes (CD19$^+$), NK cells (CD56$^+$), myeloid cells (CD33$^+$), and dendritic cells (CD11c$^+$HLA-DR$^+$).

Importantly, in accordance with previous findings[6,16], we also found low level of human neutrophils in peripheral blood of huMISTRG mice at steady state (**Figure 2A-B**), identified as huCD45$^+$Gr-1$^-$CD14$^-$CD15$^+$Lineage$^-$ (see gating strategy on **Figure 2C**), while these were amply represented in bone marrow (**Figure 2A-B**). To discriminate between the four different neutrophil developmental stages existing in the bone marrow niche, namely promyelocytes (PM), metamyelocytes (MM), band cells (BC) and segmented cells (SC) [23], we performed flow cytometry analysis based on expression of cell surface markers CD11b and CD16 on the (human) CD15$^+$ population (**Figure 2C**)[24–26]. This allowed us to confirm the presence of all neutrophil maturation stages from CD11b$^-$CD16$^-$ PM to end-stage CD11b$^+$CD16$^+$ SC in huMISTRG bone marrow samples, both phenotypically and morphologically (**Figure 2C-D**). A morphological analysis of the nucleus of each of the different FACS sorted subpopulations confirmed that the neutrophil progenitors from bone marrow of huMISTRG mice very closely resembled those that are found in human bone marrow, with the more immature cells showing a rounder and more banded nucleus, and the more mature ones already showing a number of nuclear segmentations (**Figure 2D**)[24]. When specifically analyzing the human neutrophil phenotype, we found that huMISTRG neutrophils from bone marrow acquired an elevated expression level of Fc gamma receptor CD32 (Fcγ receptor IIa), activation marker CD62L (L-selectin), and differentiation marker Siglec-9 upon maturation, with the most mature subset showing a similar phenotype as circulating neutrophils from human blood. No significant differences between neutrophil subpopulations were seen for Fc gamma receptor CD64 (Fcγ receptor I) and maturation marker EGF-like module-containing mucin-like hormone receptor-like 3 (EMR3, **Figure 2E**).

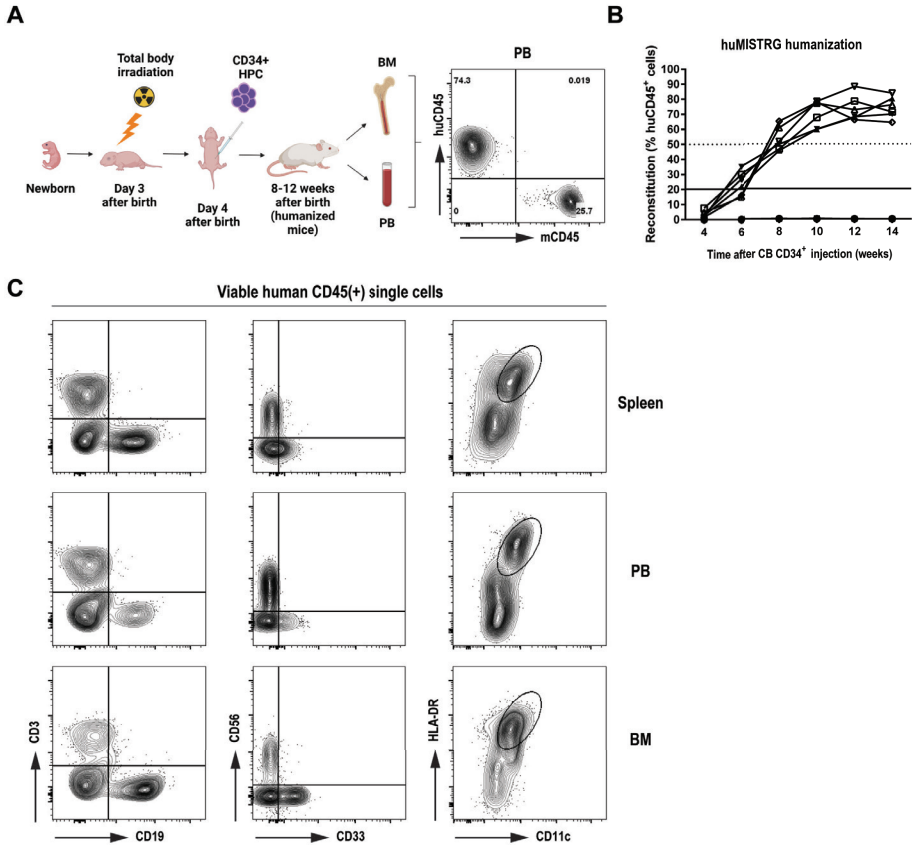


Figure 1. General characterization of human immune compartment in huMISTRG mice (A) *Schematic representation of human reconstitution procedure in huMISTRG mice until harvest of the material. Animals undergo sublethal total body irradiation on day 3 after birth, one day prior to intraperitoneal injection of CD34⁺ HPC. Adult animals of 8-12 weeks of age are checked for successful humanization (huCD45 vs mouse CD45) and organs (i.e. BM, PB) are harvested for subsequent experiments. Numbers indicate the percentages of the gated populations. Created with BioRender.com.* **(B)** *Kinetics of human reconstitution as determined by % of human CD45 cells after cord blood CD34⁺ cell injection of 10 independent mice.* **(C)** *Representative flow cytometry analysis of human immune characterization (CD3⁺ T lymphocytes, CD19⁺ B lymphocytes, CD56⁺ NK cells, CD33⁺ myeloid cells, and CD11c⁺HLA-DR⁺ dendritic cells) gated on viable human CD45⁺ single cells 8 weeks after transplantation in spleen (top), PB (middle) and BM (bottom) compartments of huMISTRG mice. HPC, hematopoietic progenitor cells; CB, cord blood; PB, peripheral blood; BM, bone marrow.*

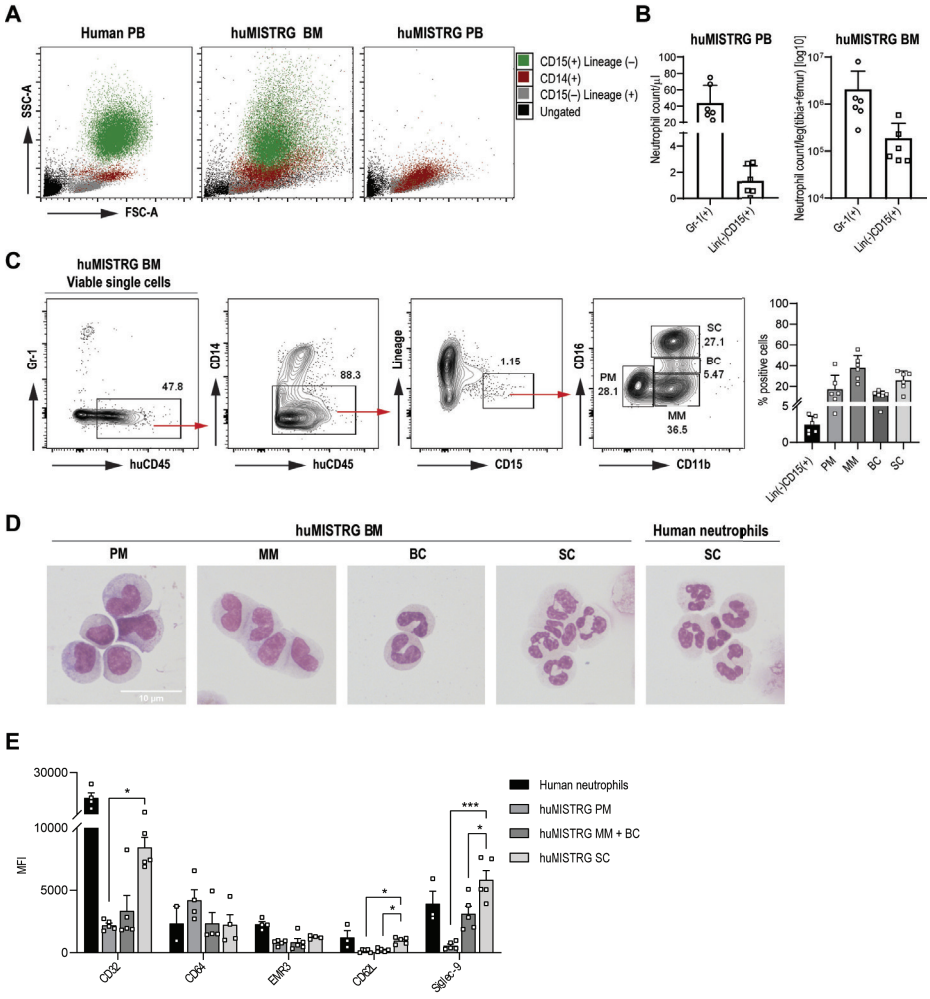


Figure 2. Human neutrophil development and phenotype in huMISTRG mice (A) *SSC-A vs FSC-A density plots for the identification of the different immune cell populations of human PB, huMISTRG BM or huMISTRG PB: ungated (black), CD15⁻Lineage⁺ (human T, B and NK cells, grey), CD14⁺ (human monocytes, red), CD15⁺Lineage⁻ (human neutrophils, green).* **(B)** *Quantification of Gr-1⁺ murine, and Lineage⁻CD15⁺ human neutrophils in bone marrow, and in peripheral blood of huMISTRG animals at steady state. N=2-4, of 2 independent experiments.* **(C)** *Representative sequential gating for identification of BM human neutrophils (huCD45⁺CD14⁻Lineage⁻CD15⁺) and subpopulations (CD11b vs CD16; PM, MM, BC, SC) in huMISTRG mice, gated on viable single cells. Human eosinophils (Siglec-8⁺), being <0.2% of the whole sample, were also excluded from the gating (not shown). Numbers indicate the percentages of the gated populations. Percentage of positive cells from BM human neutrophils as Lineage⁻CD15⁺ and subpopulations gated as in gating strategy on the left is shown for n=6, of 4 individual experiments (right).* **(D)** *Representative cytospins of each neutrophil progenitor (PM, MM, BC, SC) from magnetically enriched CD15⁺ huMISTRG BM fraction after flow cytometry sorting based on CD11b and CD16 expression after May-Giemsa staining (objective 100x). Human neutrophils from peripheral blood were used as control for*

comparison of end-stage segmented nucleus. **(E)** *Neutrophil marker expression (CD32, CD64, EMR3, CD62L and Siglec-9) on the different huMISTRG neutrophil subpopulations (PM, MM+BC, SC) and on human neutrophils. N=2-5. PM, promyelocytes; MM, metamyelocytes; BC, band cells; SC, segmented cells; MFI, mean fluorescence intensity; FSC-A, forward scatter-area; SSC-A, side scatter-area; PB, peripheral blood; BM, bone marrow.*

Together, huMISTRG mice displayed multilineage human immune reconstitution with representation of the myeloid compartment, including dendritic cells, monocytes and neutrophils. Irrespective of the minimal neutrophil numbers in blood of huMISTRG animals in steady state, we confirmed the presence of all human neutrophil maturation stages in huMISTRG bone marrow, similar to those described in human bone marrow[24].

Bone marrow neutrophils of huMISTRG mice show close to physiological *ex vivo* functionality

In order to study the functionality of human neutrophils in our model system, we isolated this population from bone marrow of huMISTRG mice by MACS using anti-human CD15 microbeads. This led to an enrichment of ~50% of $CD15^+$ MACS-sorted cells with still some $CD15^{low}$ cells present, consisting mainly of $CD14^+$ monocytes (**Figure 3A** and **Suppl. Figure 1A-B**). Despite lacking cells of the murine adaptive immune system[6], huMISTRG animals still had a considerate amount of Gr-1^+ murine neutrophils, but these were excluded completely by CD15-based MACS purification (**Suppl. Figure 1C-D**). Within the $CD15^+$ MACS-sorted samples, all four known neutrophil bone marrow subpopulations, as defined by expression of CD11b and CD16, were represented (**Figure 3A** and **Suppl. Figure 1B**)[24]. This cell suspension consisting of $CD15^{low}$ and $CD15^+$ cells, containing both immature and mature neutrophils, was the population used for subsequent functional studies.

Neutrophils are endowed with the unique capacity to engulf and subsequently kill invading microbes through phagocytosis, which is essential for the maintenance of host health[27]. Hence, we first investigated the huMISTRG neutrophil's ability to ingest fluorescently-labeled serum-opsonized zymosan (STZ) via a FACS-based phagocytosis assay. We observed that neutrophils from huMISTRG animals significantly phagocytosed FITC-conjugated zymosan particles reaching levels of 50% as early as 10 minutes post-stimulation, at which point plateau was achieved (**Figure 3B**). A similar pattern was found for human control neutrophils, although these reached higher levels of phagocytosis (>90%), likely explained by the different proportions of immature cells in these samples (**Suppl. Figure 1B**). Cytospin analysis of the samples confirmed the presence of zymosan particles overloading the neutrophil's cytoplasm at the latest timepoint, indicative of efficient phagocytosis by huMISTRG CD15-sorted samples.

Neutrophils eliminate bacteria or other pathogens by the release of highly toxic ROS via the NADPH oxidase system[28,29]. We used an Amplex Red hydrogen peroxide assay to assess the ability of neutrophils to respond to a number of microbial stimuli. As expected, *E. coli*,

zymosan, STZ, phorbol 12-myrisatate 13-acetate (PMA) and platelet-activation factor/N-formylmethionine-leucyl-phenylalanine (PAF/fMLP) were potent inducers of ROS in human control neutrophils (**Figure 3C**). huMISTRG neutrophils were also found to respond to all stimuli tested in a similar trend, indicative of a functional NADPH oxidase complex in the overall population of MACS-sorted bone marrow samples. To assess the ability of each of the neutrophil bone marrow progenitors from our samples to produce intracellular ROS, we performed a flow cytometry-based assay with prior staining for CD11b and CD16 (**Figure 3D**). The capacity of huMISTRG neutrophils to generate ROS in response to PMA stimulation positively correlated with the maturation state of the cell, with the segmented cells fraction outperforming all other less mature fractions, as would also be expected for neutrophils that develop in the human bone marrow[24], and being comparable to that of mature control neutrophils. As the end-stage segmented cells make up only 15-30% of the $CD15^+$ population used in these assays (**Figure 3A**), this likely explains the lower overall ROS production by the unfractionated population (than by human control neutrophils) tested in **Figure 3C**.

As neutrophils use degranulation of proteolytic enzymes to combat infections[30–33], we examined the presence of granule-related proteins for azurophilic (neutrophil elastase) and specific (lactoferrin) granules within the different BM neutrophil precursors by confocal imaging, and confirmed that they are indeed contained within their cytoplasm in steady state (**Suppl. Figure 2A**)[26,34]. Next, we assessed the proteolytic ability of huMISTRG neutrophils by a DQ-BSA assay. Upon full lysis with triton (Tx-100), both human and huMISTRG neutrophil populations successfully cleaved the DQ-BSA substrate, resulting in fluorescence, performed by the potent hydrolytic enzyme neutrophil elastase, among others (**Figure 3E**). Since we performed the assay using total $CD15^+$ MACS-sorted fractions, the number of mature neutrophils within our huMISTRG samples only sufficed to detect a significant DQ-BSA cleavage in response to CytoB/PMA, while significance was not achieved for CytoB/fMLP. However, when directly measuring the surface expression of CD63 and CD66b in the fractionated huMISTRG bone marrow samples thanks to prior staining for CD11b and CD16, we detected upregulation of both azurophilic (CD63) and specific (CD66b) granule markers in response to adequate activation from band cell stage onward, coinciding with initiation of FPR1 expression (fMLP receptor, **Figure 3F**)[26,31,34,35]. Once again, this suggests that the reduced response of the entire huMISTRG neutrophil population (as compared to that of human control neutrophils) to the same stimulus, when measured by the DQ-BSA assay, is explained by the relatively low proportion of end-stage segmented cells, which are the only cells exhibiting clear degranulation capacity (**Figure 3E, F**). Of note, in a parallel experiment we found that only neutrophils had proteolytic capabilities while monocytes had none (**Suppl. Figure 2B**), suggesting that only the $CD15^+$ neutrophil population, and not the $CD14^+$ cells, within the CD15-sorted samples was responsible for the protease activity that was measured.

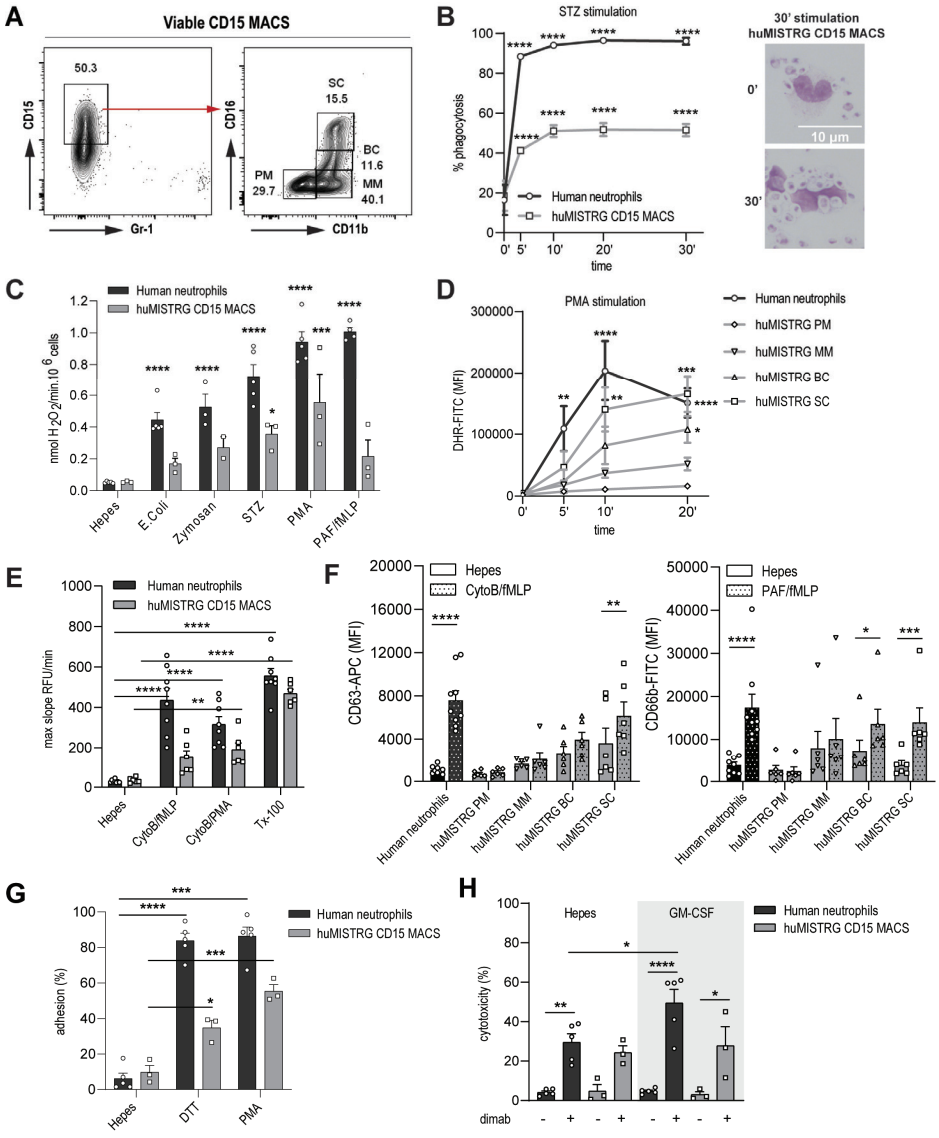


Figure 3. Close to physiological *ex vivo* functionality by neutrophils from bone marrow of huMISTRG mice. (A) *Gating strategy of viable CD15 MACS-sorted cells of huMISTRG mice showing enrichment after sorting and human neutrophil BM subpopulations based on CD11b and CD16 staining (PM, MM, BC, SC). Numbers indicate the percentages of the different populations.* **(B)** *Phagocytosis of FITC-labeled serum-opsonized (STZ) zymosan particles from 0 to 30 min by human neutrophils (black line) and CD15 MACS-sorted huMISTRG neutrophils (grey line) as assessed by flow cytometry. N= 3, of two individual experiments. Statistical differences compared to respective unstimulated condition. Representative microscopic images from cytospin slides (right) after May-Giemsa staining (objective 50x) at timepoint 0 and 30 min depicting zymosan*

particles inside the neutrophil only at 30 min timepoint. **(C)** *NADPH-oxidase activity of human neutrophils (black bars) and CD15 MACS-sorted huMISTRG neutrophils (grey bars) in the presence of the indicated stimuli expressed as nmol H_2O_2/min per 10^6 cells. N=2-5, of three individual experiments. Statistical differences compared to respective Hepes condition.* **(D)** *PMA-induced DHR oxidation from 0 to 20 min by mature human neutrophils (black line) and from the different neutrophil BM subpopulations based on CD11b and CD16 gating (PM, MM, BC, SC) within the neutrophil-characteristic FSC/SSC pattern of huMISTRG mice (grey lines). N=3-5, of three individual experiments. Statistical differences compared to respective unstimulated condition.* **(E)** *Protease activity of human neutrophils (black bars) and CD15 MACS-sorted huMISTRG neutrophils (grey bars) in the presence of the indicated stimuli, or Triton (Tx-100) for total release, expressed as max slope RFU/min. N=6-9, of six individual experiments.* **(F)** *Surface exposure of CD63 (azurophilic granules) and CD66b (specific granules) upon stimulation (dotted bars) with CytoB/fMLP and PAF/fMLP, respectively, on mature human neutrophils (black bars) and on the different neutrophil BM subpopulations based on CD11b and CD16 gating (PM, MM, BC, SC) within the neutrophil-characteristic FSC/SSC pattern of huMISTRG mice (grey bars). N=6-9, of six individual experiments.* **(G)** *Evaluation of adhesion capacity in the presence of the indicated stimuli on human neutrophils (black bars) and CD15 MACS-sorted huMISTRG neutrophils (grey bars) as determined by the percentage of total input of calcein-labeled cells. N=3-5, of three individual experiments.* **(H)** *ADCC of IMR-32 neuroblastoma cells opsonized with (+) or without (–) dinutuximab (dimab) by unstimulated (Hepes) or GM-CSF stimulated human neutrophils (black bars) and CD15 MACS-sorted huMISTRG neutrophils (grey bars). N=3-5, of three individual experiments. PM, promyelocytes; MM, metamyelocytes; BC, band cells; SC, segmented cells; RFU, relative fluorescent units; DHR, dihydrorhodamine; MFI, mean fluorescence intensity; ADCC, antibody-dependent cellular cytotoxicity.*

Neutrophil adhesion is important for the extravasation into inflamed tissues[36,37], which is a process dependent on CD11b/CD18 integrin[38,39]. huMISTRG neutrophils strongly adhered to plastic in response to both outside-in (DTT) and inside-out integrin (PMA) activation-dependent stimuli, suggesting that integrin function was fully operational in these cells (**Figure 3G**). Neutrophils can have a dual role within the tumor microenvironment, having pro-tumor activity as myeloid-suppressor cells or anti-tumor activity by ADCC[18,19,40,41]. As a read-out of anti-tumor ADCC, we assessed whether huMISTRG neutrophils could kill antibody-opsonized tumor cells. We co-incubated either unstimulated or GM-CSF stimulated neutrophils with neuroblastoma cell lines IMR-32 and NMB in the presence or absence of the therapeutic antibody dinutuximab, which binds to GD2, a target on this tumor type (**Figure 3H** and **Suppl. Figure 2C**). huMISTRG neutrophils were able to induce cytotoxicity towards dinutuximab-opsonized neuroblastoma cells within the 4-hour co-incubation with tumor cells. They did so less efficiently than human blood-derived neutrophils (**Figure 3H** and **Suppl. Figure 2C**, grey bars). This may at least partially be explained by the fact that the huMISTRG neutrophil population contained a mixture of mature and immature neutrophils, unlike the human blood derived cells, which were all mature. Of note, the possibility that the monocyte population still present within our MACS-sorted samples contributed to this effect was minimal as monocytes are known to require longer (overnight) incubation times to induce efficient cytotoxicity[42–44].

Overall, the huMISTRG bone marrow-derived, CD15 MACS-sorted neutrophils exhibited close to physiological *ex vivo* functionality, which correlated with the maturation state of the cells.

