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RE-EDUCATING MACROPHAGES TO ACTIVATE ANTITUMOR IMMUNITY

One Clever Immunotherapy

Miro Viitala

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ABSTRACT

The immune system is our constant protector against external foes but also against our own incipient malignant cells. Unfortunately, developing cancers often learn to shut down the antitumor immune response or even to manipulate the immune system to support their own growth and progression instead. Checkpoint blockers that reactivate adaptive antitumor immunity have revolutionized cancer treatment—however, benefiters are in the minority. Therefore, novel treatment options are required for patients whose cancers are refractory. Tumor-associated macrophages of the innate immune system—educated in the tumor microenvironment—have emerged as prominent supporters of cancer progression that promote nine out of ten hallmarks of cancer. However, thanks to their remarkable intrinsic plasticity, macrophages retain the capability to promote the antitumor immune response even in thrall of cancer. Consequently, significant interest has been directed to the possibility of targeting tumor-associated macrophages to promote antitumor immunity as cancer immunotherapy.

In this PhD thesis, I present the preclinical proof-of-concept, putative mechanism-of-action and results from early clinical trials of one such experimental immunotherapy: antibody blockade of the scavenger receptor Clever-1, expressed on a subset of tumor-associated macrophages. The results herein establish macrophage Clever-1 as an endogenous immune suppressor that restrains both macrophage overactivation and adaptive immunity. We show that Clever-1 blockade "re-educates" macrophages to promote antitumor immunity by activating cytotoxic T cells in preclinical tumor models. We propose this is mechanistically linked to Clever-1's association with the vacuolar ATPase, the disruption of which antagonizes antigen degradation in phagolysosomes and saves them for cross-presentation. Lastly, I present results from early clinical trials, which indicate that Clever-1 blockade may boost antitumor immunity specifically in a subset of patients with noninflamed tumors for whom checkpoint blockade is rarely efficacious.

KEYWORDS: cancer immunology, immunotherapy, innate immunity, tumor-associated macrophages, Clever-1

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TIIVISTELMÄ

Immuunijärjestelmä on herkeämätön suojamme sekä ulkoisia uhkatekijöitä että omia orastavia pahanlaatuisia solujamme vastaan. Valitettavasti kehittyvät syövät oppivat usein sammuttamaan kasvainta torjuvan immuunivasteen tai jopa keplottelevat immuunijärjestelmän edistämään niiden omaa kasvua ja etenemistä. Tarkastuspisteiden estäjät, jotka käynnistävät uudelleen kasvainta torjuvan hankitun immuniteetin, ovat mullistaneet syövän hoidon—niistä hyötyvät ovat kuitenkin vähemmistössä. Siksi tarvitaan uusia hoitovaihtoehtoja potilaille, joiden syövät ovat itsepintaisia. Synnynnäiseen immuniteettiin kuuluvat kasvaimeen liittyvät syöjäsolut—jotka koulutetaan kasvaimen mikroympäristössä—ovat syövän etenemisen keskeisiä tukijoita ja edistävät yhdeksää kymmenestä syövän ominaispiirteestä. Omaleimaisen luontaisen mukautumiskykynsä ansiosta syöjäsolut kuitenkin säilyttävät kykynsä myös vahvistaa kasvainta torjuvaa immuunivastetta jopa syövän pauloissa. Mahdollisuuteen herättää kasvainta torjuva immuniteetti käyttämällä kasvaimeen liittyviä syöjäsoluja lääkekohteena syövän immunologisessa hoidossa onkin kohdistunut merkittävää kiinnostusta.

Tässä väitöskirjassa esitän prekliinisen soveltuvuusselvityksen, oletetun vaikutusmekanismin sekä tuloksia varhaisista kliinisistä kokeista yhdelle tällaiselle kokeelliselle immunoterapialle: vasta-ainevälitteiselle Clever-1-haaskareseptorin estolle, jota ilmentää osajoukko kasvaimeen liittyviä syöjäsoluja. Nämä tulokset vakiinnuttavat Clever-1:n sisäsyntyisenä immuniteetin vaimentajana, joka hillitsee sekä syöjäsolujen tarmokkuutta että hankittua immuniteettia. Osoitamme, että Clever-1:n häiritseminen ”uudelleenkouluttaa” syöjäsolut tukemaan kasvainta torjuvaa immuniteettia herättämällä tappaja-T-soluja prekliinisissä kasvainmalleissa. Mekaanisesti ehdotamme tämän olevan seurausta Clever-1:n ja vakuolaarisen ATPaasin yhteistoiminnasta, jonka purkaminen estää antigeenien pilkkomista lysosomeissa ja pelastaa ne ristiinesittelyä varten. Viimeiseksi esitän tuloksia varhaisista kliinisistä kokeista, joiden perusteella Clever-1:n estäminen saattaa tehostaa kasvainta torjuvaa immuniteettia erityisesti osajoukossa potilaita, joiden kasvaimissa ei ole aktiivista tulehdusvastetta ja joihin tarkastuspisteiden estäjät harvoin tehoavat.