In vivo human neutrophil migration to the periphery and peritoneum in response to mobilizing agents and inflammation

Given the low number of circulating human neutrophils in steady state conditions in huMISTRG animals, we sought to determine whether huMISTRG neutrophils could be mobilized from bone marrow into the periphery. To do so, adult huMISTRG mice were treated with two well-established neutrophil mobilizing agents: G-CSF (Neupogen) and the CXCR4 antagonist Plerixafor (Mobizil)[45–47], which were administered as single agents or in combination for two consecutive days prior to analysis (**Figure 4A**). We found a pronounced mobilization of neutrophils (assessed by the appearance of $CD15^+$ cells with a characteristic FSC/SSC pattern) into peripheral blood in response to all treatment conditions, while no circulating neutrophils were detected in the control group (**Figure 4B**). Plerixafor treatment as a single agent induced the mobilization of mainly end-stage $CD11b^+CD16^+$ neutrophils, also confirmed microscopically by the presence of at least 3 nuclear lobes, in accordance with the morphology of mature human neutrophils (**Figure 4C-D**)[23]. On the other hand, treatment with G-CSF alone or in combination with Plerixafor mobilized both $CD11b^+CD16^-$ and $CD11b^+CD16^+$ neutrophils, and these cells correspondingly exhibited either a more banded nucleus or a multilobulated nucleus, respectively (**Figure 4C-D**). Of note, the end-stage $CD11b^+CD16^+$ neutrophil population was absent in the bone marrow compartments of the same mobilized mice, especially for mice treated with G-CSF as a single agent or in combination with Plerixafor (**Suppl. Figure 3A**). Importantly, it was difficult to assess the effect of the mobilizing agents on the murine neutrophils ($Gr\text{-}1^+$) in huMISTRG animals, since the vast majority of these were already circulating under steady-conditions (**Suppl. Figure 3B**). Yet, a slight decrease of $Gr\text{-}1^+$ cells was observed in the bone marrow compartment after treatment, suggesting that a small pool of murine neutrophils did mobilize to the periphery in response to G-CSF and/or Plerixafor. The fact that murine neutrophils responded to human mobilizing agents was not unexpected, since complete cross-reactivity exists between human and mouse G-CSF[48].

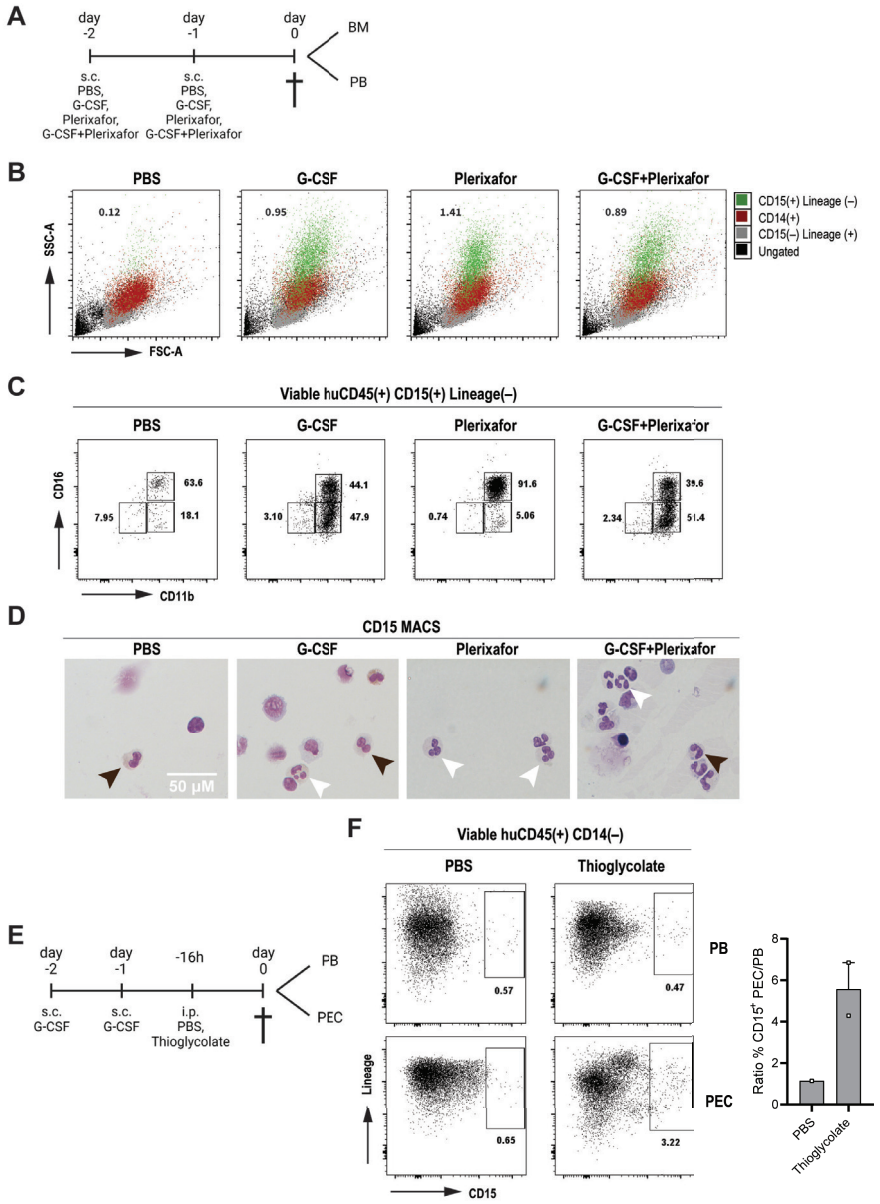


Figure 4. ***In vivo*** **human neutrophil migration to the periphery and peritoneum in response to mobilizing agents and inflammation (A)** *Schematic representation of the treatment scheme with mobilizing agents until tissue sampling. Created with BioRender.com.* **(B)** *SSC-A vs FSC-A density plots for the identification of the different immune cell populations of mobilized PB huMISTRG samples gated as viable huCD45⁺: ungated (black), CD15⁻Lineage⁺ (human T, B and NK cells, grey), CD14⁺ (human monocytes,*

red), CD15$^+$Lineage$^-$ (human neutrophils, green). Numbers indicate the percentages of mobilized human neutrophils as CD15$^+$Lineage$^-$. **(C)** *Representative CD11b vs CD16 flow cytometry plots of mobilization of PB huMISTRG neutrophils in response to different treatments, gated on viable human CD45$^+$CD15$^+$Lineage$^-$ cells. Numbers indicate the percentages of the different subpopulations.* **(D)** *Representative cytospins of mobilized CD15-MACSed PB huMISTRG neutrophils for each respective treatment condition after May-Giemsa staining (objective 50x). Black arrows indicate less mature neutrophil with round/banded nucleus, white arrows indicate mature neutrophil with segmented nucleus.* **(E)** *Schematic representation of the treatment scheme with G-CSF and thioglycolate until tissue sampling. Created with BioRender.com.* **(F)** *Representative Lineage vs CD15 flow cytometry plots of G-CSF mobilized mice in response to peritoneal injection of thioglycolate, gated on viable human CD45$^+$CD14$^-$ cells. Numbers indicate the percentages of the different subpopulations. On the right, quantification of the influx of neutrophils in the peritoneum per condition represented by the ratio of CD15$^+$ cells in the PEC suspension to those in the peripheral blood. N=1-2, of two individual experiments. PEC, peritoneal exudate cells; PB, peripheral blood; FSC-A, forward scatter-area; SSC-A, side scatter-area.*

To investigate whether the human neutrophils in huMISTRG mice could respond to inflammation *in vivo*, we subjected the mice to thioglycolate-induced acute sterile peritoneal inflammation following neutrophil mobilization with G-CSF (**Figure 4E**). This is a commonly used approach to study the development of an inflammatory reaction in mice due to the simple isolation of peritoneal exudate cells (PEC), with neutrophils being the first cells recruited to the injection site[49,50]. Flow cytometric assessment of the PEC suspension allowed us to determine the composition of the infiltrated cell population. Although we found murine neutrophils as Gr-1$^+$ cells to strongly respond to thioglycolate (**Suppl. Figure 3C**), an influx (6-fold increase) of human neutrophils as CD15$^+$ cells within the PEC suspension were also observed as compared to the control group (**Figure 4F**), indicative that the human neutrophils in huMISTRG mice have the ability to migrate towards inflammatory chemokines *in vivo*.

Taken together, not only were the human bone marrow neutrophils released into peripheral blood in response to mobilizing agents, but they also effectively responded to a local (peritoneum) sterile inflammation, suggestive of their adequate migration capacities *in vivo*.

Human immune response with neutrophil infiltration in tumors engrafted in huMISTRG mice

As above-mentioned, in addition to their roles in inflammation and infection, neutrophils are increasingly recognized as critical players during cancer progression, where they can acquire either immunosuppressive functions (pro-tumor activity) or contribute to tumor elimination through ADCC (anti-tumor activity)[18,19]. To determine whether huMISTRG animals can be used to study human intratumoral neutrophils, we engrafted huMISTRG mice with human tumors. We used patient-derived human melanoma cell lines Mel526 and NKIRTIL006 as tumor models[51], which were subcutaneously injected in the flank of adult huMISTRG mice.

Tumors grew gradually for up to 3 weeks at which point peripheral blood and tumors were harvested for analysis (**Figure 5A**).

We detected blood-circulating human neutrophils of both $CD11b^+CD16^-$ and $CD11b^+CD16^+$ phenotype in both tumor models (**Figure 5B**), although these were not significantly higher numbers than circulating human neutrophils detected at steady state, as shown by absolute cell count (**Figure 5C**). Notably, tumor engraftment was also able to mobilize murine $Gr\text{-}1^+$ neutrophils, as shown by the increased number of both blood-circulating and tumor-infiltrating murine neutrophils (**Figure 5E** and **Suppl. Figure 4A-B**). This result suggests that a chronic inflammatory setting solely generated by the presence of a tumor was sufficient to mobilize human neutrophils from bone marrow into tumor tissue. Strikingly, in addition to murine neutrophils (**Suppl. Figure 4A-B**), human neutrophils were found to infiltrate NKIRTIL006 tumors isolated from huMISTRG animals as detected by $CD15^+$ staining with flow cytometry analysis, which were further characterized for maturation markers CD11b and CD16 (**Figure 5D-E**). In addition, analysis of the entire tumor tissue via wide-field fluorescence microscopy further confirmed the presence of intratumoral neutrophils as depicted by positive staining for CD66b surrounding a banded nucleus characteristic of human neutrophils (**Figure 5F**). In fact, the human neutrophils were found in a tumor environment that contained a complete human immune infiltrate consisting of a lymphoid compartment of B cells ($CD19^+$), NK cells ($CD56^+$) and T cells ($CD3^+$) (**Figure 5G** and **Suppl. Figure 4C**), with $CD4^+$ (including regulatory T cells $CD25^+FoxP3^+$) and $CD8^+$ T cells, and a subset of $CD11c^+HLA\text{-}DR^+$ human dendritic cells, recapitulating the immune landscape in patients[52,53].

In summary, these results show that huMISTRG mice develop a human immune infiltrate in melanoma tumors, and potentially provide a model to study responses of human neutrophils in human solid tumors.

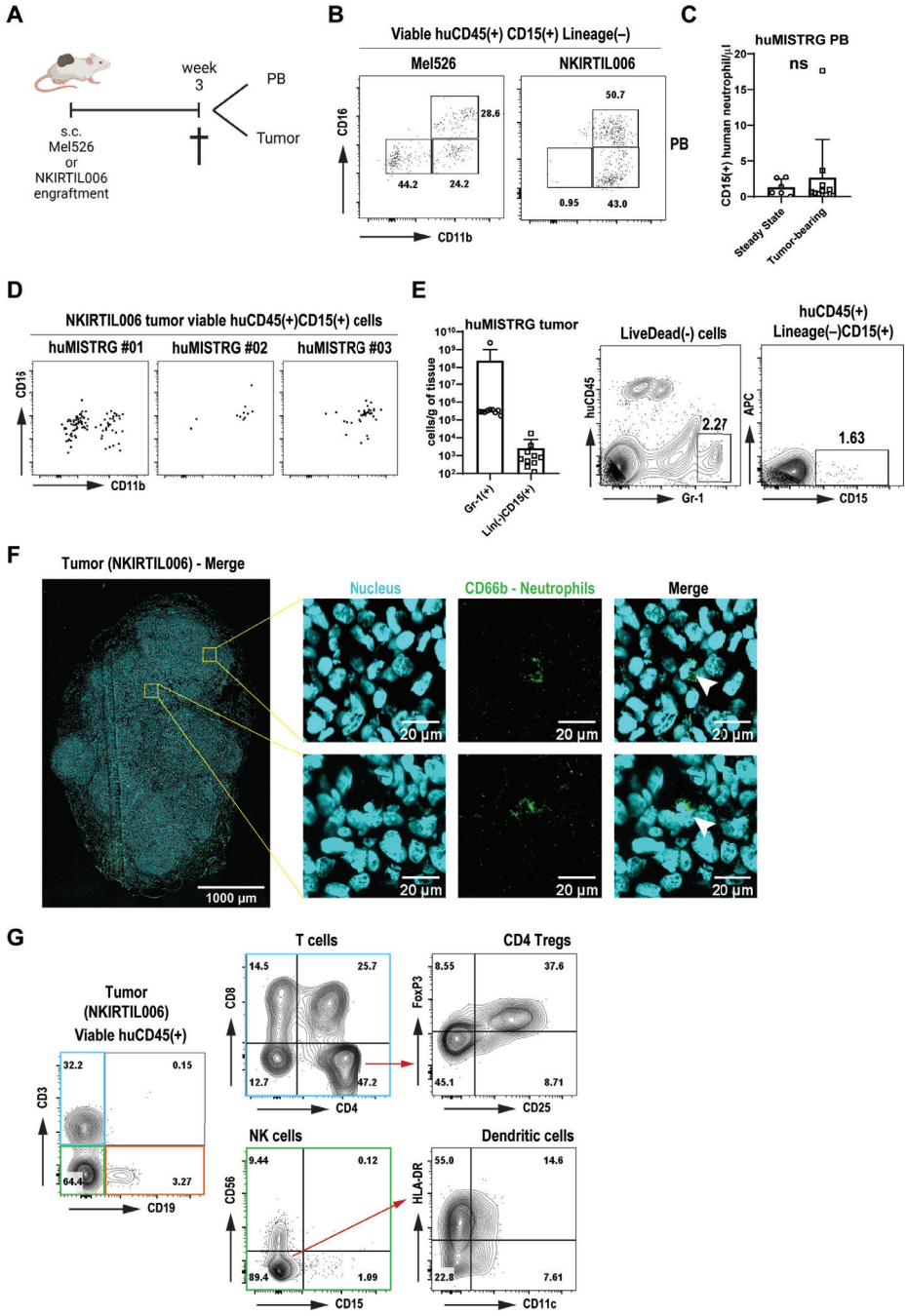

A
week 3
PB
Tumor
s.c. Mel526 or NKIRTIL006 engraftment
B
Viable huCD45(+) CD15(+) Lineage(–)
Mel526
NKIRTIL006
CD16
CD11b
28.6
44.2
24.2
50.7
0.95
43.0
PB
C
huMISTRG PB
ns
CD15(+) human neutrophil/µl
Steady State
Tumor-bearing
D
NKIRTIL006 tumor viable huCD45(+)CD15(+) cells
huMISTRG #01
huMISTRG #02
huMISTRG #03
CD15
CD11b
E
huMISTRG tumor
cells/g of tissue
Gr-1(+)
Lin(-)CD15(+)
LiveDead(-) cells
huCD45
2.27
Gr-1
huCD45(+) Lineage(–)CD15(+)
APC
1.63
CD15
F
Tumor (NKIRTIL006) - Merge
1000 µm
Nucleus
CD66b - Neutrophils
Merge
20 µm
G
Tumor (NKIRTIL006) Viable huCD45(+)
CD3
CD19
32.2
0.15
64.4
3.27
T cells
CD8
CD4
14.5
25.7
12.7
47.2
CD4 Tregs
FoxP3
CD25
8.55
37.6
45.1
8.71
NK cells
CD56
CD15
9.44
0.12
89.4
1.09
Dendritic cells
HLA-DR
CD11c
55.0
14.6
22.8
7.61

Figure 5. Human immune response with neutrophil infiltration in tumors engrafted in huMISTRG mice (A) *Schematic representation of the tumor cell engraftment of the patient-derived Mel526 and NKIR-TIL006 melanoma cells into the flank of adult huMISTRG mice until tissue sampling. Created with BioRender.com.* **(B)** *Representative CD11b vs CD16 flow cytometry plots of blood-circulating neutrophils in Mel526 (left) and NKIRTIL006 (right) tumor-bearing huMISTRG mice. Numbers indicate the percentages of the different populations.* **(C)** *Quantification of blood-circulating $CD15^+$ human neutrophils at steady state, and in tumor-bearing huMISTRG animals. N=6-10, of 2 independent experiments.* **(D)** *Representative CD11b vs CD16 flow cytometry plots of three different NKIRTIL006 tumor-bearing mice, gated on viable human $CD45^+CD3^-CD19^-CD56^-CD68^-CD15^+CD14^-$ cells.* **(E)** *Quantification of intratumoral $Gr\text{-}1^+$ murine, and $CD15^+$ human neutrophils in tumor-bearing huMISTRG animals (left). Representative flow cytometry plots of intratumoral $Gr\text{-}1^+$ murine, and $CD15^+$ human neutrophils (right). N=10, of 2 independent experiments.* **(F)** *Representative wide-field fluorescent image of a 10 µm NKIRTIL006 tumor section stained for human neutrophils (CD66b, green) and nuclear marker (Hoechst, cyan). Crops (right) were taken from the indicated Tilescan areas of the entire tumor. White arrows indicate CD66b positive staining surrounding a banded nucleus, characteristic nuclear morphology of human neutrophils.* **(G)** *Representative flow cytometry plots of the human immune infiltration in the tumor tissue of NKIRTIL006 tumor-bearing mice, distinguishing T cells (blue gate) from all other immune cells (green and orange gates), gated on viable human $CD45^+$ cells. Numbers indicate the percentages of the different populations. PB, peripheral blood.*

DISCUSSION

Mice transplanted with a human hemato-lymphoid system aim to help close the gap for translating the findings derived from rodents to humans[54]. The development of the neutrophil lineage – the most abundant circulating leukocyte and the first line of defense against infections in humans[17] – in such mice remains defective in traditional humanized mouse strains[54]. In this study, we investigated the functionality of the human neutrophil population in huMISTRG mice, a strain that allows reconstitution of a much more complete human immune system than previous models.[6] We demonstrate that this model system can be suitable for the study of neutrophil biology in human immune processes.

Previous work with huMISTRG mice has shown the presence of only a small number of human neutrophils in the peripheral blood of these mice at steady state, while these are abundantly present in the bone marrow[6]. Rongvaux *et al.* suggested that the terminal differentiation of neutrophils in this mouse environment may be suboptimal, as seen for other humanized strains such as *NSG-SGM3* – NSG mice engineered to constitutively express human stem cell factor, GM-CSF and IL-3 cytokines –, where these displayed the morphology and cell surface phenotype of immature cells[16,55]. In contrast, we found that huMISTRG mice do generate end-stage neutrophils with a $CD11b^+CD16^+$ phenotype and a segmented nucleus, which possess functional capacity in the bone marrow. Others suggested that the egress of neutrophils from the bone marrow could be impaired[6,54] due to incompatibilities in adhesion molecules and chemokine-chemokine receptor pairs

between species[56,57]. However, treatment with the CXCR4 antagonist Plerixafor or with human G-CSF resulted in the release of end-stage human neutrophils into the circulation of huMISTRG animals. This shows that at least the SDF-1α/CXCR4 chemokine axis apparently operates across species and that also the adhesion steps required for migration out of the bone marrow are functional. The dearth of human neutrophils in the circulation of huMISTRG mice at steady state might be explained by a lack of chemotactic cues to induce egress of mature neutrophils from the bone marrow in the absence of inflammation or infections. Indeed, huMISTRG animals are housed in exceptionally clean environments under specific-pathogen-free (SPF) conditions and are maintained under continuous prophylactic large spectrum antibiotics treatment, which altogether may contribute to the containment of the mature human neutrophil pool in the bone marrow niche. It is important to note in this regard that even regular SPF laboratory mice have markedly more neutropenic blood than humans[1,58].

We have shown that the huMISTRG model is amenable to studying antimicrobial properties of human neutrophils. In particular, we demonstrated neutrophil-specific responses towards physiological antimicrobial stimuli. *In vivo*, the extravasation of human neutrophils into the peritoneal cavity of huMISTRG mice after thioglycolate injection further demonstrated the potential to study (trans)migration capacities of neutrophils triggered by chemotactic cues[49,59]. The huMISTRG model could thus be used to test other qualitative human neutrophil functions in various *in vivo* experimental set ups, such as cecal ligation and puncture, ischemia-reperfusion injury, LPS nebulization, sterile heart injury, laser injury in skin or cremaster[60]. Moreover, the discovery of circulating and intratumoral human mature neutrophils in tumor-bearing mice suggests that huMISTRG animals can also be used to address neutrophil functions in cancer *in vivo*. Despite de fact that the tumor microenvironment is mostly of murine origin (*i.e.* endothelium, extracellular matrix, fibroblasts), human neutrophils are evidently still able to extravasate in response to mobilizing factors (such as G-CSF[61]) produced by the human tumor engrafted in huMISTRG mice. There is debate on the nature and function of infiltrated neutrophils in human tumors[62]. On the one hand, neutrophils may promote tumor growth as myeloid-derived suppressor cells, while on the other, they have been implicated as effectors (via ADCC) of antibody treatment of cancer, such with the use of dinutuximab in neuroblastoma patients[22,63]. The huMISTRG model could be further developed into a tool for obtaining the evidence needed to clarify their role.

As described previously[6], huMISTRG display graft-versus-host disease-related erythropenia, that ultimately leads to severe anemia. In our hands, this was not a limiting factor in our study, which involved relatively short term experiments (up to 20 weeks after birth). In addition, breeding of MISTRG animals was similar to other strains. Nevertheless, one should

consider such limitations in the case of long-term experiments. One drawback of the MISTRG mouse model remains that the number of human neutrophils is low. A recent study showed that greater numbers of blood circulating human neutrophils can be obtained in MISTRG mice by replacing the murine gene encoding G-CSF with the human version, in combination with deletion of the murine G-CSF receptor gene, which may partially be explained by the elimination of competition between murine and human neutrophils[64]. As tested in that study, it seems likely that the neutrophils in that model are functional and capable of intra- and extravasation, just as we have shown here for "regular" MISTRG mice. In these latter mice, competition with endogenous murine neutrophils could be eliminated via injection of anti-Gr-1 depleting antibodies which is known to lead to a profound and durable neutropenia[65]. Taken together, although the presence of murine neutrophils in such experimental design should be considered, our data do show that the huMISTRG provides a potential model system for the study of neutrophil biology in complex human diseases, such as the preclinical evaluation of their responses to novel immunotherapeutic approaches against solid cancer and for testing the role of genetic backgrounds or manipulations on their function *in vivo*.

ACKNOWLEDGEMENTS

We thank N. Franke for assistance with obtention of Plerixafor from the Princess Máxima Center.

AUTHOR CONTRIBUTIONS

PM-S and JK designed and performed the experiments, analyzed the data and wrote the manuscript, with the help of DA. MH, AL, ES and NB performed experiments and reviewed the manuscript. GT helped with the study design and reviewed the manuscript. DA, RF, AR, and RB contributed to data interpretation and reviewed the manuscript. TK, HM and KF designed the experiments, interpreted and evaluated the data and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST DISCLOSURE

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FUNDING

JK, DA and HM are supported by The Dutch Cancer Society (grant #13124, #13491 and #11537, respectively). This work was further supported by a grant to DA from Sanquinnovate.

REFERENCES

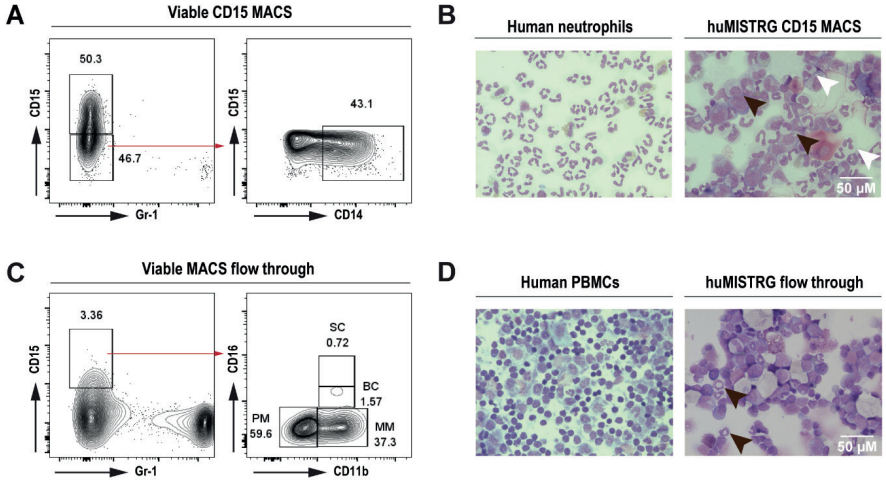
1. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol.* 2004;172(5):2731-2738.
2. Rongvaux A, Takizawa H, Strowig T, et al. Human hemato-lymphoid system mice: current use and future potential for medicine. *Annu Rev Immunol.* 2013;31:635-674.
3. Theocharides APA, Rongvaux A, Fritsch K, Flavell RA, Manz MG. Humanized hemato-lymphoid system mice. *Haematologica.* 2016;101(1):5-19.
4. Fujiwara S. Humanized mice: A brief overview on their diverse applications in biomedical research. *J Cell Physiol.* 2018;233(4):2889-2901.
5. De La Rochere P, Guil-Luna S, Decaudin D, Azar G, Sidhu SS, Piaggio E. Humanized Mice for the Study of Immuno-Oncology. *Trends Immunol.* 2018;39(9):748-763.
6. Rongvaux A, Willinger T, Martinek J, et al. Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol.* 2014;32(4):364-372.
7. Manz MG. Human-hemato-lymphoid-system mice: opportunities and challenges. *Immunity.* 2007;26(5):537-541.
8. Gille C, Orlikowsky TW, Spring B, et al. Monocytes derived from humanized neonatal NOD/SCID/IL2Rγ(null) mice are phenotypically immature and exhibit functional impairments. *Hum Immunol.* 2012;73(4):346-354.
9. Strowig T, Chijioke O, Carrega P, et al. Human NK cells of mice with reconstituted human immune system components require preactivation to acquire functional competence. *Blood.* 2010;116(20):4158-4167.
10. Drake AC, Chen Q, Chen J. Engineering humanized mice for improved hematopoietic reconstitution. *Cell Mol Immunol.* 2012;9(3):215-224.
11. Willinger T, Rongvaux A, Strowig T, Manz MG, Flavell RA. Improving human hemato-lymphoid-system mice by cytokine knock-in gene replacement. *Trends Immunol.* 2011;32(7):321-327.
12. Chen Q, Khoury M, Chen J. Expression of human cytokines dramatically improves reconstitution of specific human-blood lineage cells in humanized mice. *Proc Natl Acad Sci U S A.* 2009;106(51):21783-21788.
13. Li Y, Chen Q, Zheng D, et al. Induction of Functional Human Macrophages from Bone Marrow Promonocytes by M-CSF in Humanized Mice. *J Immunol.* 2013;191(6):3192-3199.
14. Strowig T, Rongvaux A, Rathinam C, et al. Transgenic expression of human signal regulatory protein alpha in Rag2-/-gamma(c)-/- mice improves engraftment of human hematopoietic cells in humanized mice. *Proc Natl Acad Sci U S A.* 2011;108(32):13218-13223.
15. Nguyen R, Patel AG, Griffiths LM, et al. Next-generation humanized patient-derived xenograft mouse model for pre-clinical antibody studies in neuroblastoma. *Cancer Immunol Immunother.* 2021;70(3):721-732.
16. Sippel TR, Radtke S, Olsen TM, Kiem HP, Rongvaux A. Human hematopoietic stem cell maintenance and myeloid cell development in next-generation humanized mouse models. *Blood Adv.* 2019;3(3):268-274.
17. Silvestre-Roig C, Fridlender ZG, Glogauer M, Scapini P. Neutrophil Diversity in Health and Disease. *Trends Immunol.* 2019;40(7):565-583.
18. Treffers LW, Hiemstra IH, Kuijpers TW, van den Berg TK, Matlung HL. Neutrophils in cancer. *Immunol Rev.* 2016;273(1):312-328.

19. Furumaya C, Martinez-Sanz P, Bouti P, Kuijpers TW, Matlung HL. Plasticity in Pro- and Anti-tumor Activity of Neutrophils: Shifting the Balance. *Front Immunol*. 2020;11.
20. Leliefeld PH, Koenderman L, Pillay J. How Neutrophils Shape Adaptive Immune Responses. *Front Immunol*. 2015;6:471.
21. Hosur V, Skelly DA, Francis C, et al. Improved mouse models and advanced genetic and genomic technologies for the study of neutrophils. *Drug Discov Today*. 2020;25(6):1013-1025.
22. Martinez Sanz P, van Rees DJ, van Zogchel LMJ, et al. G-CSF as a suitable alternative to GM-CSF to boost dinutuximab-mediated neutrophil cytotoxicity in neuroblastoma treatment. *J Immunother Cancer*. 2021;9(5).
23. Borregaard N. Neutrophils, from marrow to microbes. *Immunity*. 2010;33(5):657-670.
24. Aarts CEM, Hiemstra IH, Tool ATJ, et al. Neutrophils as Suppressors of T Cell Proliferation: Does Age Matter? *Front Immunol*. 2019;10:2144.
25. Singel KL, Emmons TR, Khan ANH, et al. Mature neutrophils suppress T cell immunity in ovarian cancer microenvironment. *JCI Insight*. 2019;4(5).
26. Grassi L, Pourfarzad F, Ullrich S, et al. Dynamics of Transcription Regulation in Human Bone Marrow Myeloid Differentiation to Mature Blood Neutrophils. *Cell Rep*. 2018;24(10):2784-2794.
27. Lee WL, Harrison RE, Grinstein S. Phagocytosis by neutrophils. *Microbes Infect*. 2003;5(14):1299-1306.
28. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol*. 2004;4(3):181-189.
29. Pullar JM, Vissers MC, Winterbourn CC. Living with a killer: the effects of hypochlorous acid on mammalian cells. *IUBMB Life*. 2000;50(4-5):259-266.
30. Lacy P. Mechanisms of degranulation in neutrophils. *Allergy Asthma Clin Immunol*. 2006;2(3):98-108.
31. Lacy P, Eitzen G. Control of granule exocytosis in neutrophils. *Front Biosci*. 2008;13:5559-5570.
32. Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? *Front Physiol*. 2018;9:113.
33. Häger M, Cowland JB, Borregaard N. Neutrophil granules in health and disease. *J Intern Med*. 2010;268(1):25-34.
34. Hoogendijk AJ, Pourfarzad F, Aarts CEM, et al. Dynamic Transcriptome-Proteome Correlation Networks Reveal Human Myeloid Differentiation and Neutrophil-Specific Programming. *Cell Rep*. 2019;29(8):2505-2519 e4.
35. Aarts CEM, Downes K, Hoogendijk AJ, et al. Neutrophil specific granule and NETosis defects in gray platelet syndrome. *Blood Adv*. 2021;5(2):549-564.
36. Filippi MD. Neutrophil transendothelial migration: updates and new perspectives. *Blood*. 2019;133(20):2149-2158.
37. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. 2013;13(3):159-175.
38. Kishimoto TK, Rothlein R. Integrins, ICAMs, and selectins: role and regulation of adhesion molecules in neutrophil recruitment to inflammatory sites. *Adv Pharmacol*. 1994;25:117-169.
39. Zarbock A, Ley K. Neutrophil adhesion and activation under flow. *Microcirculation*. 2009;16(1):31-42.
40. Heemskerk N, van Egmond M. Monoclonal antibody-mediated killing of tumour cells by neutrophils. *Eur J Clin Invest*. 2018;48 Suppl 2:e12962.
41. van Egmond M, Bakema JE. Neutrophils as effector cells for antibody-based immunotherapy of cancer. *Semin Cancer Biol*. 2013;23(3):190-199.
42. Boross P, Lohse S, Nederend M, et al. IgA EGFR antibodies mediate tumour killing in vivo. *EMBO Mol Med*. 2013;5(8):1213-1226.

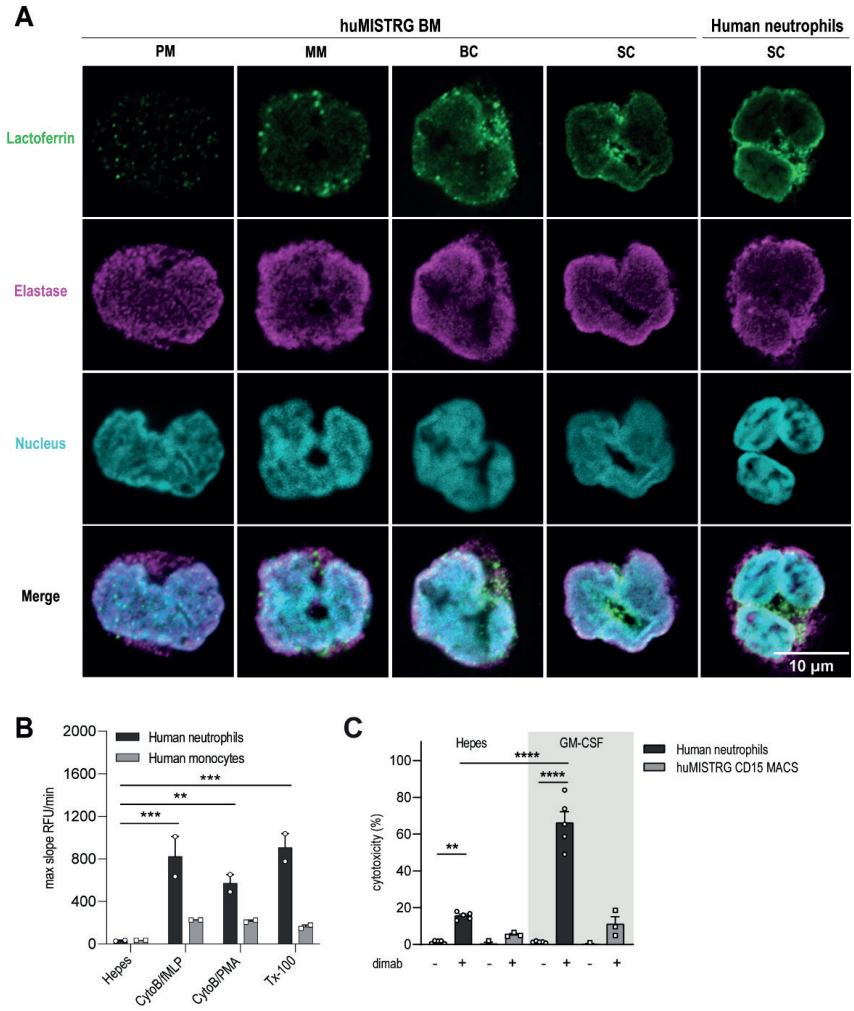
43. Brandsma AM, Ten Broeke T, Nederend M, et al. Simultaneous Targeting of FcgammaRs and FcalphaRI Enhances Tumor Cell Killing. *Cancer Immunol Res*. 2015;3(12):1316-1324.
44. Lohse S, Brunke C, Derer S, et al. Characterization of a mutated IgA2 antibody of the m(1) allotype against the epidermal growth factor receptor for the recruitment of monocytes and macrophages. *J Biol Chem*. 2012;287(30):25139-25150.
45. Bendall LJ, Bradstock KF. G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. *Cytokine Growth Factor Rev*. 2014;25(4):355-367.
46. Semerad CL, Liu F, Gregory AD, Stumpf K, Link DC. G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. *Immunity*. 2002;17(4):413-423.
47. Martin C, Burdon PC, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity*. 2003;19(4):583-593.
48. Nicola NA, Begley CG, Metcalf D. Identification of the human analogue of a regulator that induces differentiation in murine leukaemic cells. *Nature*. 1985;314(6012):625-628.
49. Baron EJ, Proctor RA. Elicitation of peritoneal polymorphonuclear neutrophils from mice. *J Immunol Methods*. 1982;49(3):305-313.
50. Alvarez-Zarate J, Matlung HL, Matozaki T, Kuijpers TW, Maridonneau-Parini I, van den Berg TK. Regulation of Phagocyte Migration by Signal Regulatory Protein-Alpha Signaling. *PLoS One*. 2015;10(6):e0127178.
51. Kelderman S, Heemskerk B, Fanchi L, et al. Antigen-specific TIL therapy for melanoma: A flexible platform for personalized cancer immunotherapy. *Eur J Immunol*. 2016;46(6):1351-1360.
52. Attrill GH, Ferguson PM, Palendira U, Long G V, Wilmott JS, Scolyer RA. The tumour immune landscape and its implications in cutaneous melanoma. *Pigment Cell Melanoma Res*. 2021;34(3):529-549.
53. Moldoveanu D, Ramsay L, Lajoie M, et al. Spatially mapping the immune landscape of melanoma using imaging mass cytometry. *Sci Immunol*. 2022;7(70):eabi5072.
54. Martinov T, McKenna KM, Tan WH, et al. Building the Next Generation of Humanized Hemato-Lymphoid System Mice. *Front Immunol*. 2021;12:643852.
55. Wunderlich M, Chou FS, Sexton C, et al. Improved multilineage human hematopoietic reconstitution and function in NSGS mice. *PLoS One*. 2018;13(12):e0209034.
56. Núñez D, Comas L, Lanuza PM, et al. A Functional Analysis on the Interspecies Interaction between Mouse LFA-1 and Human Intercellular Adhesion Molecule-1 at the Cell Level. *Front Immunol*. 2017;8:1817.
57. Zlotnik A. Chemokines and cancer. *Int J Cancer*. 2006;119(9):2026-2029.
58. Eruslanov EB, Singhal S, Albelda SM. Mouse versus Human Neutrophils in Cancer: A Major Knowledge Gap. *Trends Cancer*. 2017;3(2):149-160.
59. Chavakis T, Bierhaus A, Al-Fakhri N, et al. The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment. *J Exp Med*. 2003;198(10):1507-1515.
60. Margraf A, Ley K, Zarbock A. Neutrophil Recruitment: From Model Systems to Tissue-Specific Patterns. *Trends Immunol*. 2019;40(7):613-634.
61. Karagiannidis I, Salataj E, Said Abu Egal E, Beswick EJ. G-CSF in tumors: Aggressiveness, tumor microenvironment and immune cell regulation. *Cytokine*. 2021;142:155479.

62. Quail DF, Amulic B, Aziz M, et al. Neutrophil phenotypes and functions in cancer: A consensus statement. *J Exp Med*. 2022;219(6).
63. Barker E, Mueller BM, Handgretinger R, Herter M, Yu AL, Reisfeld RA. Effect of a Chimeric Anti-Ganglioside G D2 Antibody on Cell-mediated Lysis of Human Neuroblastoma Cells. *Cancer Res*. 1991;51:144-149.
64. Zheng Y, Sefik E, Astle J, et al. Human neutrophil development and functionality are enabled in a humanized mouse model. *Proc Natl Acad Sci U S A*. 2022;119(4):e2121077119.
65. Stackowicz J, Jönsson F, Reber LL. Mouse Models and Tools for the in vivo Study of Neutrophils. *Front Immunol*. 2020;10.

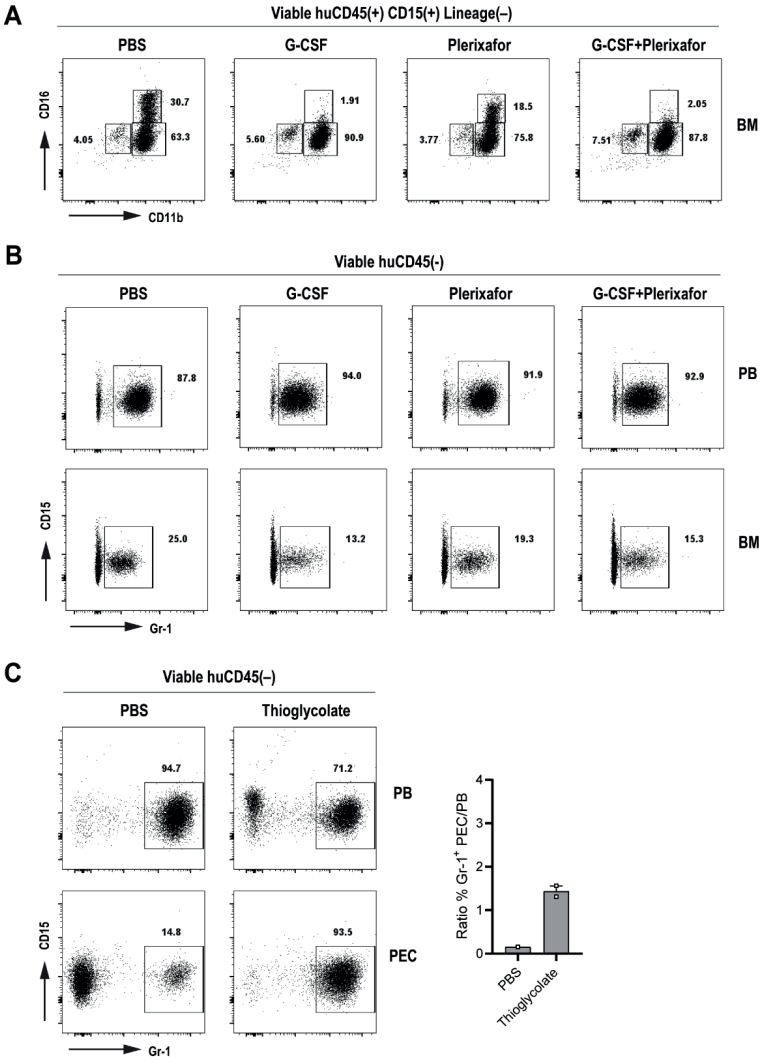
SUPPLEMENTAL DATA



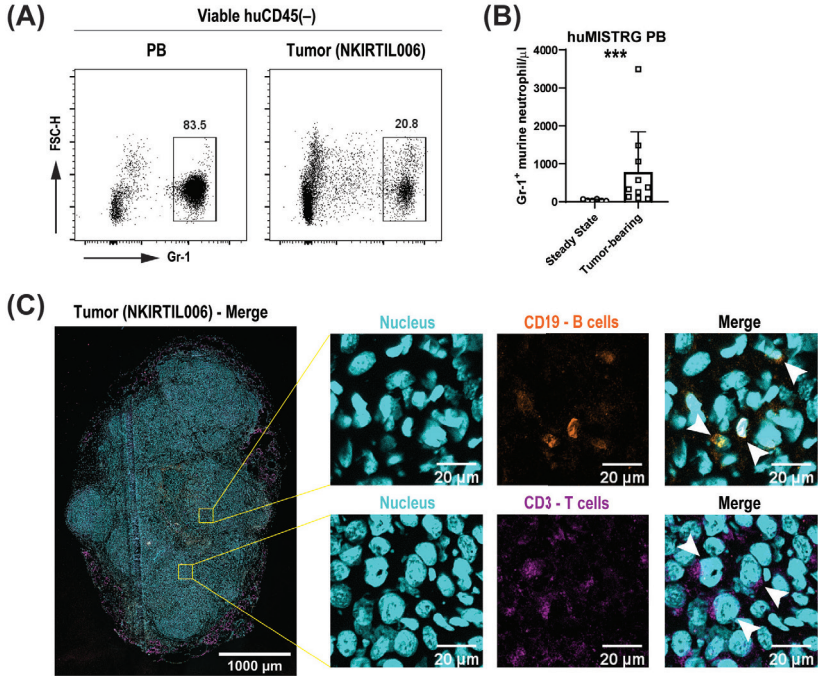
Supplemental Figure 1. (A) *Gating strategy of huMISTRG samples showing presence of CD14⁺ cells within the CD15^{low} MACS-sorted fraction. Numbers indicate the percentages of the different populations.* **(B)** *Representative cytospin of human neutrophils (left) and CD15 MACS-sorted fractions of huMISTRG samples (right) after May-Giemsa staining (objective 50x). Black arrows indicate immature neutrophil subpopulations with round/banded nucleus, white arrows indicate mature subpopulations with segmented nucleus in huMISTRG samples.* **(C)** *Gating strategy of huMISTRG samples showing absence of CD15⁺ cells as well as absence of neutrophil BM subpopulations based on CD11b and CD16 staining (PM, MM, BC, SC) in the discarded flow through after MACS sorting. Numbers indicate the percentages of the different populations.* **(D)** *Representative cytospin of human PBMCs (left) and flow through fraction after MACS sorting of huMISTRG samples (right) after May-Giemsa staining (objective 50x). Black arrows indicate murine neutrophils with circular nucleus in huMISTRG BM samples. PB, peripheral blood; BM, bone marrow; PBMC, peripheral blood mononuclear cells.*



Supplemental Figure 2. (A) *Representative confocal fluorescent image of each flow cytometry sorted neutrophil bone marrow progenitor (PM, MM, BC, SC) stained for specific granule marker lactoferrin (green), azurophilic granule marker neutrophil elastase (magenta), and nuclear marker (Hoechst, cyan). Human control samples of circulating neutrophils were used as control.* **(B)** *Protease activity of human neutrophils (black bars) and human monocytes (isolated by MACS sorting for CD14, grey bars) from human PB in the presence of the indicated stimuli, or Triton (Tx-100) for total release, expressed as max slope RFU/min. N=2, of one individual experiment.* **(C)** *ADCC of NMB neuroblastoma cells opsonized with (+) or without (–) dinutuximab (dimab) by unstimulated (Hepes) or GM-CSF stimulated human neutrophils (black bars) and CD15 MACS-sorted huMISTRG neutrophils (grey bars). N=3-5, of three individual experiments. ADCC, antibody-dependent cellular cytotoxicity; PB, peripheral blood; BM, bone marrow; RFU, relative fluorescent units.*



Supplemental Figure 3. (A) *Representative CD11b vs CD16 flow cytometry plots of BM samples in response to different mobilizing agents, gated on viable human CD45⁺CD15⁺ cells. Numbers indicate the percentages of the different subpopulations.* **(B)** *Representative Gr-1 vs CD15 flow cytometry plots of PM (top panels) and BM (bottom panels) samples in response to different mobilizing agents, gated on viable human CD45⁻ cells. Numbers indicate the percentages of the different subpopulations.* **(C)** *Representative CD15 vs Gr-1 flow cytometry plots of G-CSF mobilized mice in response to peritoneal injection of thioglycolate, gated on viable human CD45⁻ cells. Numbers indicate the percentages of the different subpopulations. On the right, quantification of the influx of murine neutrophils in the peritoneum per condition represented by the ratio of Gr-1⁺ cells in the PEC suspension to those in the PB. N=1-2, of two individual experiments. BM, bone marrow; PB, peripheral blood; PEC, peritoneal exudate cells.*



Supplemental Figure 4. (A) *Representative Gr-1 vs FSC-H flow cytometry plots of PB or NKIRTIL006 tumor tissue samples of tumor-bearing mice, gated on viable human CD45⁻ cells. Numbers indicate the percentages of the different subpopulations.* **(B)** *Quantification of blood-circulating Gr-1⁺ murine neutrophils at steady state, and in tumor-bearing huMISTRG animals. N=6-10, of 2 independent experiments.* **(C)** *Representative wide-field fluorescent image of a 10 µm NKIRTIL006 tumor section stained for human B cells (CD19, orange), T cells (CD3, magenta) and nuclear marker (Hoechst, cyan). Crops (right) were taken from the indicated Tilescan areas of the entire tumor. White arrows indicate respective CD19 and CD3 positive staining surrounding a round nucleus, characteristic nuclear morphology of human lymphocytes.*

6

CHAPTER 7

GENERAL DISCUSSION

Besides the conventional ways to treat cancer (surgery, radiation and chemotherapy, briefly introduced in **chapter 1** of this thesis), we now count with a fourth pillar, immunotherapy, as the ultimate tool to beat cancer. Immunotherapy specifically engages the body's army of soldiers (the immune cells) and turns it against tumor cells. From the broad array of existing immune cells, neutrophils are usually the last cells to be therapeutically engaged for cancer cell eradication. This seems somewhat counterintuitive since they are the largest white blood cell population circulating in our bloodstream, hence they would be the ones yielding the most powerful response, wouldn't they? Yet, their potential as cancer killing cells may have been largely eclipsed because of their crucial role at fighting off infections, as well as their generally known short lifespan, altogether contributing to making them look as less competent cells to combat cancer in comparison to other effector immune cells. Moreover, when thinking of cancer, neutrophils have often been regarded as drivers of tumor cell progression given their well-described ability to suppress anti-tumor immune responses, namely via their myeloid-derived-suppressor cell (MDSC) activity[1–3]. The MDSC activity of neutrophils has, however, always been observed in instances where therapeutic antibodies were not present. In the presence of such a therapeutic intervention, neutrophils are stimulated to actively destroy cancer cells via antibody-dependent cellular cytotoxicity (ADCC)[4–10]. Chen et al. specifically show this in the context of neuroblastoma[11]. This thesis was aimed to show just a glimpse of the road ahead on the outlook of neutrophils as cancer killing cells, which was the central theme of this thesis.