AVAINSANAT: syöpäimmunologia, immunoterapia, synnynnäinen immuniteetti, kasvaimeen liittyvät syöjäsolut, Clever-1

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Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
ALR	absent-in-melanoma-2-like receptor
ANG	angiopoietin
APC	antigen-presenting cell
ARG1	arginase 1
ATP	adenosine triphosphate
BCR	B-cell receptor
BMDM	bone-marrow-derived macrophage
cGAS	cyclic guanosine monophosphate–adenosine monophosphate synthase
CITE-seq	cellular indexing of transcriptomes and epitopes by sequencing
Clever-1	common lymphatic endothelial and vascular endothelial receptor 1
COX2	cyclo-oxygenase 2
CTL	cytotoxic T lymphocyte
CTLA4	cytotoxic T lymphocyte antigen 4
CyTOF	cytometry by time-of-flight
DAMP	danger-associated molecular pattern
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
FasL	Fas ligand
FBS	fetal bovine serum
Fcγ(R)	fragment crystallizable γ (receptor)
FDA	the United States Food and Drugs Administration
FLT3(L)	Fms-related receptor tyrosine kinase 3 (ligand)
FoB	follicular B cell
GGA	Golgi-localized, γ-adaptin-ear-containing, adenosine-diphosphate-ribosylation-factor-binding
(G)M-CSF	(granulocyte–)macrophage-colony-stimulating factor
HIF	hypoxia-inducible factor
HMGB1	high mobility group box protein 1

IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IGF-1(R)	insulin-like growth factor 1 (receptor)
IL	interleukin
ILC	innate lymphoid cell
IMDM	Iscove's modified Dulbecco's medium
INs-seq	intracellular staining and sequencing
KLH	keyhole limpet hemocyanin
LAG3	leukocyte activating gene 3
LAMP-1	lysosomal-associated membrane protein 1
LPS	lipopolysaccharide
LXR/RXR	liver X receptor/retinoid X receptor
MAPK	mitogen-activated protein kinase
MARCO	macrophage receptor with collagenous structure
MATINS	Macrophage Antibody to Inhibit Immune Suppression
MDM	monocyte-derived macrophage
MHC	major histocompatibility complex
M-MDSC	monocytic myeloid-derived suppressor cell
MMP9	matrix metalloprotease 9
mTOR	mammalian target of rapamycin
(MZ)B	(marginal zone) B cell
NK	natural killer cell
NLR	nucleotide-binding-oligomerization-domain-like receptor
NO(S2)	nitric oxide (synthase 2)
NP	4-hydroxy-3-nitrophenylacetic acid
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PD-(L)1	programmed death receptor (ligand) 1
PGE_2	prostaglandin E_2
PhD	doctor of philosophy
PI3K	phosphoinositide 3-kinase
PL	placental lactogen
PMN-MDSC	polymorphonuclear myeloid-derived suppressor cell
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
RAGE	receptor for advanced glycation end products
RLH	retinoic-acid-inducible-gene-I-like helicase
ROS	reactive oxygen species
(sc)RNA-seq	(single-cell) ribonucleic acid sequencing

SI-CLP	Stabilin-1-interacting chitinase-like protein
SIRPα	signal-regulatory protein α
SPARC	secreted protein acidic and rich in cysteine
STING	stimulator of interferon genes
TAM	tumor-associated macrophage
TAP	transporters associated with antigen processing
T_{CM}	central memory T cell
TCR	T-cell receptor
TD	thymus-dependent antigen
T_{EFF}	effector T cell
T_{FH}	follicular helper T cell
T_{EM}	effector memory T cell
TGFβ	transforming growth factor β
T_H	helper T cell
TI	thymus-independent antigen
TIE2	tyrosine kinase with immunoglobulin and endothelial growth factor homology domains 2
TIGIT	T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains
TIM3	T-cell immunoglobulin domain and mucin domain 3
TLR	Toll-like receptor
TME	tumor microenvironment
TRAIL(R)	tumor-necrosis-factor-related apoptosis-inducing ligand (receptor)
T_{REG}	regulatory T cell
TREM2	triggering receptor expressed on myeloid cells 2
TRM	tissue-resident macrophage
VAP-1	vascular adhesion protein 1
v-ATPase	vacuolar adenosine triphosphatase
VCAM-1	vascular cell adhesion molecule 1
(V)EGF(R)	(vascular) endothelial growth factor (receptor)
VISTA	V-domain immunoglobulin suppressor of T-cell activation

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Dunkel, J.*; **Viitala, M.***; Karikoski, M.; Rantakari, P.; Virtakoivu, R.; Elima, K.; Hollmén, M.; Jalkanen, S. & Salmi, M. Enhanced antibody production in Clever-1/Stabilin-1-deficient mice. *Frontiers in Immunology*, 2018; 9: 2257.