The double-side sword of neutrophils

In recent years a large body of evidence has highlighted the functional plasticity of neutrophils in the context of cancer[12,13]. It has been suggested that they represent a heterogeneous cell population with opposing functions as different factors within the tumor microenvironment may induce either a pro- or an anti-tumorigenic phenotype once recruited to the tumor site[14,15]. In **chapter 2** the different roles that neutrophils can play in cancer are extensively reviewed, where we have seen that both immunosuppressive and anti-tumor neutrophils actually have a few traits in common:

i. The need for activation via specific stimuli in order to optimally exert their function, with fMLP, LPS or TNFα for MDSC activity[16–19], or with GM-CSF, G-CSF and/or IFNγ for ADCC[8,20–22].
ii. The intimate contact mediated by CD11b/CD18 or Mac-1 integrin, either between the neutrophil and the T cell in the case of MDSC activity[17,23,24] or between the neutrophil and the antibody-opsonized tumor cell in the case of ADCC[6,21,25,26].
iii. The mechanical way to either immunosuppress T cells or to kill tumor cells which is in both cases via trogocytosis[6,17].

Conversely, degranulation and ROS production, which are traditional anti-microbial killing processes, are necessary for neutrophil MDSC function but fully dispensable for neutrophil ADCC[6,17], indicating that at least some of the cellular mechanisms needed for the opposing functions of neutrophils are directly antagonistic. Advances in the field demonstrated that there are ways to steer the effector functions of neutrophils in the tumor microenvironment from immunosuppressors to effective tumor killers.

The presence of neutrophils in tumors is in most cases negatively associated with cancer progression and clinical outcome[27–29]. Therefore, one first possibility that crosses one's mind to eliminate the tumor-promoting functions of neutrophils in tumors consists of the general depletion of neutrophils via antibodies for their neutralization. Neutrophil depletion was first proven to work in mouse models resulting in tumor growth inhibition of UV-induced skin tumors[30], the reduction of metastatic lesions in a model of liver metastasis[31] and the reactivation of T cell responses in a model of pancreatic ductal adenocarcinoma[32]. In humans, a similar approach was tested with Gemtuzumab ozogamicin, an antibody specifically designed to neutralize CD33-expressing cells commonly expressed by monocytic and granulocytic MDSCs, which showed restoration of (CAR) T cell anti-tumor response against multiple cancers[33]. Yet, from a clinical point of view a general depletion of neutrophils via antibodies is, however, not a practical option as prolonged neutropenia highly increases the risk of infections and is considered a life-threatening condition[34]. In addition to eliminating their crucial role in host defense against incoming invaders, a systemic depletion of neutrophils may also completely remove their anti-tumor effects from the picture as nicely demonstrated in an *in vivo* model by Albanesi et al[35]. Another formula aimed at targeting the pro-tumor activity of neutrophils is to inhibit their chemotactic-induced recruitment to the tumor site. This has been achieved by approaches that directly inhibit CXCR1-2 receptors expressed on neutrophils which respond to IL-8 secreted by tumors[36]. This was demonstrated to induce more T cell accumulation in tumors enhancing response to immunotherapy in mice[37], the results of which even led to the testing of CXCR1-2 inhibitors in clinical trials[38].

Potentiating neutrophil killing: cytokine stimulation

The easiest trick to redirect the detrimental MDSC activity of neutrophils toward an anti-tumorigenic one is by having them in the context of antibody therapy. Neutrophils can directly bind to the tumor targeting antibody via their Fc receptors and in this way their cytotoxic functions are engaged. In recent years, more and more research is focused on finding ways to promote the antibody-mediated effector capacity of neutrophils and to further exploit it. It is widely known in the neutrophil field that their efficacy to kill antibody-opsonized cancer cells in the case of IgG-based antibodies is potentiated by a prior stimulation and activation of these effector cells by cytokines or growth factors[20,22,39,40].

GM-CSF specifically stimulates neutrophilic granulocytes, in addition to monocytes, dendritic cells, basophils and eosinophils, because of the expression of GM-CSF receptor (CD116) on their surface to which GM-CSF binds evoking an array of effector functions by these cells[41]. In addition, neutrophils (as well as basophils and eosinophils) also express the receptor for G-CSF (CD114) and thereby they can also be activated by this cytokine[42]. These two cytokines have overlapping functions in regards to their effect on neutrophils: they stimulate granulopoiesis, phagocytosis, chemotaxis, and last but not least, also ADCC[43].

In **chapter 3**, we specifically showed how the neutrophil's ability to induce antibody-mediated cytotoxicity towards tumor cells is comparable between neutrophils that were stimulated with either G-CSF or GM-CSF. We explicitly studied this in a pediatric subtype of neuroendocrine tumor: neuroblastoma. Neuroblastoma patients count with a well-defined treatment protocol including surgery, chemotherapy, myeloablative therapy with stem cell rescue and radiotherapy[44]. However, despite intensive multimodal treatment, high-risk neuroblastoma children have high chances of relapsing[45,46]. For this reason, in 2015 the Food and Drug Administration approved the implementation of the IgG-based therapeutic antibody dinutuximab in combination with GM-CSF to increase dinutuximab responsiveness as part of the immunotherapy regimen for those patients with risk of relapsing[47], which significantly increased their prognosis[44]. Dinutuximab specifically targets GD2, a disialoganglioside antigen expressed on tumors of neuroectodermal origin including neuroblastoma[48,49], but also some sarcomas[50] and melanomas[51], and upon binding it marks the cells for immune-mediated destruction by Fc-expressing cells. The need of a widely accessible cytokine to combine with the immunotherapy regimen with IgG-based dinutuximab (dinutuximab has not yet been generated in another form for clinical use) is urgently needed for this cancer type[52,53]. This is because children with neuroblastoma outside of Northern America find themselves at risk for undergoing suboptimal treatment due to the clinical unavailability of GM-CSF, for which the pharmaceutical license is only available in Canada and the United States[54]. Given the fact that neutrophils (and not monocytes and macrophages) have been demonstrated to be the primary cells mediating the *in vitro* killing of neuroblastoma-antibody coated cells, and that in an IgG-based context these need of stimulation to perform their effector functions,[55] it becomes of great importance to find an alternative stimulating cytokine to replace GM-CSF in the regions where the latter is not accessible. Our findings demonstrating comparable cytotoxicity towards neuroblastoma cells by either G-CSF or GM-CSF stimulated neutrophils (either after *in vitro* stimulation or after *in vivo* stimulation) were explained by the similar effect the two cytokines have at influencing the expression of Fcγ receptors and integrin expression upon stimulation. FcγRIIa expression was the most relevant in our ADCC assays, being highly expressed on the surface of either G-CSF or GM-CSF stimulated neutrophils. In line with existing literature[56], FcγRI expression was slightly increased in stimulated neutrophils

to a similar extent regardless of the cytokine used for stimulation. Yet, this receptor was not found to be of relevance at mediating the cytotoxic functions of neutrophils, probably explained by the relatively low expression levels compared to the other Fcγ receptor subtypes[6,57]. With regards to CD11b/CD18 integrin, another important molecule being involved in the immunological synapse between the antibody-opsonized cell and the neutrophil, we found it to be directly responsible of mediating neuroblastoma tumor cell killing both by G-CSF or GM-CSF stimulated neutrophils in our blocking experiments, which aligned to previous findings from other cancer types[6,21,26]. The combination of all these factors gave neutrophils the room to trogocytose the dinutiximab-opsonized cells by tearing off small pieces of the cancer cell membrane resulting in the lysis of the cancer cells, which was performed to the same extent by the differentially stimulated neutrophils. Although a direct causal relation between cell-mediated trogocytosis and tumor cell killing has been demonstrated for solid tumors, for hematological cancers (particularly for malignant B cell cancers), trogocytosis has been described as an immune escape mechanism. In the latter, trogocytosis is often seen as an antigen-modulatory mechanism, instead, as it contributes to the shaving of the targeted antigen making the tumor cells resistant to killing by neutrophils[58–60].

Some studies claimed that G-CSF can directly contribute to enhancing neuroblastoma's tumor cell proliferation and metastasis formation, thereby enhancing tumorigenesis[61–63], when used as a mean to treat chemotherapy-induced neutropenia in these patients. After thorough investigation, we found no indications that G-CSF was affecting neuroblastoma cell's proliferation capacities, the cell's phenotype (epithelial-to-mesenchymal transition was not facilitated after long-term exposure to G-CSF), nor their susceptibility to be killed by neutrophils, irrespective of the expression of (non-functional) G-CSF receptors on the tumor cell's surface, as detected by us and others[64]. Altogether, we strongly believe that our *in vitro* findings regarding the efficacy and safety of G-CSF, combined with substantial experience with G-CSF in the clinics in different pediatric malignancies, encourage direct clinical evaluation of G-CSF to improve immunotherapy in neuroblastoma patients.

Potentiating neutrophil killing: checkpoint inhibition

Although the recent addition of dinutuximab (with or without cytokine administration) to the treatment protocol for high-risk neuroblastoma patients improved their 5-year survival rate from 20% to 50%, there is still significant room for improvement as still half of the patients relapse or die from it. Pediatric tumors are characterized as extremely aggressive tumors with a highly immunosuppressive microenvironment[65,66]. In addition to the low mutational rates that these tumors display, it all contributes to the generation of tumors of low immunogenic profiles with regards to T cell infiltration, such that they are often considered to be "cold tumors"[67,68]. As a result, the T cell-directed therapies

that in adult tumors have resulted in great therapeutic outcomes, do not seem to be of benefit for childhood malignancies[69]. Instead, neuroblastoma tumors have high infiltration rate of myeloid cells, including neutrophils, monocytes and NK cells[70–72], which would probably explain the fact that neutrophils are among the main mediators of ADCC in this particular cancer type. Therefore, it seems logical that immunotherapeutic strategies aimed at targeting these effector cells, rather than T cells, may result in better responses.

Considering that neutrophils may indeed act as a relevant effector cells in pediatric malignancies and solid tumors in general, their tumor cell killing capacity may benefit from additional myeloid checkpoint inhibition. In this study we focused on the most well-studied myeloid immune checkpoint CD47/SIRPα that has already been shown to render tumor cells vulnerable to neutrophil-mediated cytotoxicity upon disruption, particularly in the presence of cancer-opsonizing antibodies, in other solid cancers both *in vitro* and *in vivo*[6,73–76]. For macrophages, a similar effect is seen for antibody-dependent cellular phagocytosis (ADCP)[75,77,78]. In **chapter 4**, we set out to explore whether different strategies aimed at disrupting CD47/SIRPα axis would improve dinutuximab responsiveness. We first illustrated the clinical relevance of neuroblastoma tumors as potential targets for myeloid checkpoint inhibition strategies by providing evidence that neuroblastoma cells try to evade clearance by neutrophils through the overexpression of the phagocytic surface protein CD47 ("don't eat me" signal), which was not found in adrenal gland healthy tissue. Reports have shown that CD47 transcription is induced by *MYC(N)* proto-oncogene[79]. This could perhaps explain CD47 overexpression specifically by neuroblastoma cells, since genomic amplification of *MYCN* is the most frequent genetic aberration detected in high-risk neuroblastoma[80,81]. Although relevant, this has not yet been investigated and it would be a factor to further examine.

Next, we demonstrated potent therapeutic synergy resulting from the targeting of GD2 with dinutuximab and the inhibition of CD47/SIRPα axis. We showed significantly augmented anti-tumor efficacy of neutrophils, demonstrating that the removal of this break unleashed the effector cell's potential to the fullest (in some instances the levels of cytotoxicity reached around 80-100%). This was shown by the two different approaches tested: i) by genetic ablation of CD47 on the tumor cells or ii) by monoclonal antibody blockade of SIRPα on the neutrophils. Monoclonal antibodies to either of the molecules have been developed to block the interaction[82,83]. Yet, therapeutically speaking, considering the more restricted expression of SIRPα on myeloid cells versus the CD47's ubiquitous expression also in healthy cells (*i.e.* for red blood cells, CD47 is a regulator of their lifespan to which macrophages bind for their clearance upon aging), it seems advantageous to focus on targeting SIRPα with a blocking agent. In fact, a number of drugs targeting CD47 have been tested in clinical trials, and although some have only shown moderate toxic

effects (which was the case for the already clinically validated Magrolimab[84]), others had to be stopped due to reaching life-threatening anemia levels[83,85]. Antibodies to SIRPα, instead, only showed mild effects including infusion-related reactions, fatigue, headache and diarrhea, while avoiding anemia, when used as monotherapy[86].

The above-mentioned results were found in instances where the tumor cells expressed high levels of GD2 antigen and thereby could be strongly opsonized with dinutuximab. However, although not too frequent[87], GD2 loss has been described in a number of patients[88], which is believed to be explained by the outgrowth of GD2-negative clones after intensive therapy giving rise to mesenchymal-like cells[89]. Since we found no enhanced neutrophil-mediated anti-tumor effect upon CD47/SIRPα blockade in the absence of opsonizing antibody, it is important to keep in mind that patients lacking GD2 expression will most likely not benefit from this therapeutic strategy as monotherapy (the monotherapy effect has only been recently described in the case of macrophage-mediated phagocytosis[90]). In the field of neuroblastoma, efforts at finding alternative targetable molecules with antibody therapy to engage neutrophil-mediated killing are currently being investigated[91,92]. An intriguing and promising target is the B7-H3 (B7 homolog 3 protein, also known as CD276), a newly discovered protein of the B7 family that is overexpressed in tumor tissues – including neuroblastoma – while having limited expression in normal tissues[93]. Among other functions, B7-H3 has been found to exert a protective role from NK cell-mediated cytotoxicity of neuroblastoma cells *in vitro*, acting as a sort of novel immune checkpoint[94]. Together with other preclinical evidence pointing in this direction[95], this resulted in the design of a phase I clinical trial where the safety and tolerability of an anti-B7-H3 antibody was tested in a number of childhood solid tumors, including neuroblastoma (NCT02982941). It would be of interest to assess whether therapeutically targeting B7-H3 with or without CD47/SIRPα checkpoint blockade would also result in more robust neutrophil-mediated killing in cases where GD2 targeting is not an option due to its absence. Moreover, there are also other means of escaping immunosurveillance harnessed by tumor cells, apart from CD47/SIRPα (and perhaps also B7-H3). A common one is hypersialylation of proteins on their cell surfaces. Recently, a study performed in mice that investigated the effect of a CD47 blocking agent in combination with dinutuximab on macrophage phagocytosis, showed that the therapeutic synergy found with these two agents was driven by GD2 specific factors that reoriented the balance of macrophage activity towards phagocytosis[96]. Amongst these, was the newly described GD2/Siglec-7 axis that appeared to be hampering the macrophage's phagocytic capacities towards tumor cells (GD2 contains two sialic acid residues with which it can bind to Siglecs expressed on immune cells). It would be of interest to test whether a similar phenomenon would take place in the context of neutrophils, which express their own set of Siglec molecules (*i.e.* Siglec-5, -9, -14)[97,98].

Alternative ways of exploiting anti-tumor neutrophil functions

Next to Fcγ receptors, neutrophils also express the activating myeloid Fcα receptor (also known as FcαRI or CD89)[99]. FcαR specifically crosslinks with antibodies of an IgA isotype which is the predominant antibody subclass present in mucosal areas having a key role in mucosal defense. IgA antibodies have also been demonstrated to be a promising therapeutic option in cancer treatment[100,101], where they engage the neutrophil's killing capacities in a superior way than when compared to IgG in the absence of neutrophil stimulation[102–105]. Although the signaling pathways downstream of either activating receptors are comparable for the different isotypes, the superior ability of IgA at inducing antibody-mediated cytotoxic responses has been linked to the higher avidity with its receptor, which recruits up to four ITAMs due to bivalent binding (1:2 stoichiometry)[106,107]. In the case of IgG antibodies, these can only bind in a 1:1 conformation[108]. Therefore, the use of IgA-based antibodies could be a possible alternative to steer the neutrophil's phenotype into a tumor-eliminating one. An important downside of IgA antibodies is, however, their rapid clearance because of their inability to bind the recycling neonatal Fc receptor (FcRn). This directly influences their serum half-life which is estimated to be of 4-6 days in humans[100,109], whereas IgG antibodies stay in circulation up to about 21 days[110]. The fast catabolism of IgA-based antibodies greatly restricts their application for clinical use and scientists in the field are actively looking for strategies to prolong its half-life (*i.e.* by introducing FcRn-binding domains)[111]. In addition, alternative approaches aimed at engaging the powerful FcαR-mediated responses of neutrophils should be investigated.

In **chapter 5** we aimed to show proof of another possible way to circumvent the above-mentioned limitation to further improve the neutrophil's tumor cell killing ability. We made use of genetic engineering to generate a GD2-directed chimeric antigen receptor (CAR) that was coupled to the FcαR signaling moiety to specifically redirect the Fcα-mediated effector functions of neutrophils to the target of interest. The construct was designed for its insertion in NB4-differentiated neutrophils which allow gene editing while maintaining the standard NB4 neutrophil functions. Based on the evidence mentioned above, we hypothesized that the signaling moiety of such an activating receptor would induce amplified responses than when using that of a FcγR. Indeed, upon target cell recognition the cytolytic activity of neutrophils was directly triggered towards GD2-expressing cells without the need of target cell opsonization. In contrast, this was not observed when testing a FcγRIIa-bearing CARs directed to the same antigen (data not shown in this thesis). We believe that the results in this study serve as a first step to allow the rational design of innovative immunotherapeutic strategies to harness the neutrophil's most powerful cytotoxic abilities in a CAR-mediated context. This should redirect their functions as immunosuppressors to tumor cell killers in the tumor microenvironment, which could be highly advantageous to treat tumor indications characterized by a high neutrophil (or

myeloid) infiltration rate, such as that of neuroblastoma[70]. This is especially needed because of the limited clinical successes of adoptive cell transfer with CAR T cells shown in this specific tumor type[112].

Similar results were found for constructs designed to recognize two other major solid tumor antigens: EGFR (overexpressed in epithelial carcinomas[113]) and HER2/neu (overexpressed in breast cancers[114]), highlighting the potential of our FcαR-based CAR approach for these tumor indications as well. Nonetheless, it is worth mentioning that finding an ideal target antigen in solid tumors for CAR therapy purposes appears to be more challenging than finding one in hematological malignancies. In contrast to the latter, most solid tumors tend to overexpress tumor-associated antigens which are also generally expressed at low levels in normal tissues[115]. This is the case for the three tumor antigens targeted in the study presented in this thesis (GD2, EGFR and HER2/neu). This underlines the relevance of finding a safe antigen considering that even modest levels of the target antigen on healthy tissue may cause significant toxicities which should be carefully monitored[116,117]. Several studies on CAR T cells have shown that the affinity by which the CAR binds to the target should also be taken in consideration when thinking of the safety profile of such an approach, and highlight that a higher binding affinity does not always translate into better efficacy[118–122]. Off-target toxicities would perhaps be of less concern for myeloid-based CARs given their rapid turnover in comparison to T lymphocytes. One could speculate that the infusion-related complications of these ready-to-kill myeloid-based CAR cells would be much milder given the evidence from phase I clinical trials with anti-SIRPα agents, which are believed to fully unleash the anti-tumor effector functions of myeloid cells without causing severe adverse reactions[86]. As already mentioned earlier in this chapter, B7-H3 might be a promising molecule to target neuroblastoma tumors because of its restricted expression in cancer cells of a range of solid tumors, while exhibiting little or no protein surface expression in most normal tissue[95,123,124]. In fact, a recent study found that B7-H3-directed CAR-T cells have significant anti-tumor efficacy in solid tumor preclinical models, including neuroblastoma and other pediatric cancers[125]. It would be worth testing whether a similar outcome would result from myeloid cell-based therapies.

Given that neutrophils are short-lived cells that have lost their capacity to further proliferate, making it impossible to expand in culture or to apply gene editing techniques, other alternatives to primary neutrophils or the immortal neutrophil-like cells (*i.e.* NB4 or HL60 cell lines) have been explored. Fully functional and well-differentiated human neutrophils have been generated from either induced-pluripotent stem cells (iPSCs) or cord-blood/peripheral blood-derived $CD34^+$-hematopoietic stem cells (HSCs). In some instances these have been contemplated as a neutrophil source for cell immunotherapy purposes after genetic engineering[126,127]. The engineering of such cells with synthetic CAR

molecules for their differentiation into neutrophils has already been shown and these worked effectively at lysing tumor cells preclinically[128,129]. Next step in line for the FcαR-based CAR described in our study would be to test its effectiveness in such a setting with the goal to one day generate a clinically applicable CAR-neutrophil product that could be infused into cancer patients. Further genetic modifications may need to be taken into account when developing such a product to try to prevent adverse events such as HLA alloimmunization, which is a well-recognized complication of granulocyte transfusions[130]. Additionally, another technical consideration to think about during manufacturing is the large number of cells that would need to be infused into the patients in order to expect efficacy. For granulocyte transfusions this number ranges from 1.5-6 × 10^9 granulocytes/kg[131].

Most importantly, growing evidence suggests that neutrophils are capable of controlling and modulating adaptive immune responses through cellular crosstalk, either at sites of infection or in the context of cancer[132,133]. With regards to the latter, one study elegantly showed enhanced neutrophil-induced T cell activation upon administration of Fc/IL-2+TA99 antibody in an adoptive T cell transfer setting, which induced robust cures of established melanoma tumors[134]. In light of this, neutrophils strongly resonate as promising effector cells in cancer therapy, owing to the now widely acknowledged believe that in order for immunotherapies to be curative, they must not only directly destroy tumor cells but also initiate adaptive immune responses to achieve long-lasting tumor-specific immunity[135].

Advancing in the preclinical study of neutrophils *in vivo*

The preclinical use of mouse models allow to properly study the systemic effect of specific immunotherapeutic approaches. *In vivo* experimentation with these laboratory animals makes it possible to better investigate the interrelation of all body cells with each other in an organismal setting. But, by definition, findings derived from mouse models cannot be directly extrapolated to humans, especially when studying neutrophils, one of the immune cell types showing the largest fundamental differences between these two species – the most remarkable being the predominant presence of neutrophils in human circulation (50-70%), whereas these are way less abundant in mouse blood (10-30%)[136,137]. Added to the fact that experimental therapy in humans is constrained by technical and ethical considerations, this creates a need for more suitable models in which human hemato-lymphoid cells can be specifically studied to allow better translatability of preclinical findings. The development of humanized mice generated from engrafting human $CD34^+$ HSCs in highly immunodeficient mice just after sublethal total body irradiation have gained a lot of attention in the last decades[138]. Depending on which cell compartment of the immune system one aims to study, a variety of available humanized strains are available, nonetheless, with regards to neutrophil research, the options are limited to only a few

models. The humanized MISTRG (huMISTRG) mice are considered an improved model compared to other traditional humanized strains as they have shown complete human immune system reconstitution, with a robust representation of a myeloid compartment including neutrophils. Nonetheless, the incapacity of the human neutrophils to leave the bone marrow niche and enter the bloodstream has probably contributed to the focus on characterizing other cells of the myeloid compartment, including monocytes, macrophages and NK cells[139]. However, this thesis is a clear example of the relevance of neutrophils as effectors cells in cancer especially when engaged with immunotherapy, which prompted us to find out whether huMISTRG animals could be a suitable model to study neutrophil involvement in health and disease.

In **chapter 6** we carried out an exhaustive phenotypic and functional characterization of the neutrophil compartment in huMISTRG mice. While other studies only used size (FSC) and granularity (SSC) parameters together with CD16 or CD66b markers to trace human neutrophils in huMISTRG animals, we made use of additional neutrophil specific markers. These included CD15, which help distinguish neutrophils from other myeloid cells (*i.e.* monocytes), together with CD11b and CD16 maturation markers, which aid to further subdivide the different neutrophil progenitor bone marrow stages. EMR3 and Siglec-9 were also included as differentiation markers, for which the expression was found to be higher in the most mature subpopulations. In addition, we showed the morphological nuclear changes characteristic of neutrophil differentiation with the acquisition of increased number of nuclear segmentations upon maturation. To date, this constitutes the most thorough neutrophil phenotypic characterization in these animals.

Functionally, we have also shown for the first time that human neutrophils, isolated from the bone marrow samples of huMISTRG mice via magnetic anti-CD15 beads, shared functional similarities with human blood-derived neutrophils in phagocytosis, ROS production, degranulation and proteolytic activity, adhesion capacities and ADCC of tumor cells when tested *ex vivo*. Most of the functional assays required reasonably high number of cells which made it impossible to perform some of the studies upon further purification into the different progenitors and fully differentiated neutrophils. *In vivo*, the fact that we were able to mobilize (mostly mature) neutrophils to the periphery and tissues (*i.e.* peritoneum, tumor) in response to mobilizing factors, inflammation or tumors contradicted the statements of all previous reports claiming a suboptimal environment in these animals to survive and migrate to tissues[139,140]. In fact, these experiments highlighted that neutrophils are able to overcome any of the barriers posed by the murine endothelium and that they were dotted with excellent migration capacities *in vivo*. Some neutrophil functions that were not tested in our study, such as NET formation and chemotaxis *ex vivo*, and response to LPS nebulization *in vivo*, were confirmed recently by others[141]. Importantly, Zheng and colleagues used an

improved version of huMISTRG mice aimed to create a more suitable environment for neutrophils, termed "huMISTRGGR". These mice were modified to additionally harbor the human version of G-CSF cytokine at the same time that the murine receptor for G-CSF was ablated. This was aimed at reducing competition between the two different neutrophil species present in these humanized mice.

Now that we have shown that the huMISTRG mice represent a unique mouse model permitting the study of neutrophil-related immune processes, this opens up a world of possibilities in which to test their involvement in complex human diseases, such as cancer (but also in infectious diseases and inflammation). In fact, since the huMISTRGGR model greatly improved human neutrophil numbers in peripheral blood and tissues, this may represent a more suitable model in which to further examine the role of neutrophils in tumors. When further optimizing these models, even a personalized model wherein the interaction of the patient's immune system with his/her own cancer cells could be studied, where the effect of relevant immunotherapeutic approaches could be tailored "on the spot". Given that the standard of care of neuroblastoma patients allows the acquisition of autologous tumor matched $CD34^+$ HSCs with which huMISTRG or huMISTRGGR animals could be reconstituted, added to the fact that neutrophils have a prominent role in dinutuximab-mediated tumor cell killing, this seems to be a potentially interesting tumor model in which to study neutrophil-mediated immunotherapies in these animals.

Still there is much to improve in the model. When testing neutrophil-directed immunotherapies, one should bear in mind that murine neutrophils may also be engaged by those. To circumvent the need to generate mutant mice, a systemic administration of anti-Gr-1 antibodies prior to treatment is an already established methodology leading to a profound neutropenia[142] that could be exploited to make sure that only the contribution of the human neutrophil population is being studied. Moreover, as briefly touched upon in the first chapter of this thesis, it is important to consider the possibility that the neutrophil responses in these humanized mice may not occur exactly as they do in humans. Although huMISTRG mice have been claimed to reproduce human tumor pathology successfully[139,143], implanted tumors might still reflect the "escape phase" where mostly the tumor-promoting mechanisms of neutrophils are dominant[144,145]. Lastly, when aiming to experiment with huMISTRG mice, the fact that mice will eventually develop severe anemia, due to an enhanced human myeloid function inducing high levels of mouse RBC phagocytosis, should be contemplated[146]. This might restrict the longevity of the experiments significantly[139]. As the field advances, these models will begin to incorporate more and more human physiological elements that will contribute to the rapid preclinical evaluation of novel immunotherapeutic agents, which ultimately may aid to the achievement of more cures of patients with cancer.

Thinking outside the box

At the end of the day, the ultimate recurring question that we all ask ourselves is whether the cancer's end is conceivable in the (*near*) future, and whether it is possible to eradicate this disease from our body and society forever. The latter is quite an ambitious and seemingly unrealistic goal, since up to today only one disease affecting humans has been fully eradicated (smallpox was considered eradicated in 1980)[147]. Nevertheless, the whole scientific community is clearly devoted to find a code that will enable the human system to fight, and perhaps even beat or cure cancer one day. From my *almost naïve* perspective, I feel that the cure for cancer is, unfortunately, still far from being discovered. I strongly believe, however, that we have reached a turning moment in our battle against cancer. Huge advancements have certainly been made in the field. We have learnt that given the right conditions, the human immune system is capable of recognizing and killing cancer. In this thesis we have shown this for one relevant subtype of effector immune cell – the neutrophil –, but researchers all around the globe are thoroughly investigating this for every other existing immune cell type. I am certain that we are on the right track and that immunotherapy is probably the best tool to get closer to a possible "cure", most likely when used in combination with the other conventional therapies. Regardless if we get there or not, I believe that the current goal should be focused on changing what it means to have cancer, and work toward making it a chronic but controllable condition instead. A window of opportunity has just been opened and we now can start to reinvent the relationship we have with a condition that has defined us for far too long. I don't know about you, but I find that exciting!

REFERENCES

1. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9(3):162-174.
2. Veglia F, Perego M, Gabrilovich D. Myeloid-derived suppressor cells coming of age. *Nat Immunol*. 2018;19(2):108-119.
3. Aarts CEM, Kuijpers TW. Neutrophils as myeloid-derived suppressor cells. *Eur J Clin Invest*. 2018;48(S2):e12989.
4. Gale RP, Zighelboim J. Polymorphonuclear leukocytes in antibody-dependent cellular cytotoxicity. *J Immunol*. 1975;114(3):1047-1051.
5. Albanesi M, Mancardi DA, Jönsson F, et al. Neutrophils mediate antibody-induced antitumor effects in mice. *Blood*. 2013;122(18):3160-3164.
6. Matlung HL, Babes L, Zhao XW, et al. Neutrophils Kill Antibody-Opsonized Cancer Cells by Trogoptosis. *Cell Rep*. 2018;23(13):3946-3959 e6.
7. Rosner T, Kahle S, Montenegro F, et al. Immune Effector Functions of Human IgG2 Antibodies against EGFR. *Mol Cancer Ther*. 2019;18(1):75-88.
8. Siders WM, Shields J, Garron C, et al. Involvement of neutrophils and natural killer cells in the anti-tumor activity of alemtuzumab in xenograft tumor models. *Leuk Lymphoma*. 2010;51(7):1293-1304.
9. Hernandez-Ilizaliturri FJ, Jupudy V, Ostberg J, et al. Neutrophils Contribute to the Biological Antitumor Activity of Rituximab in a Non-Hodgkin's Lymphoma Severe Combined Immunodeficiency Mouse Model. *Clin Cancer Res*. 2003;9(16):5866-5873.
10. Heemskerk N, van Egmond M. Monoclonal antibody-mediated killing of tumour cells by neutrophils. *Eur J Clin Invest*. 2018;48 Suppl 2:e12962.
11. Chen RL, Reynolds CP, Seeger RC. Neutrophils are cytotoxic and growth-inhibiting for neuroblastoma cells with an anti-GD2 antibody but, without cytotoxicity, can be growth-stimulating. *Cancer Immunol Immunother*. 2000;48(11):603-612.
12. Treffers LW, Hiemstra IH, Kuijpers TW, van den Berg TK, Matlung HL. Neutrophils in cancer. *Immunol Rev*. 2016;273(1):312-328.
13. Grecian R, Whyte MKB, Walmsley SR. The role of neutrophils in cancer. *Br Med Bull*. 2018;128(1):5-14.
14. Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? *Carcinogenesis*. 2012;33(5):949-955.
15. Rakic A, Beaudry P, Mahoney DJ. The complex interplay between neutrophils and cancer. *Cell Tissue Res*. 2018;371(3):517-529.
16. Fridlender ZG, Sun J, Kim S, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell*. 2009;16(3):183-194.
17. Aarts CEM, Hiemstra IH, Béguin EP, et al. Activated neutrophils exert myeloid-derived suppressor cell activity damaging T cells beyond repair. *Blood Adv*. 2019;3(22):3562-3574.
18. Lechner MG, Liebertz DJ, Epstein AL. Characterization of Cytokine-Induced Myeloid-Derived Suppressor Cells from Normal Human Peripheral Blood Mononuclear Cells. *J Immunol*. 2010;185(4):2273-2284.
19. Pylaeva E, Lang S, Jablonska J. The Essential Role of Type I Interferons in Differentiation and Activation of Tumor-Associated Neutrophils. *Front Immunol*. 2016;7:629.

20. Tamamori Y, Sawada T, Nishihara T, et al. Granulocyte-colony stimulating factor enhances chimeric antibody Nd2 dependent cytotoxicity against pancreatic cancer mediated by polymorphonuclear neutrophils. *Int J Oncol*. 2002;21(3):649-654.
21. Metelitsa LS, Gillies SD, Super M, Shimada H, Reynolds CP, Seeger RC. Antidisialoganglioside/granulocyte macrophage-colony-stimulating factor fusion protein facilitates neutrophil antibody-dependent cellular cytotoxicity and depends on FcgammaRII (CD32) and Mac-1 (CD11b/CD18) for enhanced effector cell adhesion and azurophil granule exocytosis. *Blood*. 2002;99(11):4166-4173.
22. Cheung IY, Hsu K, Cheung NK. Activation of peripheral-blood granulocytes is strongly correlated with patient outcome after immunotherapy with anti-GD2 monoclonal antibody and granulocyte-macrophage colony-stimulating factor. *J Clin Oncol*. 2012;30(4):426-432.
23. Singel KL, Emmons TR, Khan ANH, et al. Mature neutrophils suppress T cell immunity in ovarian cancer microenvironment. *JCI Insight*. 2019;4(5).
24. Pillay J, Kamp VM, van Hoffen E, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. 2012;122(1):327-336.
25. Bouti P, Zhao XW, Verkuijlen P, et al. Kindlin3-Dependent CD11b/CD18-Integrin Activation Is Required for Potentiation of Neutrophil Cytotoxicity by CD47-SIRPα Checkpoint Disruption. *Cancer Immunol Res*. 2021;9(2):147-155.
26. Kushner BH, Cheung NK v. Absolute Requirement of CD11/CD18 Adhesion Molecules, FcRII, and the Phosphatidylinositol-Linked FcRIII for Monoclonal Antibody-Mediated Neutrophil Antihuman Tumor Cytotoxicity. *Blood*. 1992;79(6):1484-1490.
27. Gentles AJ, Newman AM, Liu CL, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med*. 2015;21(8):938-945.
28. Templeton AJ, McNamara MG, Seruga B, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst*. 2014;106(6):dju124.
29. Guthrie GJ, Charles KA, Roxburgh CS, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: experience in patients with cancer. *Crit Rev Oncol Hematol*. 2013;88(1):218-230.
30. Pekarek LA, Starr BA, Toledano AY, Schreiber H. Inhibition of tumor growth by elimination of granulocytes. *J Exp Med*. 1995;181(1):435-440.
31. Coffelt SB, Kersten K, Doornebal CW, et al. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature*. 2015;522(7556):345-348.
32. Stromnes IM, Brockenbrough JS, Izeradjene K, et al. Targeted depletion of an MDSC subset unmasks pancreatic ductal adenocarcinoma to adaptive immunity. *Gut*. 2014;63(11):1769.
33. Fultang L, Panetti S, Ng M, et al. MDSC targeting with Gemtuzumab ozogamicin restores T cell immunity and immunotherapy against cancers. *EBioMedicine*. 2019;47:235-246.
34. Ford A, Marshall E. Neutropenic sepsis: a potentially life-threatening complication of chemotherapy. *Clin Med (Lond)*. 2014;14(5):538-542.
35. Albanesi M, Mancardi DA, Jönsson F, et al. Neutrophils mediate antibody-induced antitumor effects in mice. *Blood*. 2013;122(18):3160-3164.
36. Alfaro C, Teijeira A, Onate C, et al. Tumor-Produced Interleukin-8 Attracts Human Myeloid-Derived Suppressor Cells and Elicits Extrusion of Neutrophil Extracellular Traps (NETs). *Clin Cancer Res*. 2016;22(15):3924-3936.
37. Sun L, Clavijo PE, Robbins Y, et al. Inhibiting myeloid-derived suppressor cell trafficking enhances T cell immunotherapy. *JCI Insight*. 2019;4(7).

38. Schott AF, Goldstein LJ, Cristofanilli M, et al. Phase Ib Pilot Study to Evaluate Reparixin in Combination with Weekly Paclitaxel in Patients with HER-2–Negative Metastatic Breast Cancer. *Clin Cancer Res*. 2017;23(18):5358-5365.
39. Kushner BH, Cheung NK. GM-CSF enhances 3F8 monoclonal antibody-dependent cellular cytotoxicity against human melanoma and neuroblastoma. *Blood*. 1989;73(7):1936-1941.
40. Michon J, Moutel S, Barbet J, et al. In Vitro Killing of Neuroblastoma Cells by Neutrophils Derived From Granulocyte Colony-Stimulating Factor-Treated Cancer Patients Using an Anti-Disialoganglioside/Anti-FcγRI Bispecific Antibody. *Blood*. 1995;86(3):1124-1130.
41. Carulli G. Effects of recombinant human granulocyte colony-stimulating factor administration on neutrophil phenotype and functions. *Haematologica*. 1997;82(5):606-616.
42. Bendall LJ, Bradstock KF. G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. *Cytokine Growth Factor Rev*. 2014;25(4):355-367.
43. Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol*. 2008;8(7):533-544.
44. Smith V, Foster J. High-Risk Neuroblastoma Treatment Review. *Children (Basel)*. 2018;5(9).
45. Maris JM, Hogarty MD, Bagatell R, Cohn SL. Neuroblastoma. *The Lancet*. 2007;369(9579):2106-2120.
46. Park JR, Bagatell R, London WB, et al. Children's Oncology Group's 2013 blueprint for research: neuroblastoma. *Pediatr Blood Cancer*. 2013;60(6):985-993.
47. Food and Drug Admministration. Drug Trials Snapshot: UNITUXIN. Published June 11, 2015. Accessed March 21, 2023. https://www.fda.gov/drugs/drug-approvals-and-databases/drug-trials-snapshots-unituxin
48. Yanagisawa M, Yoshimura S, Yu RK. Expression of GD2 and GD3 gangliosides in human embryonic neural stem cells. *ASN Neuro*. 2011;3(2):e00054.
49. Cheung NK, Saarinen UM, Neely JE, Landmeier B, Donovan D, Coccia PF. Monoclonal antibodies to a glycolipid antigen on human neuroblastoma cells. *Cancer Res*. 1985;45(6):2642-2649.
50. Chang HR, Cordon-Cardon C, Houghton AN, Cheung NK, Brennan MF. Expression of disialogangliosides GD2 and GD3 on human soft tissue sarcomas. *Cancer*. 1992;70(3):633-638.
51. Hamilton WmB, Helling F, Lloyd KO, Livingston PO. Ganglioside expression on human malignant melanoma assessed by quantitative immune thin-layer chromatography. *Int J Cancer*. 1993;53(4):566-573.
52. McGinty L, Kolesar J. Dinutuximab for maintenance therapy in pediatric neuroblastoma. *Am J Health Syst Pharm*. 2017;74(8):563-567.
53. Baldwin GC, Chung GY, Kaslander C, Esmail T, Reisfeld RA, Golde DW. Colony-stimulating factor enhancement of myeloid effector cell cytotoxicity towards neuroectodermal tumour cells. *Br J Haematol*. 1993;83(4):545-553.
54. LEUKINE® (sargramostim) is the first and only FDA-approved GM-CSF. Accessed March 2, 2023. https://www.leukine.com/
55. Barker E, Mueller BM, Handgretinger R, Herter M, Yu AL, Reisfeld RA. Effect of a chimeric anti-ganglioside GD2 antibody on cell-mediated lysis of human neuroblastoma cells. *Cancer Res*. 1991;51(1):144-149.
56. Repp R, Valerius T, Sendler A, et al. Neutrophils express the high affinity receptor for IgG (Fc gamma RI, CD64) after in vivo application of recombinant human granulocyte colony- stimulating factor. *Blood*. 1991;78(4):885-889.

57. Treffers LW, Zhao XW, van der Heijden J, et al. Genetic variation of human neutrophil Fcgamma receptors and SIRPalpha in antibody-dependent cellular cytotoxicity towards cancer cells. *Eur J Immunol*. 2018;48(2):344-354.
58. Bonavida B. Postulated Mechanisms of Resistance of B-Cell Non-Hodgkin Lymphoma to Rituximab Treatment Regimens: Strategies to Overcome Resistance. *Semin Oncol*. 2014;41(5):667-677.
59. Taylor RP, Lindorfer MA. Fcγ-receptor–mediated trogocytosis impacts mAb-based therapies: historical precedence and recent developments. *Blood*. 2015;125(5):762-766.
60. Valgardsdottir R, Cattaneo I, Klein C, Introna M, Figliuzzi M, Golay J. Human neutrophils mediate trogocytosis rather than phagocytosis of CLL B cells opsonized with anti-CD20 antibodies. *Blood*. 2017;129(19):2636-2644.
61. Gay A, Chang S, Rutland L, Yu L, Byeseda S. Granulocyte Colony Stimulating Factor (GCSF) Alters the Phenotype of Neuroblastoma Cells: Implications for disease free survival of high-risk patients. *J Pediatr Surg*. 2008;43(5):837-842.
62. Agarwal S, Lakoma A, Chen Z, et al. G-CSF Promotes Neuroblastoma Tumorigenicity and Metastasis via STAT3-Dependent Cancer Stem Cell Activation. *Cancer Res*. 2015;75(12):2566-2579.
63. Kim ES, Agarwal S, Shohet JM. G-CSF Is a Cancer Stem Cell-Specific Growth Factor-Response. *Cancer Res*. 2015;75(18):3992.
64. Hsu DM, Agarwal S, Benham A, et al. G-CSF receptor positive neuroblastoma subpopulations are enriched in chemotherapy-resistant or relapsed tumors and are highly tumorigenic. *Cancer Res*. 2013;73(13):4134-4146.
65. Raffaghello L, Prigione I, Airoldi I, et al. Mechanisms of immune evasion of human neuroblastoma. *Cancer Lett*. 2005;228(1-2):155-161.
66. Wienke J, Dierselhuis MP, Tytgat GAM, Kunkele A, Nierkens S, Molenaar JJ. The immune landscape of neuroblastoma: Challenges and opportunities for novel therapeutic strategies in pediatric oncology. *Eur J Cancer*. 2020;144:123-150.
67. Lawrence MS, Stojanov P, Polak P, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature*. 2013;499(7457):214-218.
68. Grobner SN, Worst BC, Weischenfeldt J, et al. The landscape of genomic alterations across childhood cancers. *Nature*. 2018;555(7696):321-327.
69. Park JA, Cheung N V. Limitations and opportunities for immune checkpoint inhibitors in pediatric malignancies. *Cancer Treat Rev*. 2017;58:22-33.
70. Verhoeven BM, Mei S, Olsen TK, et al. The immune cell atlas of human neuroblastoma. *Cell Rep Med*. 2022;3(6):100657.
71. Asgharzadeh S, Salo JA, Ji L, et al. Clinical Significance of Tumor-Associated Inflammatory Cells in Metastatic Neuroblastoma. *J Clin Oncol*. 2012;30(28):3525-3532.
72. Zhong X, Zhang Y, Wang L, Zhang H, Liu H, Liu Y. Cellular components in tumor microenvironment of neuroblastoma and the prognostic value. *PeerJ*. 2019;7:e8017-e8017.
73. Zhao XW, van Beek EM, Schornagel K, et al. CD47-signal regulatory protein-α (SIRPα) interactions form a barrier for antibody-mediated tumor cell destruction. *Proc Natl Acad Sci U S A*. 2011;108(45):18342-18347.
74. Treffers LW, ten Broeke T, Rosner T, et al. IgA-Mediated Killing of Tumor Cells by Neutrophils Is Enhanced by CD47-SIRPalpha Checkpoint Inhibition. *Cancer Immunol Res*. 2020;8(1):120-130.

75. Matlung HL, Szilagyi K, Barclay NA, van den Berg TK. The CD47-SIRPalpha signaling axis as an innate immune checkpoint in cancer. *Immunol Rev*. 2017;276(1):145-164.
76. Ring NG, Herndler-Brandstetter D, Weiskopf K, et al. Anti-SIRPα antibody immunotherapy enhances neutrophil and macrophage antitumor activity. *Proc Natl Acad Sci U S A*. 2017;114(49):E10578-e10585.
77. Kim D, Wang J, Willingham SB, Martin R, Wernig G, Weissman IL. Anti-CD47 antibodies promote phagocytosis and inhibit the growth of human myeloma cells. *Leukemia*. 2012;26(12):2538-2545.
78. Chao MP, Alizadeh AA, Tang C, et al. Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell*. 2010;142(5):699-713.
79. Casey SC, Tong L, Li Y, et al. MYC regulates the antitumor immune response through CD47 and PD-L1. *Science (1979)*. 2016;352(6282):227-231.
80. Matthay KK, Maris JM, Schleiermacher G, et al. Neuroblastoma. *Nat Rev Dis Primers*. 2016;2(1):16078.
81. Gabay M, Li Y, Felsher DW. MYC activation is a hallmark of cancer initiation and maintenance. *Cold Spring Harb Perspect Med*. 2014;4(6):a014241.
82. Voets E, Paradé M, Lutje Hulsik D, et al. Functional characterization of the selective pan-allele anti-SIRPα antibody ADU-1805 that blocks the SIRPα–CD47 innate immune checkpoint. *J Immunother Cancer*. 2019;7(1):340.
83. Maute R, Xu J, Weissman IL. CD47–SIRPα-targeted therapeutics: status and prospects. *Immunooncol Technol*. 2022;13:100070.
84. Fisher GA, Lakhani NJ, Eng C, et al. A phase Ib/II study of the anti-CD47 antibody magrolimab with cetuximab in solid tumor and colorectal cancer patients. *J Clin Oncol*. 2020;38(4_suppl):114.
85. Kaur S, Cicalese K v, Bannerjee R, Roberts DD. Preclinical and Clinical Development of Therapeutic Antibodies Targeting Functions of CD47 in the Tumor Microenvironment. *Antib Ther*. 2020;3(3):179-192.
86. Delord JP, Kotecki N, Marabelle A, et al. A Phase 1 Study Evaluating BI 765063, a First in Class Selective Myeloid Sirpa Inhibitor, As Stand-Alone and in Combination with BI 754091, a Programmed Death-1 (PD-1) Inhibitor, in Patients with Advanced Solid Tumours. *Blood*. 2019;134(Supplement_1):1040.
87. Kramer K, Gerald WL, Kushner BH, Larson SM, Hameed M, Cheung NK. Disialoganglioside GD2 loss following monoclonal antibody therapy is rare in neuroblastoma. *Med Pediatr Oncol*. 2001;36(1):194-196.
88. Schumacher-Kuckelkorn R, Hero B, Ernestus K, Berthold F. Lacking immunocytological GD2 expression in neuroblastoma: report of 3 cases. *Pediatr Blood Cancer*. 2005;45(2):195-201.
89. Terzic T, Cordeau M, Herblot S, et al. Expression of Disialoganglioside (GD2) in Neuroblastic Tumors: A Prognostic Value for Patients Treated With Anti-GD2 Immunotherapy. *Pediatr Dev Pathol*. 2018;21(4):355-362.
90. Sakamoto M, Murata Y, Tanaka D, et al. Anticancer efficacy of monotherapy with antibodies to SIRPα/SIRPβ1 mediated by induction of antitumorigenic macrophages. *Proc Natl Acad Sci U S A*. 2022;119(1):e2109923118.
91. Pulido R, Nunes-Xavier CE. Hopes on immunotherapy targeting B7-H3 in neuroblastoma. *Transl Oncol*. 2023;27:101580.
92. Li N, Spetz MR, Li D, Ho M. Advances in immunotherapeutic targets for childhood cancers: A focus on glypican-2 and B7-H3. *Pharmacol Ther*. 2021;223:107892.