II **Viitala, M.**; Virtakoivu, R.; Tadayon, S.; Rannikko, J.; Jalkanen, S. & Hollmén, M. Immunotherapeutic blockade of macrophage Clever-1 reactivates the $CD8^+$ T-cell response against immunosuppressive tumors. *Clinical Cancer Research*, 2019; 25 (11): 3289–3303.

III Virtakoivu, R.*; Rannikko, J.*; **Viitala, M.***; Vaura, F.; Takeda, A.; Lönnberg, T.; Koivunen, J.; Jaakkola, P.; Pasanen, A.; Shetty, S.; de Jonge, M.; Robbrecht, D.; Ting Ma, Y.; Skyttä, T.; Minchom, A.; Jalkanen, S.; Karvonen, M.; Mandelin, J.; Bono, P. & Hollmén, M. Systemic blockade of Clever-1 elicits lymphocyte activation alongside checkpoint molecule downregulation in patients with solid tumors: results from a phase I/II clinical trial. *Clinical Cancer Research*, 2021; 27 (15): 4205–4220.

*, equal contribution.

1 Introduction

The immune system, a tight-knit ensemble of molecules, cells and organs, has evolved to protect its host organism from harmful substances of both external and internal origin. Complex immune systems have evolved even in the "simplest"—for lack of a better word—multicellular species (Beutler, 2004), and mechanisms of immunity are present even in bacteria and some viruses that protect them from being overtaken by other micro-organisms (Barrangou *et al.*, 2007; Levasseur *et al.*, 2016; Meselson & Yuan, 1968). In vertebrate animals, including human beings, the immune system is classically divided into two main branches: innate and adaptive immunities (Cooper & Alder, 2006). The ever-vigilant innate immune system is our first line of defense against pathogenic micro-organisms and recruits the adaptive immune system, which provides an additional layer of specific and long-lasting protection against detrimental agents attempting to re-emerge. Notably, the immune system protects us also from our own cells should they take a turn towards tumorigenicity. However, the sophisticated mechanisms in place cannot completely contain all the ways in which it is possible for things to go wrong. Sometimes such aberrant cells emerge that will, eventually, become tumorous or even cancerous. Often, cancer even becomes able to turn the immune system against the host to promote its own development and progression—but as our understanding of the fundamental immunology behind this unfortunate phenomenon has increased, we have also invented means of turning the tables. Over the last decade, cancer immunotherapies targeted at T cells of the adaptive immune system have made revolutionary breakthroughs in the treatment of previously incurable malignancies. These so-called checkpoint blockers are inhibitors of inhibitors that reactivate the existent but suppressed antitumor immune response in some inflamed or "hot" tumors but, regrettably, benefiters are in the minority. Therefore, intense effort has been directed towards discovering novel approaches to help the remainder, especially those with noninflamed or "cold" tumors that lack an antitumor immune response. Given the innate immune system's ability to dispatch abnormal cells and stimulate adaptive immunity on the one hand and promote cancer development and progression on the other, its manipulation as cancer immunotherapy has attracted significant interest. Macrophages, highly adaptable phagocytes of the innate immune system with the ability to both

stimulate and suppress immune responses, have become the focus of many experimental therapies. Tumor-associated macrophages (TAMs) are typically the most abundant immune cells present in the tumor microenvironment (TME), wherein the bombardment of pathological signals distorts TAMs to foster the hallmarks of cancer. Yet, macrophages also have their own intrinsic antitumor activity in addition to their capacity to stimulate other innate and adaptive immune cells. And, importantly, macrophages' extraordinary adaptability allows them to alter between stimulatory and suppressive activation states, a capability they appear to retain even under the influence of cancer. Thus, experimental immunotherapies have been developed to the end of depleting, activating or "re-educating" TAMs from suppressive to stimulatory in order to restart the antitumor immune response. Some of these investigational treatments have already undergone clinical trials with varying degrees of success. The results I present in this PhD thesis describe the preclinical proof-of-concept, putative mechanism-of-action and results from early clinical trials of one such experimental immunotherapy: the antibody-mediated interference of Clever-1, a scavenger receptor enriched on a subset of immunosuppressive macrophages, including TAMs. We discovered that in addition to its previously described ability to inhibit T-cell responses, Clever-1 also inhibits the activity of B cells, and concluded that myeloid Clever-1 is an endogenous immunosuppressive molecule that limits overactivation of the immune system. Moreover, we explicated that, in the context of cancer, Clever-1 regulates the suppressive activation state of a subset of TAMs that inhibit antitumor immune responses. We found that Clever-1 deficiency re-educates immunosuppressive TAMs to become more stimulatory, allowing them to reactivate antitumor immunity executed by killer T cells. Importantly, we could achieve similar results with immunotherapeutic Clever-1 blockade in several preclinical tumor models. Additionally, we connected Clever-1^+ TAMs' reduced capacity to activate T cells to the association of Clever-1 with vacuolar adenosine triphosphatase (v-ATPase), through which Clever-1 promotes the lysosomal degradation of its cargo, including protein antigens, to putatively limit cross-presentation. Lastly, we analyzed the effects of immunotherapeutic Clever-1 blockade on the immune responses of patients with treatment-resistant cancers participating in a combined phase I and II clinical trial investigating the safety and efficacy of the human Clever-1 antibody, bexmarilimab, and reported the first clinical evidence of macrophage re-education by antibody-mediated Clever-1 interference. Significantly, our results indicated that Clever-1 blockade may increase antitumor immunity in a subset of patients with cold tumors, warranting the continuation of bexmarilimab's clinical development.

2 Review of the Literature

2.1 The Immune System

Every day, we are challenged by innumerable small attacks that originate outside and within ourselves. The ubiquitous microbes all around and inside us cause infection and disease in unfavorable circumstances, while mutations that accumulate in our own cells through the years, because of unavoidable exposure to radiation and hostile substances in the environment, disrupt their well-regulated behavior and eventually lead to cancer. Still, for many of us, these daily violations can go mostly unnoticed thanks to the immune system, which has evolved to prevent infections and protect us from diseases. The immune system accomplishes this through the efficient recognition, containment and elimination of substances by launching an immune response, a powerful onslaught aimed at molecular patterns that the immune system can discriminate as "foreign" or, more specifically, as non-self. A successful immune response leads not only to the eradication of the substance that triggered it, but can also grant the host long-lasting immunity against later encounters with the same substance. Because of its strength, the immune system must be strictly controlled, as overactive immune responses can cause massive collateral damage, while misdirected immune responses unleashed against the host's innocuous self-molecules can lead to debilitating and even fatal autoimmune diseases (Davies *et al.*, 1975). On the other hand, weak immune responses leave the host vulnerable and can be equally deadly (Glanzmann & Riniker, 1950; Gottlieb *et al.*, 1981; Holmes *et al.*, 1966). Therefore, to ensure an appropriate immune response, various checks and balances have evolved at every tier of the immune system that can either stimulate or suppress its activity as required. In addition to defending the host from harmful substances, the immune system is now recognized also to be an integral regulator of ontogeny and physiology (Mor *et al.*, 2017; Rankin & Artis, 2018)—one of its important tasks is to survey and cull the body of potentially cancerous cells that have become abnormal due to infection or mutations (Burnet, 1970). Paradoxically, it has also long since become apparent that the immune system can have a completely backwards role in promoting cancer development, progression and therapeutic resistance (Balkwill & Mantovani, 2001; Sharma *et al.*, 2017).

All cells of the human immune system—called white blood cells, leukocytes or simply immune cells—originate from a common pluripotent progenitor, the hematopoietic stem cell (Baum *et al.*, 1992). In adult humans, new immune cells are produced in the bone marrow, where daughter cells of the hematopoietic stem cell differentiate into either common myeloid or common lymphoid progenitors. Through several intermediaries, common myeloid progenitors produce red blood cells, platelets and the majority of innate immune cells, which include neutrophils, dendritic cells (DCs) and monocytes, the precursors of macrophages. Likewise, common lymphoid progenitors produce the lymphocytes of the adaptive immune system—B and T cells—as well as natural killer (NK) cells, which are classified as innate immune cells. The innate and adaptive immune systems are by no means separate, but actually must engage one another at multiple checkpoints to co-ordinate the local and systemic status of the immune system. Communication between cells of the immune system occurs by specific ligand:receptor interactions in direct cell-to-cell contact and through secreted signaling molecules, including growth factors, cytokines and chemokines, that regulate cellular behavior by autocrine, juxtacrine, paracrine and endocrine mechanisms. The final effects of triggering an immune receptor are often diverse and contextual, as they depend on the time and place of receptor stimulation and the type and state of the responding cell. In this literature review, I first introduce the components that make up our immune system and how they come together to generate an immune response. I also discuss some ways in which cancer is able to manipulate the immune system to benefit its own ends. Finally, I discuss some pharmacological interventions that have been developed to overcome this manipulation.