93. Zhou WT, Jin WL. B7-H3/CD276: An Emerging Cancer Immunotherapy. *Front Immunol*. 2021;12. https://www.frontiersin.org/articles/10.3389/fimmu.2021.701006
94. Castriconi R, Dondero A, Augugliaro R, et al. Identification of 4Ig-B7-H3 as a neuroblastoma-associated molecule that exerts a protective role from an NK cell-mediated lysis. *Proc Natl Acad Sci U S A*. 2004;101(34):12640-12645.
95. Loo D, Alderson RF, Chen FZ, et al. Development of an Fc-enhanced anti-B7-H3 monoclonal antibody with potent antitumor activity. *Clin Cancer Res*. 2012;18(14):3834-3845.
96. Theruvath J, Menard M, Smith BAH, et al. Anti-GD2 synergizes with CD47 blockade to mediate tumor eradication. *Nat Med*. 2022;28(2):333-344.
97. Jiang KY, Qi LL, Kang FB, Wang L. The intriguing roles of Siglec family members in the tumor microenvironment. *Biomark Res*. 2022;10(1):22.
98. Fraschilla I, Pillai S. Viewing Siglecs through the lens of tumor immunology. *Immunol Rev*. 2017;276(1):178-191.
99. Monteiro RC, Van De Winkel JG. IgA Fc receptors. *Annu Rev Immunol*. 2003;21:177-204.
100. Leusen JHW. IgA as therapeutic antibody. *Mol Immunol*. 2015;68(1):35-39.
101. Dechant M VT. IgA antibodies for cancer therapy. *Crit Rev Oncol Hematol*. 2001;39:69-77.
102. Lohse S, Derer S, Beyer T, et al. Recombinant dimeric IgA antibodies against the epidermal growth factor receptor mediate effective tumor cell killing. *J Immunol*. 2011;186(6):3770-3778.
103. Lohse S, Loew S, Kretschmer A, et al. Effector mechanisms of IgA antibodies against CD20 include recruitment of myeloid cells for antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. *Br J Haematol*. 2018;181(3):413-417.
104. Borrok MJ, Luheshi NM, Beyaz N, et al. Enhancement of antibody-dependent cell-mediated cytotoxicity by endowing IgG with FcalphaRI (CD89) binding. *MAbs*. 2015;7(4):743-751.
105. Dechant M, Beyer T, Schneider-Merck T, et al. Effector Mechanisms of Recombinant IgA Antibodies against Epidermal Growth Factor Receptor1. *J Immunol*. 2007;179(5):2936-2943.
106. Herr AB, White CL, Milburn C, Wu C, Bjorkman PJ. Bivalent Binding of IgA1 to FcαRI Suggests a Mechanism for Cytokine Activation of IgA Phagocytosis. *J Mol Biol*. 2003;327(3):645-657.
107. Herr AB, Ballister ER, Bjorkman PJ. Insights into IgA-mediated immune responses from the crystal structures of human FcαRI and its complex with IgA1-Fc. *Nature*. 2003;423(6940):614-620.
108. Brandsma AM, Bondza S, Evers M, et al. Potent Fc Receptor Signaling by IgA Leads to Superior Killing of Cancer Cells by Neutrophils Compared to IgG. *Front Immunol*. 2019;10:704.
109. Rifai A, Fadden K, Morrison SL, Chintalacharuvu KR. The N-glycans determine the differential blood clearance and hepatic uptake of human immunoglobulin (Ig)A1 and IgA2 isotypes. *J Exp Med*. 2000;191(12):2171-2182.
110. Kim J, Hayton WL, Robinson JM, Anderson CL. Kinetics of FcRn-mediated recycling of IgG and albumin in human: pathophysiology and therapeutic implications using a simplified mechanism-based model. *Clin Immunol*. 2007;122(2):146-155.
111. van Tetering G, Evers M, Chan C, Stip M, Leusen J. Fc Engineering Strategies to Advance IgA Antibodies as Therapeutic Agents. *Antibodies (Basel)*. 2020;9(4):70.
112. Richards RM, Sotillo E, Majzner RG. CAR T Cell Therapy for Neuroblastoma. *Front Immunol*. 2018;9:2380.
113. Nicholson RI, Gee JMW, Harper ME. EGFR and cancer prognosis. *Eur J Cancer*. 2001;37:9-15.

114. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/neu Oncogene. *Science (1979)*. 1987;235(4785):177-182.
115. Martinez M, Moon EK. CAR T Cells for Solid Tumors: New Strategies for Finding, Infiltrating, and Surviving in the Tumor Microenvironment. *Front Immunol*. 2019;10:128.
116. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther*. 2010;18(4):843-851.
117. Richman SA, Nunez-Cruz S, Moghimi B, et al. High-Affinity GD2-Specific CAR T Cells Induce Fatal Encephalitis in a Preclinical Neuroblastoma Model. *Cancer Immunol Res*. 2018;6(1):36-46.
118. Park S, Shevlin E, Vedvyas Y, et al. Micromolar affinity CAR T cells to ICAM-1 achieves rapid tumor elimination while avoiding systemic toxicity. *Sci Rep*. 2017;7(1):14366.
119. Magee MS, Abraham TS, Baybutt TR, et al. Human GUCY2C-Targeted Chimeric Antigen Receptor (CAR)-Expressing T Cells Eliminate Colorectal Cancer Metastases. *Cancer Immunol Res*. 2018;6(5):509-516.
120. Olson ML, Mause ER vander, Radhakrishnan S v, et al. Low-affinity CAR T cells exhibit reduced trogocytosis, preventing rapid antigen loss, and increasing CAR T cell expansion. *Leukemia*. 2022;36(7):1943-1946.
121. Arcangeli S, Rotiroti MC, Bardelli M, et al. Balance of Anti-CD123 Chimeric Antigen Receptor Binding Affinity and Density for the Targeting of Acute Myeloid Leukemia. *Mol Ther*. 2017;25(8):1933-1945.
122. Singh AP, Zheng X, Lin-Schmidt X, et al. Development of a quantitative relationship between CAR-affinity, antigen abundance, tumor cell depletion and CAR-T cell expansion using a multiscale systems PK-PD model. *MAbs*. 2020;12(1):1688616.
123. Seeger RC, Rosenblatt HM, Imai K, Ferrone S. Common antigenic determinants on human melanoma, glioma, neuroblastoma, and sarcoma cells defined with monoclonal antibodies. *Cancer Res*. 1981;41(7):2714-2717.
124. Flem-Karlsen K, Fodstad Ø, Tan M, Nunes-Xavier CE. B7-H3 in Cancer: Beyond Immune Regulation. *Trends Cancer*. 2018;4(6):401-404.
125. Majzner RG, Theruvath JL, Nellan A, et al. CAR T Cells Targeting B7-H3, a Pan-Cancer Antigen, Demonstrate Potent Preclinical Activity Against Pediatric Solid Tumors and Brain Tumors. *Clin Cancer Res*. 2019;25(8):2560-2574.
126. Newcomb JD, Sanberg PR, Klasko SK, Willing AE. Umbilical cord blood research: current and future perspectives. *Cell Transplant*. 2007;16(2):151-158. https://pubmed.ncbi.nlm.nih.gov/17474296
127. Pineault N, Abu-Khader A. Advances in umbilical cord blood stem cell expansion and clinical translation. *Exp Hematol*. 2015;43(7):498-513.
128. Roberts MR, Cooke KS, Tran AC, et al. Antigen-specific cytolysis by neutrophils and NK cells expressing chimeric immune receptors bearing zeta or gamma signaling domains. *J Immunol*. 1998;161(1):375-384.
129. Chang Y, Syahirah R, Wang X, et al. Engineering chimeric antigen receptor neutrophils from human pluripotent stem cells for targeted cancer immunotherapy. *Cell Rep*. 2022;40(3):111128.

130. Estcourt LJ, Stanworth SJ, Hopewell S, Doree C, Trivella M, Massey E. Granulocyte transfusions for treating infections in people with neutropenia or neutrophil dysfunction. *Cochrane Database Syst Rev.* 2016;4(4):CD005339.
131. Price TH, Boeckh M, Harrison RW, et al. Efficacy of transfusion with granulocytes from G-CSF/dexamethasone–treated donors in neutropenic patients with infection. *Blood*. 2015;126(18):2153-2161.
132. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol*. 2011;11(8):519-531.
133. Deniset JF, Kubes P. Recent advances in understanding neutrophils. *F1000Res*. 2016;5:2912.
134. Zhu EF, Gai SA, Opel CF, et al. Synergistic innate and adaptive immune response to combination immunotherapy with anti-tumor antigen antibodies and extended serum half-life IL-2. *Cancer Cell*. 2015;27(4):489-501.
135. Moynihan KD, Opel CF, Szeto GL, et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. *Nat Med*. 2016;22(12):1402-1410.
136. Doeing DC, Borowicz JL, Crockett ET. Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC Clin Pathol*. 2003;3(1):3.
137. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol*. 2004;172(5):2731-2738.
138. Mian SA, Anjos-Afonso F, Bonnet D. Advances in Human Immune System Mouse Models for Studying Human Hematopoiesis and Cancer Immunotherapy. *Front Immunol*. 2021;11. https://www.frontiersin.org/articles/10.3389/fimmu.2020.619236
139. Rongvaux A, Willinger T, Martinek J, et al. Development and function of human innate immune cells in a humanized mouse model. *Nat Biotechnol*. 2014;32(4):364-372.
140. Sippel TR, Radtke S, Olsen TM, Kiem HP, Rongvaux A. Human hematopoietic stem cell maintenance and myeloid cell development in next-generation humanized mouse models. *Blood Adv*. 2019;3(3):268-274.
141. Zheng Y, Sefik E, Astle J, et al. Human neutrophil development and functionality are enabled in a humanized mouse model. *Proc Natl Acad Sci U S A*. 2022;119(4):e2121077119.
142. Stackowicz J, Jönsson F, Reber LL. Mouse Models and Tools for the in vivo Study of Neutrophils. *Front Immunol*. 2019;10:3130.
143. Nguyen R, Patel AG, Griffiths LM, et al. Next-generation humanized patient-derived xenograft mouse model for pre-clinical antibody studies in neuroblastoma. *Cancer Immunol Immunother*. 2021;70(3):721-732.
144. Eruslanov EB, Singhal S, Albelda SM. Mouse versus Human Neutrophils in Cancer: A Major Knowledge Gap. *Trends Cancer*. 2017;3(2):149-160.
145. Sagiv JY, Michaeli J, Assi S, et al. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep*. 2015;10(4):562-573.
146. Ito R, Takahashi T, Katano I, et al. Establishment of a Human Allergy Model Using Human IL-3/GM-CSF–Transgenic NOG Mice. *J Immunol*. 2013;191(6):2890-2899.
147. WHO. Eradication of Smallpox. Accessed February 16, 2023. https://www.who.int/health-topics/smallpox#tab=tab_1

ANNEXES

ENGLISH SUMMARY

Cancer is currently considered a major public health issue globally, and it will continue to be so as long as the advances in the medical field continue to contribute to the extension of our life expectancy, and as a consequence the increased chance of developing cancer increase with age. The term "oncos", which was first used by a Greek physician to describe tumors around the years 130-200 AD, means mass or load in Greek. It is therefore not surprising that the branch of medicine that specializes in the diagnosis and treatment of cancer was given the name "oncology". Within the oncology field, it is believed that around 40% of the people worldwide will receive a cancer diagnosis during their lifetime. Although in the early years developing cancer very often meant a death sentence, this is thankfully no longer the case today. In this day of medical advances and innovations, many different ways to treat cancer have been discovered and developed along the years which are aimed at preventing this from happening. Depending on the type, location and stage of the cancer, a physician may recommend one treatment plan or another. This treatment can be focused on tackling the tumor locally with surgery or radiotherapy, or systemically with chemotherapy or immunotherapy (or more commonly with a combination of these). This thesis focuses on immunotherapy, which is a highly specific therapeutic approach that harnesses the body's immune system to control the tumor.

The study of the human immune system began with the examination of a blood sample under a simple microscope. At first sight, two types of cells could be distinguished: red blood cells and white blood cells. The latter were called "leukocytes" as derived from the Greek roots (leukos- meaning white and -cyt meaning cell), and this term refers to any cell that is part of the immune system. As medical technology and equipment developed over time, it became possible to see that our bloodstream actually consists of a whole system of specialized players who work together to form a powerful army for protection against invading pathogens or against altered self-cells, such as cancer cells. The immune system can be divided in the innate and the adaptive arm. The innate immune system constitutes the first line of defense forming a rapid but non-specific response, while the adaptive arm provides a more specific (custom-made) response, which is a bit more delayed in time but is well capable of adapting to any new challenge. Neutrophils, the main immune cell studied in this thesis, are part of the innate arm of the human immune system and they make up the largest circulating white blood cell population in our bloodstream (50-70%). Given their short half-life in circulation and the important role they play in host defense, 10^{11} neutrophils are produced in the bone marrow daily which are released in circulation in slightly smaller or slightly larger amounts depending on whether they are needed for an immune attack elsewhere in the body. The involvement of neutrophils in cancer is, however, rather controversial as they can have both beneficial as well as adverse functions: they are able to eliminate cancer cells via antibody-dependent cellular cytotoxicity (ADCC) when the

cancer cells are opsonized with a therapeutic antibody, but they can also oppose immune control by differentiating into myeloid-derived suppressor cells (MDSCs). The latter may explain why neutrophils are sometimes overlooked as cancer killing cells. In **chapter 2**, we first introduce the dual role of neutrophils in cancer and further highlight therapeutic ways in which the immune suppressor activity of neutrophils can be shifted to a tumor-eliminating one, which specifically occurs in the context of antibody therapy. Since the main topic of this thesis is focused on exploring the neutrophil's effector functions against cancer, only these were further elaborated, and not their role as immune suppressor cells.

Antibodies (or immunoglobulins) are Y-shaped molecules produced naturally in our body by a different type of immune cells (the plasma cells) that upon binding to their targets, they serve as a tag for the complement or the immune system to recognize those targets and neutralize them. Several years after the discovery of antibodies, scientists also found ways to generate such proteins in the laboratory. These man-made monoclonal antibodies, which could be specifically directed to any antigen of interest, have since become well-established forms of targeted therapy for different diseases. In cancer, antibody therapy constitutes one of the main existing immunotherapies targeting tumor antigens that are specifically (over)expressed on cancer cells. Upon binding, they may kill the cancer cells directly or they may help the immune system kill the cancer cell upon crosslinking of the Fc tail of the antibody to Fc receptors that are expressed on specific immune cells, such as neutrophils, macrophages and NK cells. While the cytotoxic mechanisms by which macrophages and NK cells – each with their own unique mechanism – kill antibody-opsonized cancer cells were already well-established years ago, that of neutrophils remained somewhat unclear until recently. We cannot disregard the fact that neutrophils are also excellent phagocytes, an ability they often use to fight off small bacteria or fungi. One may think that neutrophils could also make use such abilities to engulf tumor cells, yet, the large size of the latter seems to exceed the neutrophil's phagocytic capacities. Instead, we recently found that neutrophils kill their targets by capturing target cell membrane fragments until the cancer cell's integrity is lost, giving place to a necrotic type of cell death that received the name of "trogoptosis" (Greek: trogo – to gnaw). The process gnawing or nibbling pieces off from the targeted cell membrane by neutrophils could be considered some sort of "frustrated phagocytosis" being unable to phagocytose the whole tumor cell in once, and it has been reported to occur against cancers of different origins, from hematological cancers to epidermoid carcinomas and breast cancers. In this thesis we mainly focused on studying the ability of neutrophils to trogocytose and kill neuroblastoma cells.

Neuroblastoma is an aggressive neuroendocrine cancer originating from neuroblasts (nerve cells) that is considered the most common extracranial solid tumor affecting children. Despite intensive multimodal treatment, the prognosis of high-risk neuroblastoma

children remained low, and it was not until 2015 when the Food and Drug Administration approved the therapeutic antibody dinutuximab that the survival rate started to improve. Dinutuximab specifically targets GD2 ganglioside which is expressed on the surface membrane of neuroblastoma cells, and in fact, it was soon discovered that the main effector cell population inducing to dinutuximab-mediated killing of neuroblastoma cells *in vitro* were neutrophils, above other Fc-receptor expressing leukocytes. Moreover, their killing efficacy could be significantly improved after prior stimulation with a cytokine that drives neutrophil activation: GM-CSF (granulocyte-macrophage colony-stimulating factor). Indeed, in the absence of stimulation by growth factors the neutrophil's cytotoxic capacities is known to be limited when in the context of IgG-mediated tumor cell killing, hence, it was rapidly decided to include GM-CSF administration during the dinutuximab regimen for neuroblastoma patients. Nonetheless, access to GM-CSF for clinical use is limited (it is only approved in Northern America), which poses a clear risk of suboptimal treatment for patients elsewhere in the globe. Given the circumstance, we aimed at finding a suitable and widely available surrogate that could induce similar effects as GM-CSF. G-CSF (granulocyte colony-stimulating factor), another myeloid growth factor with almost overlapping functions with GM-CSF, is often used to treat neutropenia as it boosts neutrophil proliferation and activation, and we hypothesized this could be the substitute to GM-CSF that we aimed for. In **chapter 3** we directly compared the cytotoxic ability of G-CSF versus GM-CSF stimulated neutrophils towards dinutuximab-opsonized targets and found no differences between the two in terms of antibody-mediated killing capacity and mechanism of action (trogocytosis). This was tested both with *ex vivo* stimulated healthy donor neutrophils as well as with neutrophils from either GM-CSF-injected neuroblastoma patients or G-CSF-injected healthy volunteers. In addition, as a safety check we also monitored whether G-CSF would induce a change in the neuroblastoma cell's phenotype or proliferative capacities that could make them more immune-resistant to neutrophil-mediated killing. This was investigated in view of the recent literature raising such a concern, yet fortunately, we found no detectable effect on the neuroblastoma cell's phenotype nor on their susceptibility towards neutrophil-mediated ADCC.

Besides neutrophil stimulation with cytokines, there are other ways to potentiate their cytotoxic capacities in an antibody-related context. But before we dive into that, the concept of "immune checkpoints" needs to be briefly touched upon. In order prevent an overreaction by cells of the immune system towards for instance a misidentified "self" on cells and tissues (such as in the case of an autoimmune disease), immune cells have intrinsic regulatory mechanisms that prevent this reactivity against "self" from happening: immune cells express immune inhibitory receptors on their surface which upon binding to their ligands (expressed on other body cells) they halt or inhibit the immune cell's effector functions towards the bound cell so as to maintain self-tolerance when needed, conforming

an “immune checkpoint”. Therapeutically, the molecules involved in these checkpoints are highly interesting targets in some immune diseases, but more especially in cancer. Very often, tumors like to play “hide and seek” with our immune system: they have found ways to trick the immune system by (over)expressing the ligands of these immune checkpoints on their cell surface, which make them look as normal healthy cells. In this way they can prevent their clearance or destruction by an immune attack. The discovery of these immune evasive mechanisms harnessed by cancer cells to protect themselves led to the development of blocking antibodies directed against such inhibitory receptors. The blockade of these interactions with “checkpoint inhibitors” was the ultimate way to release the brakes on the immune cells to fully unleash their phagocytic or cytotoxic potential towards cancer cells. These immune inhibitory checkpoints are expressed in a broad spectrum of immune cells, however, in regards to neutrophils (and other myeloid cells), the most well-studied immune inhibitory receptor is SIRPα (or Signal regulatory protein alpha), which negatively regulates the neutrophil’s phagocytic or cytotoxic capacities upon binding to its ligand, CD47. Besides being expressed on other healthy body cells (*i.e.* red blood cells), CD47 is also often overexpressed in certain tumor types. Indeed, the blockade of SIRPα/CD47 axis with checkpoint inhibitors further potentiated the antibody-mediated killing of neutrophils against a number of cancer subtypes in preclinical studies, which led to the clinical trial testing of a number of drugs directed to this checkpoint. Given that neutrophils are the main effectors in neuroblastoma and that antibody therapy with dinutuximab is already well-established, we aimed at investigating if this cancer indication could also be a potential tumor subtype that could benefit from SIRPα/CD47 disruption. In **chapter 4**, we first showed evidence of CD47 overexpression in neuroblastoma tumors in particular, when compared to the levels in the corresponding healthy tissue (the adrenal gland), which remained high even after disease stage stratification. This served as the first hint to believe that neuroblastoma tumors could most likely benefit from SIRPα/CD47 axis disruption. And so we further assessed the effect of SIRPα/CD47 inhibition by either a genetic disruption or by using an antagonistic agent for SIRPα, which as expected allowed neutrophils to more efficiently kill dinutuximab-opsonized neuroblastoma cells of adrenergic phenotype *in vitro*. This was in sharp contrast when compared to neuroblastoma cell lines of mesenchymal phenotype, which could be explained by the insufficient ability to opsonize the targets with the dinutuximab due to the low GD2 expression, further limiting the cytotoxic capacity of neutrophils. Our findings aimed to provide a rational basis for targeting SIRPα/CD47 interactions to further improve dinutuximab responsiveness in neuroblastoma.

More recently, as the menu of cancer immunotherapies rapidly expands, neutrophils have started to be considered as cells of which their effector functions can be engaged in different immunotherapeutic strategies, especially in the case of solid tumors. Neutrophils represent a significant portion of the tumor-infiltrating cells, yet, as explained in **chapter 2**

of this thesis, in the absence of therapeutic antibodies they often drive tumor cell progression by suppressing anti-tumor functions of other tumor-infiltrating immune cells, among others, where they are known as granulocytic myeloid-derived-suppressor cells (MDSCs). A general depletion of neutrophils via antibodies is, however, not a feasible option clinically speaking, as this may not only eliminate the pro-tumor functions of neutrophils but also the anti-tumor ones when in the presence of antibodies. Moreover, neutropenia highly increases the risk of infections, which is certainly not beneficial for the already immune-compromised cancer patients. Consequently, new alternatives involving the targeting of neutrophils to bring in their full potential in cancer treatment are required. Chimeric Antigen Receptor (CAR) therapy consist of the engineering of human immune cells (T cells and NK cells are the more common cell-vehicles used for CAR therapy) which are specifically tweaked in the laboratory to seek and destroy the cancer cells expressing the matching antigen once they are infused back in the patient after *ex vivo* expansion. It is often called the "most complex drug ever created" as it is not just a molecule or protein that has been generated but a whole cell. CAR-T cell therapy has revolutionized the field of cancer immunotherapy, especially for the treatment of hematological cancers, by boosting the anti-tumor functions of T lymphocytes, specifically, towards the selected targets. Also, some attempts were done with NK cells, yet, the efficacy of CAR-T cell or CAR-NK cell proved to be limited when tested for the treatment of solid cancers in the clinics, and thus other CAR vehicles started to be explored. As neutrophils are largely present in solid tumors, we aimed at redirecting the neutrophil's cytotoxic capacities towards tumor cells by equipping them with a CAR directed to the tumor antigen of interest. In this way, the need for a therapeutic antibody, as well as the limitations that come with it, could be circumvented. A proof of concept for this is shown in **chapter 5**, where we specifically evaluated the killing potential of neutrophil-like NB4 cells engineered to express CARs directed towards three different solid tumor antigens: GD2 (tumors of neuroectodermal origin), EGFR (epidermoid carcinomas) and HER2/neu (breast cancers). Particularly, the single chain variable fragment (scFv) of a tumor-specific antibody was linked to the signaling moiety of the Fcα-receptor (FcαR), which was found to drive potent neutrophil-mediated cytotoxicity by the NB4 CAR neutrophils. By integrating antigen recognition specificity with the lethality of these powerful cytotoxic innate cells, our observations provide the rationale of using neutrophils (and neutrophil-related signaling domains) as an alternative vehicle for CAR therapy, opening the door to immunotherapeutic strategies targeting myeloid cells that could replace T cell-based therapies in cases where these are ineffective.

In vitro studies, where the tests and experiments occur outside of a living organism in a controlled environment, such as a test tube or a petri dish, are a great first step for the early study and development of new therapeutics, as well as for doing fundamental research. Cell lines derived from animals or humans have an infinite lifespan allowing relatively cheap

and simple manipulation, and thereby enable a more rapid development of new drugs and treatments. *In vivo* studies, instead, refer to the testing of experimental procedures performed within a whole living organism, such as a laboratory animal. Despite *in vivo* studies may take longer (up to months or years), these are most of the times a necessary last step for the translation of specific *in vitro* findings into the clinics. The most commonly used laboratory animal used in biomedical research is often the mouse, likely due to the similarities that have been conserved between species despite evolution. These similarities have allowed researchers to study the main aspects of human (patho)physiology in mice. Nowadays, scientists can choose from thousands of laboratory mouse strains that are available worldwide to further continue their preclinical investigations in an organismal setting. For neutrophil research this becomes somewhat more challenging since the neutrophil biology of mice and humans differs significantly. While neutrophils are the most abundant cell type found in circulation in humans, mice are mostly neutropenic, highlighting already that the role that neutrophils may play in mice is most likely less noteworthy than the role they play in humans. Also, many other differences in the intrinsic overall biology of neutrophils have been observed (*i.e.* structure and signaling of key molecules, expression of cytokines, activation pathways and effector functions towards invading pathogens or cancer). In order to tackle these differences, researchers have generated what are now called "humanized mouse strains". These mice are generated from the transplantation of human hematopoietic stem cells (HSCs) into highly immunodeficient mice leading to a slightly more or less complete human immune reconstitution once the mice grow old. At the end of the day this allows the possibility to specifically study the human immune compartments inside a mouse organism. The humanized MISTRG mouse strain is known as the strain with the most complete human immune system, also allowing neutrophil development (other more traditional models of humanized mice were not able to establish a reliable myeloid compartment). Yet, owing to the inability of the human neutrophils to leave the bone marrow in these mouse environment, little efforts were done to investigate this population in further detail within the research community. For this reason, in **chapter 6** we aimed to fully characterize the neutrophil compartment in humanized MISTRG mice, both phenotypically and functionally, for the first time. We demonstrated that the human neutrophils in these mice are well-able to perform most (if not all) of the neutrophil's anti-microbial and anti-tumor functions. In addition, we successfully managed to mobilize them to the periphery and even found extravascular infiltration of human neutrophils in tissues in response to inflammation or tumors. Overall, we demonstrated that the humanized MISTRG mouse strain could be exploited for the study of neutrophil-related immune processes in both inflammatory diseases as well as in cancer.

In conclusion, this thesis was aimed at expanding the knowledge on the different ways of how the neutrophil's effector functions can be exploited in immunotherapeutic strategies

to improve cancer treatment. Not only we have showed that neutrophil stimulation with cytokines such as G-CSF and checkpoint inhibition of the SIRPα/CD47 axis can enhance the neutrophil's ability to recognize and kill (neuroblastoma) cancer cells, always in the context of antibody therapy, but also in more intricate and innovative approaches by equipping them with a tumor-specific CAR that redirected the anti-tumor functions of neutrophils, circumventing the need for a therapeutic antibody. Further efforts should however be made for the translation of the *in vitro* findings presented in this thesis. Here, we showed how the humanized MISTRG mouse model may hold great potential for this, as an organismal model that satisfactorily allows the development of functional human neutrophils. The functional plasticity of neutrophils within the tumor microenvironment can be strategically used to our advantage to combat cancer, highlighting once more that neutrophils cannot be overlooked as potent cancer killing cells.

NEDERLANDSE SAMMENVATTING

Kanker wordt momenteel wereldwijd beschouwd als een groot probleem voor de volksgezondheid, en dat zal zo blijven zolang de vooruitgang op medisch gebied blijft bijdragen aan de verlenging van onze levensverwachting, en daarmee ook de kans op het ontwikkelen van kanker toeneemt met leeftijd. De term “oncos”, die voor het eerst werd gebruikt door een Griekse arts om tumoren te beschrijven rond de jaren 130-200 na Christus, betekent massa of lading in het Grieks. Het is dan ook niet verwonderlijk dat de tak van de geneeskunde die gespecialiseerd is in de diagnose en behandeling van kanker de naam “oncologie” kreeg. Binnen de oncologie wordt aangenomen dat ongeveer 40% van de mensen wereldwijd tijdens hun leven de diagnose kanker zal krijgen. Hoewel het ontwikkelen van kanker in de beginjaren vaak een doodvonnis betekende, is dat vandaag gelukkig niet meer het geval. In deze tijd van medische vooruitgang en innovaties zijn er in de loop der jaren veel verschillende manieren ontdekt en ontwikkeld om kanker te behandelen en die erop gericht zijn dit te voorkomen. Afhankelijk van het type, de locatie en het stadium van de kanker, kan een arts een bepaald behandelplan aanbevelen. Deze behandeling kan gericht zijn op het lokaal aanpakken van de tumor met chirurgie of radiotherapie, of systemisch met chemotherapie of immunotherapie (of vaker een combinatie hiervan). Dit proefschrift richt zich op immunotherapie, een zeer specifieke therapeutische benadering die het immuunsysteem gebruikt om de tumor onder controle te krijgen.

De studie van het menselijke immuunsysteem begon met het onderzoeken van een bloedmonster onder een eenvoudige microscoop. Op het eerste gezicht waren er twee soorten cellen te onderscheiden: rode bloedcellen en witte bloedcellen. Deze laatste werden “leukocyten” genoemd, afgeleid van het Grieks (leukos betekent wit en -cyt betekent cel), en deze term verwijst naar elke cel die deel uitmaakt van het immuunsysteem. Naarmate medische technologie en apparatuur zich in de loop van de tijd ontwikkelden, werd het mogelijk om te zien dat onze bloedbaan eigenlijk bestaat uit een heel systeem van gespecialiseerde spelers die samenwerken om een krachtig leger te vormen ter bescherming tegen binnendringende ziekteverwekkers of tegen veranderde lichaamseigen cellen, zoals kankercellen. Het immuunsysteem kan worden onderverdeeld in de aangeboren en de adaptieve arm. Het aangeboren immuunsysteem vormt de eerste verdedigingslinie en vormt een snelle maar niet-specifieke reactie, terwijl de adaptieve arm een meer specifieke (op maat gemaakte) reactie geeft, die wat meer vertraagd is in de tijd, maar goed in staat is zich aan te passen aan elke situatie. Neutrofielen, de belangrijkste immuuncel die in dit proefschrift worden bestudeerd, maken deel uit van de aangeboren arm van het menselijke immuunsysteem en vormen de grootste populatie circulerende witte bloedcellen in onze bloedbaan (50-70%). Gezien hun korte halfwaardetijd in de bloedsomloop en de belangrijke rol die ze spelen in de afweer van de gastheer, worden er dagelijks 10^{11} neutrofielen

geproduceerd in het beenmerg, die in iets kleinere of iets grotere hoeveelheden in de bloedsomloop worden vrijgegeven, afhankelijk van of ze nodig zijn voor een immuunreactie elders in het lichaam. De betrokkenheid van neutrofielen bij kanker is echter nogal controversieel, aangezien ze zowel gunstige als nadelige functies kunnen hebben: ze zijn in staat om kankercellen te elimineren via antilichaamafhankelijke cellulaire cytotoxiciteit (ADCC) wanneer de kankercellen worden geopsoniseerd met een therapeutisch antilichaam, maar ze kunnen zich ook verzetten tegen immuuncontrole door te differentiëren in zogenaamde myeloïde suppressorcellen (MDSC's). Dit laatste zou kunnen verklaren waarom neutrofielen soms over het hoofd worden gezien als kankerdodende cellen. In **hoofdstuk 2** introduceren we eerst de dubbele rol van neutrofielen in kanker en belichten we verder de therapeutische manieren waarop de immuunonderdrukkende activiteit van neutrofielen kan worden omgebogen naar een tumor-eliminerende activiteit, wat specifiek gebeurt in de context van antilichaamtherapie. Aangezien het hoofdonderwerp van dit proefschrift gericht is op het onderzoeken van de effectorfuncties van neutrofielen tegen kanker, werden alleen deze verder uitgewerkt, en niet hun rol als immuunonderdrukkende cellen.

Antilichamen (of immunoglobulinen) zijn Y-vormige moleculen die van nature in ons lichaam worden geproduceerd door een ander type immuuncellen (de plasmacellen). Antilichamen dienen na binding aan hun antigenen als een tag voor het complement- of het immuunsysteem om die antigenen te herkennen en te neutraliseren. Enkele jaren na de ontdekking van antilichamen vonden wetenschappers ook manieren om dergelijke eiwitten in het laboratorium te genereren. Deze door de mens gemaakte monoklonale antilichamen, die specifiek gericht kunnen worden op elk antigeen van interesse, zijn sindsdien gevestigde vormen van gerichte therapie voor verschillende ziekten geworden. Bij kanker vormt antilichaamtherapie een van de belangrijkste bestaande immunotherapieën gericht op tumorantigenen die specifiek tot (over)expressie komen op kankercellen. Na binding kunnen ze de kankercellen direct doden of ze kunnen het immuunsysteem helpen de kankercel te doden door binding van de Fc-staart van het antilichaam aan Fc-receptoren die tot expressie worden gebracht op specifieke immuuncellen, zoals neutrofielen, macrofagen en NK-cellen. Terwijl de cytotoxische mechanismen waarmee macrofagen en NK-cellen - elk met hun eigen unieke mechanisme - door antilichamen geopsoniseerde kankercellen doden al jaren geleden goed ingeburgerd waren, bleef die van neutrofielen tot voor kort enigszins onduidelijk. We kunnen niet voorbijgaan aan het feit dat neutrofielen ook uitstekende fagocyten zijn, een vermogen dat ze vaak gebruiken om kleine bacteriën of schimmels te bestrijden. Men zou kunnen denken dat neutrofielen dergelijke vermogens ook zouden kunnen gebruiken om tumorcellen te verzwelgen, maar de grote omvang van deze cellen lijkt de fagocytische capaciteiten van de neutrofielen te overtreffen. In plaats daarvan hebben we onlangs ontdekt dat neutrofielen hun doelwitten doden door fragmenten van het tumorcelmembraan af te scheuren totdat de integriteit van de kankercel

verloren gaat. Dit proces leidt uiteindelijk tot een necrotische soort van celdood dat de naam “trogoptosis” (Grieks: trogo - knagen) kreeg. Het proces waarbij neutrofielen stukjes van het beoogde celmembraan knagen of knabbelen, zou kunnen worden beschouwd als een soort “gefrustreerde fagocytose”, waarbij de neutrofiel niet in staat is om de hele tumorcel in één keer te fagocyteren. Tot nu toe is gebleken dat dit neutrofiel gemedieerde trogocytose proces bijdraagt aan het doden van kankers van verschillende oorsprong, van hematologische kankers tot epidermoïde carcinomen en borstkankers. In dit proefschrift hebben we ons voornamelijk gericht op het bestuderen van het vermogen van neutrofielen om neuroblastoomcellen te trogocyteren en te doden.

Neuroblastoom is een agressieve neuro-endocriene kanker die ontstaat uit neuroblasten (zenuwcellen) en wordt beschouwd als de meest voorkomende extracraniële solide tumor die kinderen treft. Ondanks intensieve multimodale behandeling bleef de prognose van kinderen met een hoog risico neuroblastoom laag, en pas in 2015, toen de Food and Drug Administration het therapeutische antilichaam dinutuximab goedkeurde, begon het overlevingspercentage te verbeteren. Dinutuximab richt zich specifiek op GD2-ganglioside, dat tot expressie komt op het oppervlaktemembraan van neuroblastoomcellen. Al snel werd ontdekt dat de belangrijkste effectorcelpopulatie die leidde tot dinutuximab-gemedieerde doding van neuroblastoomcellen *in vitro* neutrofielen waren, boven andere Fc-receptor tot expressie brengende leukocyten. Bovendien zou hun dodende werkzaamheid aanzienlijk kunnen worden verbeterd na voorafgaande stimulatie met een cytokine dat neutrofielen activeert: GM-CSF (granulocyt-macrofaag-koloniestimulerende factor). Het is inderdaad bekend dat bij afwezigheid van stimulatie door groeifactoren de cytotoxische capaciteiten van neutrofielen beperkt zijn in de context van IgG-gemedieerde tumorceldoding, daarom werd snel besloten om GM-CSF-toediening op te nemen in het dinutuximab-regime voor neuroblastoompatiënten. Desalniettemin is de toegang tot GM-CSF voor klinisch gebruik beperkt (het is alleen goedgekeurd in Noord-Amerika), wat een risico tot suboptimale behandeling voor patiënten elders in de wereld oplevert. Gezien de omstandigheden wilden we een geschikt en algemeen verkrijgbaar surrogaat vinden dat vergelijkbare effecten zou kunnen veroorzaken als GM-CSF. G-CSF (granulocyt-kolonie-stimulerende factor), een andere myeloïde groeifactor met bijna overlappende functies met GM-CSF, wordt vaak gebruikt om neutropenie te behandelen omdat het de proliferatie en activering van neutrofielen stimuleert, en we veronderstelden dat dit de vervanging zou kunnen zijn voor GM-CSF waar we naar op zoek waren. In **hoofdstuk 3** hebben we het cytotoxische vermogen van G-CSF versus GM-CSF gestimuleerde neutrofielen tegen dinutuximab-geopsonizeerde tumor cellen vergeleken en vonden we geen verschillen tussen de twee in termen van antilichaam-gemedieerde dodingscapaciteit en werkingsmechanisme (trogocytose). Dit werd zowel getest met *ex vivo* gestimuleerde gezonde donor-neutrofielen als met neutrofielen van hetzij GM-CSF-geïnjecteerde neuroblastoompatiënten of G-CSF-

geïnjecteerde gezonde vrijwilligers. Bovendien hebben we als veiligheidscontrole ook gecontroleerd of G-CSF een verandering in het fenotype of de proliferatieve capaciteiten van de neuroblastoomcel zou veroorzaken, waardoor ze immuunresistenter zouden kunnen worden tegen neutrofiel- gemedieerde doding. Dit werd onderzocht met het oog op recente literatuur, maar gelukkig vonden we geen detecteerbaar effect van G-CSF op het fenotype van de neuroblastoomcel, noch op hun gevoeligheid voor door neutrofielen gemedieerde ADCC.

Naast neutrofielenstimulatie met cytokines, zijn er andere manieren om hun cytotoxische capaciteiten te versterken in een antilichaamgerelateerde context. Maar voordat we daarop ingaan, moet het concept van “immune checkpoints” kort worden besproken. Om een overdreven reactie van cellen van het immuunsysteem op bijvoorbeeld een verkeerd geïdentificeerd ‘zelf’ op cellen en weefsels te voorkomen (zoals in het geval van een auto-immuunziekte), hebben immuuncellen intrinsieke regulerende mechanismen die voorkomen dat deze reactiviteit tegen het ‘zelf’ gebeuren: immuuncellen brengen immuunremmende receptoren op hun oppervlak tot expressie die, na binding aan hun liganden (tot expressie gebracht op andere lichaamscellen), de effectorfuncties van de immuuncel tegen de gebonden cel stoppen of remmen om zo nodig zelftolerantie te behouden, conform een “immune checkpoint”. Therapeutisch zijn de moleculen die betrokken zijn bij deze checkpoints zeer interessante doelwitten bij sommige immuunziekten, maar meer in het bijzonder bij kanker. Heel vaak spelen tumoren graag “verstoppertje” met ons immuunsysteem: ze hebben manieren gevonden om het immuunsysteem voor de gek te houden door de liganden van deze immune checkpoints tot (over)expressie te brengen op hun celoppervlak, waardoor ze eruitzien als normale, gezonde cellen. Op deze manier kunnen ze hun opruiming of vernietiging door immuuncellen voorkomen. De ontdekking van deze immuunontwijkende mechanismen die door kankercellen worden gebruikt om zichzelf te beschermen, leidde tot de ontwikkeling van blokkerende antilichamen die gericht zijn tegen dergelijke remmende receptoren. De blokkade van deze interacties met “checkpoint-remmers” was de ultieme manier om de remmen op de immuuncellen los te laten om hun fagocytische of cytotoxische potentieel tegen kankercellen volledig te ontketenen. Deze immune checkpoints komen tot uiting in een breed spectrum van immuuncellen, maar met betrekking tot neutrofielen (en andere myeloïde cellen) is de best bestudeerde immuunremmende receptor SIRPα (of signaalregulerend eiwit alfa), die de fagocytische of cytotoxische capaciteiten van neutrofielen remt na binding aan zijn ligand, CD47. Behalve dat het tot expressie wordt gebracht op andere gezonde lichaamscellen (d.w.z. rode bloedcellen), wordt CD47 ook vaak tot overexpressie gebracht in bepaalde tumortypes. De blokkade van de SIRPα/CD47-as met checkpoint-remmers versterkte inderdaad de door antilichamen gemedieerde doding van neutrofielen tegen een aantal subtypes van kanker in preklinische studies, wat leidde tot de klinische studies van een aantal geneesmiddelen

gericht op dit checkpoint. Gezien het feit dat neutrofielen de belangrijkste effector cellen zijn bij antilichaamtherapie met dinutuximab in neuroblastoom, wilden we onderzoeken of deze behandeling van neuroblastoom baat zou kunnen hebben bij remming van SIRPα/CD47 interacties. In **hoofdstuk 4** toonden we voor het eerst bewijs van CD47-overexpressie in met name neuroblastoomtumoren, in vergelijking met de niveaus in het corresponderende gezonde weefsel (de bijnier), ook na stratificatie van het ziektestadium. Dit diende als de eerste aanwijzing om aan te nemen dat neuroblastoomtumoren hoogstwaarschijnlijk baat zouden kunnen hebben bij verstoring van de SIRPα/CD47-as. En dus hebben we het effect van SIRPα/CD47-remming verder onderzocht door gebruik te maken van ofwel een genetische manipulatie ofwel door het gebruik van een antagonistische antistof tegen SIRPα. Onder die omstandigheden waren neutrofielen, zoals verwacht, *in vitro* efficiënter in het doden van dinutuximab geopsoniseerde neuroblastoomcellen van het adrenerge fenotype. Dit in tegenstelling tot neuroblastoomcellijnen van het mesenchymale fenotype, wat verklaard zou kunnen worden door de onvoldoende mate van dinutuximab opsonisatie vanwege de lage GD2-expressie, waardoor de cytotoxische capaciteit van neutrofielen verder werd beperkt. Onze bevindingen hebben hiermee bijgedragen aan een rationele basis voor het remmen van SIRPα/CD47-interacties om de respons op dinutuximab bij neuroblastoom verder te verbeteren.

Meer recentelijk, naarmate het menu van kankerimmunotherapieën zich snel uitbreidt, worden neutrofielen langzaamaan beschouwd als cellen waarvan hun effectorfuncties kunnen worden gebruikt in verschillende immunotherapeutische strategieën, vooral in het geval van solide tumoren. Neutrofielen vertegenwoordigen een aanzienlijk deel van de tumor-infiltrerende cellen, maar, zoals uitgelegd in **hoofdstuk 2** van dit proefschrift, stimuleren ze bij afwezigheid van therapeutische antilichamen vaak de tumorcelprogressie door de antitumorfuncties van andere tumor-infiltrerende immuuncellen te onderdrukken, waarmee ze bekend staan als granulocytische myeloïde suppressorcellen (MDSC's). Een algemene depletie van neutrofielen via antilichamen is echter klinisch gezien geen haalbare optie, aangezien dit niet alleen de pro-tumorfuncties van neutrofielen kan uitschakelen, maar ook de antitumorfuncties in aanwezigheid van antilichamen. Bovendien verhoogt neutropenie het risico op infecties sterk, wat zeker niet gunstig is voor de toch al immuungecompromitteerde kankerpatiënten. Daarom zijn er nieuwe alternatieven nodig waarbij neutrofielen hun volledige potentieel in de behandeling van kanker kunnen benutten. Chimere antigeenreceptor therapie (CAR) bestaat uit het manipuleren van menselijke immuuncellen (T-cellen en NK-cellen zijn de meest voorkomende cel-vehikels die momenteel worden gebruikt voor CAR-therapie) die specifiek in het laboratorium worden aangepast om de kankercellen die het overeenkomende antigeen tot expressie brengen, op te sporen en te vernietigen. Deze vorm van therapie wordt vaak het "meest complexe medicijn ooit gemaakt" genoemd, omdat het niet alleen een molecuul of eiwit is dat is

gegenereerd, maar een gehele cel. CAR-T-celtherapie heeft een revolutie teweeggebracht op het gebied van kankerimmunotherapie, met name voor de behandeling van hematologische kankers, door de antitumorfuncties van T-lymfocyten te stimuleren. Daarnaast worden ook pogingen ondernomen met CAR-NK cellen als effector cellen, maar de werkzaamheid van CAR-T-cellen of CAR-NK-cellen bleek beperkt te zijn bij testen voor de behandeling van solide kankers in de kliniek, en dus worden momenteel ook andere CAR-effector cellen onderzocht. Aangezien neutrofielen grotendeels aanwezig zijn in solide tumoren, probeerden we de cytotoxische capaciteiten van de neutrofielen op tumorcellen te richten door ze uit te rusten met een CAR tegen het betreffende tumorantigeen. Op deze manier zou de behoefte aan een therapeutisch antilichaam, evenals de beperkingen die daarmee gepaard gaan, kunnen worden omzeild. Een proof of concept hiervoor wordt getoond in **hoofdstuk 5**, waar we specifiek het dodingspotentieel onderzochten van neutrofielachtige NB4-cellen die zijn ontworpen om CAR's tot expressie te brengen die zijn gericht tegen drie verschillende solide tumorantigenen: GD2 (tumoren van neuro-ectodermale oorsprong), EGFR (epidermoïde carcinomen) en HER2/neu (borstkanker). Hiervoor werd het 'single chain' variabele fragment (scFv) van een tumorspecifiek antilichaam gekoppeld aan het signaalgedeelte van de Fcα-receptor (FcαR). Door antigeenherkenningsspecificiteit te integreren met de cytotoxische capaciteit van neutrofielen, bieden onze waarnemingen de grondgedachte voor het gebruik van neutrofielen (en neutrofiel-gerelateerde signaaldomeinen) als een alternatief celtype voor CAR-therapie. Hiermee wordt de deur geopend voor immunotherapeutische strategieën gericht op myeloïde cellen die de op T-cellen gebaseerde therapieën zouden kunnen vervangen in gevallen waarin ze niet effectief zijn.

In vitro-onderzoeken, waarbij de experimenten plaatsvinden buiten een levend organisme in een gecontroleerde omgeving, zoals een reageerbuis of een petrischaal, zijn een geweldige eerste stap voor de vroege studie en ontwikkeling van nieuwe therapieën, evenals voor het doen van fundamenteel onderzoek. Cellijnen die zijn afgeleid van dieren of mensen hebben een oneindige levensduur waardoor relatief goedkope en eenvoudige manipulatie mogelijk is, en maken daardoor een snellere ontwikkeling van nieuwe medicijnen en behandelingen mogelijk. *In vivo*-onderzoeken verwijzen in plaats daarvan naar het testen van experimentele procedures die worden uitgevoerd binnen een heel levend organisme, zoals een proefdier. Ondanks dat *in vivo* studies langer kunnen duren (maanden of jaren), is dit meestal een noodzakelijke laatste stap voor de vertaling van specifieke *in vitro* bevindingen naar de kliniek. Het meest gebruikte laboratoriumdier dat in biomedisch onderzoek wordt gebruikt is de muis, waarschijnlijk vanwege de overeenkomsten die ondanks de evolutie tussen soorten zijn behouden. Door deze overeenkomsten konden onderzoekers de belangrijkste aspecten van de menselijke (patho)fysiologie bij muizen bestuderen. Tegenwoordig kunnen wetenschappers kiezen uit duizenden muizenstammen in het laboratorium die wereldwijd

beschikbaar zijn om hun preklinische onderzoeken voort te zetten in de context van een organisme. Voor neutrofielenonderzoek wordt dit iets uitdagender aangezien de neutrofielbiologie van muizen en mensen aanzienlijk verschilt. Hoewel neutrofielen het meest voorkomende celtype zijn dat bij mensen in de circulatie voorkomt, zijn muizen meestal neutropenisch, wat al benadrukt dat de rol die neutrofielen bij muizen kunnen spelen hoogstwaarschijnlijk minder opmerkelijk is dan de rol die ze spelen bij mensen. Er zijn ook veel andere verschillen in de intrinsieke algehele biologie van neutrofielen waargenomen (d.w.z. structuur en signalering van sleutelmoleculen, expressie van cytokines, activeringsroutes en effectorfuncties naar binnendringende pathogenen of kanker). Om deze verschillen aan te pakken, hebben onderzoekers zogenaamde "gehumaniseerde muizenstammen" gegenereerd. Deze muizen worden gegenereerd door de transplantatie van menselijke hematopoëtische stamcellen (HSC's) in zeer immunodeficiënte muizen, wat leidt tot een min of meer volledige reconstitutie van het menselijk immuunsysteem zodra de muizen ouder worden. Uiteindelijk biedt dit de mogelijkheid om specifiek de menselijke immuuncompartimenten in een muizenorganisme te bestuderen. De gehumaniseerde MISTRG-muizenstam staat bekend als de stam met het meest complete menselijke immuunsysteem, waardoor ook de ontwikkeling van neutrofielen mogelijk is (andere, meer traditionele modellen van gehumaniseerde muizen waren niet in staat om een betrouwbaar myeloïde compartiment tot stand te brengen). Maar vanwege het onvermogen van de menselijke neutrofielen om het beenmerg in deze muizenomgeving te verlaten, werden er binnen de onderzoeksgemeenschap weinig inspanningen gedaan om deze populatie nader te onderzoeken. Om deze reden hebben we in **hoofdstuk 6** voor het eerst getracht het neutrofielencompartiment in gehumaniseerde MISTRG-muizen volledig te karakteriseren, zowel fenotypisch als functioneel. We hebben aangetoond dat de menselijke neutrofielen in deze muizen goed in staat zijn om de meeste (zo niet alle) antimicrobiële en antitumorfuncties van de neutrofielen uit te voeren. Bovendien slaagden we erin om ze naar de periferie te mobiliseren en vonden we zelfs extravasculaire infiltratie van menselijke neutrofielen in weefsels als reactie op ontsteking of tumoren. Over het algemeen hebben we aangetoond dat de gehumaniseerde MISTRG-muisstam kan worden gebruikt voor het bestuderen van neutrofiel-gerelateerde immuunprocessen bij zowel ontstekingsziekten als bij kanker.

Concluderend, dit proefschrift was gericht op het uitbreiden van de kennis over de verschillende manieren waarop de effectorfuncties van de neutrofielen kunnen worden benut in immunotherapeutische strategieën om de behandeling van kanker te verbeteren. We hebben niet alleen aangetoond dat stimulatie van neutrofielen met cytokines zoals G-CSF en checkpointremming van de SIRPα/CD47-as het vermogen van de neutrofielen om (neuroblastoom) kankercellen te herkennen en te doden kan verbeteren, altijd in de context van antilichaamtherapie, maar ook in meer ingewikkelde en innovatieve

benaderingen door ze uit te rusten met een tumorspecifieke CAR die de antitumorfuncties van neutrofielen omleidt, waardoor de behoefte aan een therapeutisch antilichaam wordt omzeild. Er zullen echter verdere inspanningen nodig zijn voor de vertaling van de *in vitro* bevindingen die in dit proefschrift worden gepresenteerd. We hebben laten zien hoe het gehumaniseerde MISTRG-muismodel hiervoor van groot nut kan zijn, als een *in vivo* model dat op bevredigende wijze de ontwikkeling van functionele menselijke neutrofielen mogelijk maakt. De functionele plasticiteit van neutrofielen in het micro-milieu van de tumor kan strategisch in ons voordeel worden gebruikt om kanker te bestrijden, wat eens te meer benadrukt dat neutrofielen niet over het hoofd mogen worden gezien als krachtige kankerdodende cellen.

CONTRIBUTING AUTHORS

Amsen, D. (Derk)
Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Bąbała, N. (Nikolina)
Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Bouti, P. (Giota)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Dierselhuis, M.P. (Miranda)
Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

Flavell, R.A. (Richard)
Dept. of Immunobiology, Yale University School of Medicine, New Haven, Connecticut, United States of America
Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut, United States of America

Franke, K. (Katka)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Furumaya, C. (Charita)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Hoogenboezem, M. (Mark)
Department of Research Facilities, Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Hoogendijk, A.J. (Arjan)
Department of Molecular Hematology, Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Karrich, J.J. (Julien)
Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Kholosy, W.M. (Waleed)
Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

Klein, B. (Bart)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Kok, I. (Ivana)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Kuijpers, T.W. (Taco)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands
Department of Pediatric Immunology, Reumatology and Infectious Diseases, Emma Children's Hospital, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands

Langerak, I. (Iris)
Department of Blood Cell Research (Molecular Hematology,) Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Laurent, A.R.G. (Adrien)
Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Leeuwenburg, K. (Kira)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Matlung, H.L. (Hanke)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Molenaar, J.J. (Jan)
Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

Olsman, H. (Hugo)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Rongvaux, A. (Anthony)
Department of Immunology, University of Washington, Seattle, Washington, United States of America
Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, United States of America

Schornagel, K. (Karin)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Slot, E. (Edith)
Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Sunak, A. (Ali)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Timmerman, I. (Ilse)
Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands
Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

Tytgat, G.A.M. (Lieve)
Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

Van Bruggen, R. (Robin)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Van den Berg, T.K. (Timo)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands
Department of Molecular Cell Biology and Immunology, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, the Netherlands

Van Rees, D. (Dieke)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

Van Zogchel, L.M.J. (Lieke)
Department of Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands
Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

Verkuijlen, P.J.J.H. (Paul)
Department of Blood Cell Research (Molecular Hematology), Sanquin Research and Landsteiner Laboratory, University of Amsterdam Amsterdam, the Netherlands

LIST OF PUBLICATIONS

Plasticity in pro- and anti-tumor activity of neutrophils: Shifting the balance
Furumaya C, **Martinez-Sanz P**, Bouti P, Kuijpers TW, Matlung HL.
Frontiers in Immunology 2020 Sep 2; 11:2100
*shared authorship

G-CSF as a suitable alternative to GM-CSF to boost dinutuximab-mediated neutrophil cytotoxicity in neuroblastoma treatment
Martinez-Sanz P, van Rees DJ, van Zogchel LMJ, Klein B, Bouti P, Olsman H, Schornagel K, Kok I, Sunak A, Leeuwenburg K, Timmerman I, Dierselhuis MP, Kholosy WM, Molenaar JJ, van Bruggen R, van den Berg TK, Kuijpers TW, Matlung HL, Tytgat GAM, Franke K.
Journal for ImmunoTherapy of Cancer 2021 May 28; 9(5):e002259
*shared authorship

Response to: Correspondence on "G-CSF as a suitable alternative to GM-CSF to boost dinutuximab-mediated neutrophil cytotoxicity in neuroblastoma treatment" by Mora *et al*
Martinez-Sanz P, van Rees DJ, Matlung HL, Tytgat GAM, Franke K.
Journal for ImmunoTherapy of Cancer 2021 Dec 10; 9(12):e003983

CD47-SIRPα checkpoint inhibition enhances neutrophil-mediated killing of dinutuximab-opsonized neuroblastoma cells
Martínez-Sanz P, Hoogendijk AJ, Verkuijlen PJJH, Schornagel K, van Bruggen R, van den Berg TK, Tytgat GAM, Franke K, Kuijpers TW, Matlung HL.
Cancers 2021 Aug 24; 13(17):4261

Humanized MISTRG as a preclinical *in vivo* model to study human neutrophil-mediated immune processes
Martinez-Sanz P, Laurent ARG, Slot E, Hoogenboezem M, Bąbała N, van Bruggen R, Rongvaux A, Flavell RA, Tytgat GAM, Franke K, Matlung HL, Kuijpers TW, Amsen D*, Karrich JJ*.
Frontiers in Immunology 2023 March 8; 14:1105103
*shared authorship

PhD PORTFOLIO

Courses and workshops	**Year**	**ECTS**
o Bridging Basic & Clinical Immunology (SIICA-FOCIS, Florence, Italy)	2019	1.0
o Laboratory Animal Science (article 9, AMC Graduate School)	2019	3.9
o Scientific Ethics and Integrity workshop (Sanquin Research)	2019	0.6
o Advanced Immunology (VUmc and Sanquin Research)	2020	2.9
o How to write research papers (Oncology Graduate School)	2020	1.0
o Practical Biostatistics (AMC Graduate School)	2021	1.4
o R2 introduction workshop	2021	0.5
o Biobusiness Summer School (Hyphen Projects)	2022	1.5
o Personal Development (Sanquin Research)	2019-2022	1.2
Seminars, meetings and presentations		
o Sanquin Research Meetings	2019-2023	4.0
o Sanquin Landsteiner Lectures	2019-2023	1.0
o Sanquin Science Course	2021	0.5
o Department research meetings	2019-2023	4.0
o Department Journal Club	2019-2023	2.0
o Sanquin Science Day	2019-2023	1.0
(Inter)national conferences		
o AII Annual Meeting (Amsterdam, online)	2019-2022	1.5
o OAA PhD Retreat (Renesse) *1x poster presentation*	2019	0.5
o NVVI (Noordwijkerhout, online) *2x poster presentation, 1x oral presentation*	2019-2022	1.5
o Advances in Neuroblastoma Research (online) *1x poster presentation*	2020→2021	0.5
o International Neutrophil Symposium (online, Mexico City, Mexico) *1x poster presentation, 1x oral presentation*	2021, 2022	1.0
o Dutch Tumor Immunology Meeting KWF (online)	2021	0.5

o	Sanquin Science Day (Amsterdam, online) *3x poster presentation*	2019-2022	1.5

Teaching

o	HLO student Ivana Kok *internship, 6 months*	2019	2.0
o	Master student Ali Sunak *internship, 6 months*	2019	2.0
o	HLO student Kira Leeuwenburg *internship, 8 months*	2019-2020	2.5
		Total:	**40**

Parameters of Esteem

o	Poster awards: 2x International Neutrophil Symposium (poster, oral)	2021
o	Travel grant: AII travel grant	2022

ABOUT THE AUTHOR

Paula Martínez Sanz was born on February 15th, 1993 in Barcelona, Spain. She grew up with her parents and two brothers in Sant Cugat del Vallés (Barcelona, Spain). During her secondary school studies she developed an interest in biology of the human body and in 2011 she started her Bachelor degree in Biomedical Science at Universitat de Barcelona (Facultat de Biologia). In her last Bachelor year, she moved to Gent (Belgium) with an Erasmus grant where she completed her final Bachelor internship at the Inflammation Research Center within the Vlaams Instituut voor Biotechnologie (Universiteit Gent). While working on a project focused on the understanding of lung cancer progression, she discovered her passion for oncological research. This first experience abroad led her to continue her studies in Amsterdam where she followed a Master of Science in Oncology from 2016 to 2018 at the Vrije Universiteit. In the first year of her master's degree, she did a research internship in the department of Molecular Oncology and Immunology at the Netherlands Cancer Institute (NKI-AvL). Here she aimed at identifying drivers in a specific type of gastric cancer. In the second year, she performed a research internship at the department of Blood Cell Research (now called Molecular Hematology) at Sanquin Research and Lansteiner Laboratory, during which she focused on how to enhance the neutrophil's ability to destroy antibody-opsonized tumor cells.

After completing her master's degree, in 2019 Paula was offered a PhD position at the same department under the mentorship of Prof. dr. Taco W. Kuijpers with co-supervision by Katka Franke at first, and then by Hanke L. Matlung and Godelieve A. M. Tytgat. Here, she further investigated immunotherapeutic strategies aimed at exploiting the neutrophil's potential as cancer killing cells for cancer treatment. During her PhD period, she got the opportunity to present her work at several (inter)national scientific conferences and the findings of her research have led to this thesis.

Currently, Paula is looking for new opportunities within the pharmaceutical industry, where she would like to apply the knowledge she gained during her PhD. Whether she will stay in the Netherlands or will go back to Spain (or any other country), future will tell!

ACKNOWLEDGEMENTS

The time has come for me to express my gratitude and appreciation to everybody that has directly or indirectly supported me throughout this research journey, what I have often called "my PhD adventure", without whom this doctoral thesis would not have been possible.

Sanquin:

Hanke, my co-promotor and daily supervisor. I fell under your supervision after a year almost by surprise but what a great team we created! We have laughed, we have cried (or at least I did…), but you have been there for me week after week, giving me the room to grow as a researcher and helping me brainstorm with ideas that eventually led to this thesis. More importantly, I want to thank you for making everything look so easy when I felt overwhelmed (which was almost the whole time haha). Your calmed nature and your belief in my potential have helped me enormously through this academic journey and I feel incredibly lucky to have had you as my daily mentor. But it has not all been about scientific research. You have also been there to listen to my crazy stories and adventures and have supported me in a more personal level too. For this and all of the above, I am truly thankful.

Taco, my promotor. Through your excellent guidance and exceptionally quick feedback on my thesis chapters I managed to finish and submit a beautiful thesis just on time for my life-time trip. I will be forever grateful for that! Your expertise, encouragement and constructive feedback have been invaluable in shaping the direction and quality of this thesis, as well as in shaping the scientist I am today. Thank you for your trust and for encouraging me to think outside the box and embrace new challenges. I would also like to take this opportunity to thank you, and Hanke, for the patience and compassion you both had with me when recovering from my personal issues.

My beloved **Katka**, where to start? Perhaps from the instant "click" we both felt the very first time we met? I am not really sure what you saw in me in that first interview but since day one you became a true believer of my best qualities (even of those I often don't see myself). So much that you even gave your all to create a PhD position from scratch for me! I will always be thankful for that because without you none of this would have become a reality. You gave me the courage and the wings to fly in this adventure, even when I didn't think I was made for it. It was tough seeing you leave after my first year but I seriously think this made our relationship even stronger. Your constant encouragement since has been a driving force to finish this thesis in the best way possible. Thank you for sticking around and taking care of our friendship, for all the professional advice that you still give me today and for accompanying me during my worse struggles. You are my role model and I will always carry your wisdom and inspiration with me, wherever I go.

Lieve, my second co-promotor, thank you for always making yourself available for a meeting with us despite your busy physician schedule, but more importantly, thank you for all your clinical input on neuroblastoma. It has certainly contributed to the rigor and success of my thesis.

Robin, thank you for your very valuable and instructive feedback during the weekly work discussions and in the research articles of this thesis. Thank you for your support during this journey.

Timo, thank you for opening me the doors to the department when I was a naïve second-year master student and for believing in my internship project.

The **members of the promotion committee**: prof. dr. **T.D. de Gruijl**, prof. dr. **M.D. Hazenberg**, prof. dr. **E.F. Eldering**, dr. **M.C. Wolkers**, dr. **J.J. Molenaar** and dr. **G. de Haan.** Thank you for being part of my committee and for the time that you have spent during the reading, evaluation and preparation of my thesis defense. I hope you have enjoyed it as much as I did.

Derk and **Theo**, it has been a pleasure to have you as my PhD committee at Sanquin. Thank you for all the scientific discussions during the various stages of my research, your input has always been much appreciated. And thank you Derk, for giving me the opportunity to be part of the MISTRG project and for being there until it was completed.

My paranymphs or "mis amuletos de la suerte" of my PhD, as we would say in Spanish: **Giota**, my beloved Greek desk mate! You had to put up with me the longest, and surprisingly enough you are is still around, haha! And I really couldn't be more grateful for that! I just cannot think of a single moment in this academic adventure in which you aren't in it. We have gone through rough patches, where we have always tried to be each other's best confident, but we have also shared incredible adventures together, most of them traveling (Kefalonia, Serifos, México) where we have built unforgettable memories. Thank you for opening your heart to me since day one, for your genuine caring nature and for standing by my side through thick and thin. Today I feel the luckiest for being able to call you a true friend. Efharistó poly, Giotita! **Aafke**, I still remember the first day I saw you come in the office as a first year PhD student who started only a few days after me. Don't ask me why but from that moment I already knew we were going to become great friends. And I wasn't wrong, that is exactly what happened! I cannot imagine what I would have done without you, without our countless coffee breaks at the NKI that somehow always turned out to be the best therapy sessions, haha! You have been an incredible pilar and I have loved sharing every step of the way with you. Thank you for always offering a listening

ear and for giving back the wisest advice. Giota, Aaf, having you around has certainly been the greatest gift of this PhD. Thanks for accompanying me in this journey, you will forever hold a special place in my heart!

Sanquin, for providing me with the resources, facilities, and academic environment necessary for conducting my research. Especial thanks to the donors from "**buisjesdonoren system**" who made it possible to have fresh blood available daily for our experiments.

Mark, how can I thank you for all the patience you have had with me, starting from the animal trainings and ending with the last (always last minute) immunohistochemistry experiments and imaging to complete the last chapter? Thank you for making time for me where there seemed to be none, and for making everything look so easy! Ik ga je missen, viejo!

Erik and **Simon**, thanks a lot for all your theoretical and practical knowledge with the flow cytometers and cell sorters throughout these years, and for always being willing to help. You guys, together with Mark, are really indispensable assets for Sanquin Research.

Corry and **Suzanne**, thank you for always doing your best to make sure every last minute order came in on time. Your work is truly essential and it has been wonderful to have your joyful souls around.

The wonderful people I shared the office with: **Silvia** and **Naoual**, we laughed so much, we cried so much, but we have always been there for each other, conditionally or unconditionally! It has been a great pleasure having you around and sharing so many parties, meals, trips and adventures together all these years. You will be sooooo missed! **Karin** and **Boukje**, thank you for having the sixth sense to notice when either a good talk or a delicious sweet were needed around the office! **Anna**, the latest to join Y412 but what a great acquisition! Even though it was only for a few months, it has been wonderful to work and connect with you. Thank you for all the insightful scientific (and not scientific) talks!

Everybody else at the department. PhD students and technicians along the way: **Dieke**, it was such a pleasure to write my first research article, now Chapter 3, with you, thank you for everything you taught me. **Charita**, I still cannot understand how we managed to write a review, now Chapter 2, within 10 days (together with Giota)! I very much enjoyed working with you on it and other projects. What a great team we made! **Colin**, **Bart** and **Hugo**, the three musketeers of the hallway! Thank you for always cheering up the atmosphere. I will miss you! **Ines**, **Evelien, Cathelijn**, **Steven**, **Lidian**, **Paula**, **Angela**, you have all contributed to the amazing time I spent at the department. The senior analysts: a special thanks for **Paul**

and **Karin** for your infinite patience when asking for the million (last minute) cell requests (Paul) and for running thousands of ADCCs with infinite conditions (Karin). It has been great working with you! **Anton** and **Dirk**, thank you for your critical insights, suggestions, and expertise during the weekly work discussions that have greatly contributed to the rigor of my research.

And everybody else at Y4 I haven't mentioned up until now, which mainly includes the **Red Cell Diagnostics** department, especially **Martijn** for sharing your happiness all around the hallway, and **Maud** for always willing to give a hand as a "prikker" in the event of a last minute need of blood.

Marion, I would like to thank you for your endless help at organizing meetings, sending reminders and arranging paperwork. Also for always being so caring for our well-being.

People from U2: **Caro** and **Carlijn**, my Sanquin latin lovers! How lucky I am to have met you, I will always recall our salsa/latin parties together! I couldn't think of two more caring souls on this planet, thank you for always being willing to give a hand whenever it was needed. **Arthur** (my running mate!), **Michel** and **Riv** (yes, Riv, I consider you from U2 too haha), the party animals! You have been the indispensable asset in every borrel, birthday party or any other get-together I can think off! Thank you for being the "soul of the party" as we would say in Spanish. **Florencia**, **Nordin** (my buddy!) and **Tamara**, we all started at the same time and despite all the bumps on the road, we are all still alive and with a smile in the face! I wish you all the best in the last steps of your respective PhDs! **Adrien** and **Nika**, thank you not only for all your logistic, scientific and experimental help to move forward on the MISTRG chapter, but also for always helping me find and chase Julien every time I needed him along the way haha. **Max**, **Casper**, **Valeria**, **Giuglia**, **Timon**, **Dorien**, thank you all for the nice times I spent around your department as well as during the extra-professional experiences (trips, parties, etc) outside Sanquin.

And a special mention to **Julien**... Still today, I regret the day that Katka put us to work together in the MISTRG project... Noooo, just kidding haha! It has seriously been the greatest pleasure to work on this fascinating project with you (now Chapter 6). Even when everything else seemed to be against us, we pushed each other to make this chapter become a reality. I seriously feel incredibly proud and happy of what we have achieved together, and at least half of the merit is yours! Thank you very much for teaching me so much along the way and for helping me become a better researcher (and I hope I was also able to teach you something at the end of the day :P). I wish you the very best on what is still to come! See you in Toulouse, Barcelona or the Pyrenees mon ami!

A big thanks to our collaborators at the **Princess Máxima Center** (**Ilse**, **Miranda**, **Waleed**, **Judith**, **Lieke** and **Niels**, including also **Lieve** and **Jan**), who provided us with cell lines, patient samples, clinical grade material and their valuable input. In addition, also thanks to the neuroblastoma **patients** and **families** who gave their consent to participate in our research projects.

To all other **collaborators** and **co-authors**, it has been a pleasure to work with you all.

Now I want to take the chance to thank everyone else that has more or less indirectly contributed to the completion of my thesis.

Friends:

Andrea y **Julia**, las que habéis estado de principio a fin (y espero que estéis durante mucho tiempo más!). Desde el primer día que pusimos el pie en esta ciudad extraña os convertisteis en mi casa en Ámsterdam. Incontables son las experiencias vividas juntas en esta ciudad, donde empezamos compartiendo apuntes del máster y pasando horas y horas en la biblioteca, y donde a día de hoy seguimos apoyándonos mutuamente cada vez que tropezamos con un bache en el camino en cada una de nuestras respectivas vidas o PhDs. Pero no todo han sido baches! 7 años también han dado para muchas alegrías y me siento super agradecida de haberlas podido compartir con vosotras. Como ya os he dicho un millón de veces, no sé qué habría sido de mi si no os hubierais cruzado en mi camino. GRACIAS, en mayúsculas!

Marit, my beloved Maritposa. I still remember the day we met in a random bar in Barcelona… Who would have thought that 8 years later we would still be living 10 minute cycling from each other in your home country? Your caring and cheerful nature was what finally convinced me to move to the Netherlands (or maybe it was the delicious patatas bravas you made for me shortly after we met? :P). I could finally say I had my first Dutch friend in the Netherlands! And how great it has been to have such an wonderful human being like you around all these years! I couldn't thank you enough for everything you have done for me, you know you have been one of my most important pillars in this foreign country. No quiero perderte por nada en el mundo!

Sara, òbviament mereixes un apartat en aquesta tesis perquè sense el teu recolzament imprescindible, tot i haver estat des de la distancia, aquest camí hagués estat deu mil vegades més difícil. En especial vull remarcar els últims anys on has estat SEMPRE que t'he necessitat. Gràcies per sempre fer temps per les mil trucades espontànies i interminables a hores random. Fa 12 anys que ens vam conèixer a la uni, dels qual només 4-5 hem viscut

a la mateixa ciutat. Tot i així et sento més propera que mai, i estic segura que els anys que venen encara reforçaran més (si es pot) aquesta amistat que tant ens agrada cuidar i mimar.

Isa, otro de mis pilares fundamentales en este trayecto. Gracias por tu interés y apoyo incondicional y genuino desde el día que conectamos bailando, por estar en las duras y en las maduras y por haber sido mi vía de escape cuando necesitaba un poco de Barcelona en Ámsterdam. Te aprecio muchísimo.

Anna, porque sin ti esta etapa tampoco hubiera tenido sentido. Me siento increíblemente agradecida de haberte tenido cerca, de haber compartido momentos en Holanda juntas pero también muchos, muchísimos, en Barcelona. Gracias por ayudarme siempre a ver las partes positivas y bonitas de Holanda cuando lo veía todo negro.

My salsa treasures: **Debbie** and **Maaike**, you wouldn't think so but you have certainly been an indispensable asset for me to complete and close this chapter. Sometimes dancing was the only thing that helped me disconnect when work was too much and sharing these beautiful moments, dances and talks with you have been the best therapy. Thank you for every gesture of love we have given each other. You both are the most wonderful human beings I've ever met!

Marieke, I feel so grateful that our paths crossed in my very first internship at the NKI. Despite the distance during the hardest years of my PhD, I have always felt we were connected in some way. Thank you for all your unconditional support!

Durfis, per ser l'estímul extern que m'ha mantingut viva en aquesta intensa aventura acadèmica des de l'inici dels inicies i fins a dia d'avui. Gràcies per ser-hi sempre!

Mambos, el vostre recolzament i companyia al llarg dels anys han estat una font de força i alegria imprescindible en aquest camí. Gràcies per sempre fer-me sentir a prop. Us estimo molt a totes i cada una de vosaltres.

Family:
Quiero empezar agradeciendo a varios miembros de mi familia (**tía Arancha**, **tío Graham**, **Dani**, **Laura**, **tía Menchu**, **Lucia, Victoria**, **Sara**, **tía Merche**, etc) por vuestro apoyo incondicional y por estar al tanto de todos mis triunfos académicos. Nunca he dejado de sentir vuestro apoyo aunque fuera en la lejanía.

Ahinara, mi querida y tan apreciada cuñi, la que me ha sostenido en los momentos más difíciles. ¿Qué habría hecho sin ti? Gracias por las mil horas al teléfono, por tu

confidencialidad y cariño, pero sobre todo sobre todo por ser el motor que me ha dado la fuerza necesaria para superar cualquier obstáculo. El afecto que me has dado ha logrado ir más allá de los lazos familiares y me siento increíblemente afortunada de poder llamarte amiga!

Y por último, mi más sincero agradecimiento va dedicado a mi familia más directa: **mamá**, **papá**, **Nacho**, **Alex** (y **Ona**!). Mamá, papá, desde muy joven me inculcasteis el valor del conocimiento y la perseverancia, valores sin los cuales no habría conseguido llegar hasta aquí. Gracias por brindarme con todas las oportunidades posibles para lograrlo y por creer en mí en cada paso de mi carrera académica. Sé que ha sido duro tenerme lejos, sobre todo en los momentos más desafiantes, pero yo siempre os he sentido cerca. Nacho y Alex, los mejores hermanos que podría tener! Seremos diferentes y viviremos cada uno en un lugar del mundo pero aun así sé que siempre voy a poder contar con vuestro apoyo, estemos donde estemos. Nacho (y Ahinara), gracias por acogerme mil y un fines de semana en Bruselas cuando necesitaba una dosis familiar. Me ha encantado teneros cerca. Alex, *subi*, gracias por ponerle diseño y color a la ciencia contenida en esta tesis doctoral. Ha sido la guinda del pastel para cerrar este capítulo. Todos (incluida Ona): esta tesis doctoral es un reflejo del amor y apoyo que he recibido de vuestra parte a lo largo de los años. Gracias por ser mi red de contención en los momentos duros y por celebrar conmigo cada uno de mis éxitos. Os quiero!