2.1.1 The Innate Immune System

The innate or “natural” immune system constitutes several ancient defensive mechanisms of host protection. It also provides the explosive rapid response against harmful substances. Innate immunity is built upon three main lines of defense:

- The body’s physical surface barriers, namely healthy skin and mucous membranes made up of various types of specialized epithelial cells.
- Soluble, secreted factors such as enzymes, host defense peptides and the complement system.
- Distinct subsets of specialized immune cells whose function is to search and destroy substances deemed non-self, that is, foreign and potentially dangerous.

Healthy skin and mucous membranes of the digestive, respiratory and reproductive tracts provide physical barriers against infection. Additional protection at these barriers is provided by the body’s various secretions, including mucus, tears and saliva,

which contain soluble factors such as lytic enzymes and host defense peptides that work as natural antibiotics (Salton, 1957; Zhao *et al.*, 1996). Moreover, the complement system, a collection of more than two dozen proteins produced mainly by the liver (Pillemer, 1943), continuously monitors the blood and tissues. Activation of the complement system triggers an enzymatic cascade whose end product is the multiprotein membrane attack complex, which can destroy invading micro-organisms by literally punching holes through their surface membranes (Hadders *et al.*, 2007). Additionally, activated complement proteins provide potent stimulation for innate immune cells (Eisner *et al.*, 1994; Morgan *et al.*, 1993).

The innate immune system—both its soluble and cellular constituents—relies on pattern recognition receptors (PRRs) to distinguish non-self-agents from harmless self-molecules. PRRs are expressed by all innate immune cells, as well as epithelial cells and even some adaptive immune cells, and recognize a fixed number of conserved molecular patterns that have become hardwired in the germline during the course of our evolution (Kimbrell & Beutler, 2001). These include pathogen-associated molecular patterns (PAMPs), which are conserved and common structural molecules of microbes and include proteins, carbohydrates, fatty acids and nucleic acids, as well as danger-associated molecular patterns (DAMPs) that are normally hidden inside host cells but translocate to the cell surface or extracellular space in response to stress and damage. Because PRRs are coded by a numbered set of genes, the molecular patterns that can be sensed by the innate immune system are correspondingly limited and not altogether specific. Thus, the innate immune system recognizes broader categories of substances and is able to differentiate between, for example, bacteria, fungi and viruses (Oshiumi *et al.*, 2003; Takeuchi *et al.*, 1999a; Underhill *et al.*, 1999)—although broad overlap exists between these responses, too (Hoebe *et al.*, 2003; Yamamoto *et al.*, 2003). On the other hand, the innate immune system is constitutively active and incredibly fast, as it takes only hours for the innate immune response to peak, which is typically observed as inflammation and characterized by redness, heat, swelling, pain and loss-of-function in the inflamed area.

2.1.1.1 Cells of the Innate Immune System

Most of the 3.0–11.6 billion immune cells per liter of blood in a full-grown healthy human being are innate immune cells. Of these, neutrophils are the most numerous and comprise 40–80 % of all blood-borne immune cells, followed by monocytes at 1–10 %. Neutrophils are actually only one of four types of innate immune cells collectively referred to as polymorphonuclear cells or granulocytes, the other two found in the blood being eosinophils and basophils, present at 1–5 % and 0–1 %, respectively. The low-frequency eosinophils and basophils are mainly involved in immune responses against parasitic worms, and especially the latter mediates allergic reac-

tions. The fourth type of granulocyte, the tissue-resident mast cell, has a similar function. In comparison, neutrophils are far more numerous and well-studied—in fact, these "smaller ameboid cells which can be easily stained, with a largely polynuclear and fragmented nucleus" were described by Élie Metchnikoff already in 1887, when he named them microphages, along with cells that "generally possess a simple non-polymorphic nucleus which is round or frequently oval," which he named macrophages (Metchnikoff, 1887). According to Metchnikoff's original definition, neutrophils and macrophages are "phagocytes," that is, cells that are capable of consuming foreign bodies—even whole microbes and weakened cells of the organism itself—and ingesting them completely. The troop of phagocytes was completed by the discovery of the DC almost nine decades later (Steinman & Cohn, 1974; Steinman & Cohn, 1973; van Voorhis *et al.*, 1982). Phagocytosis, which uses PRRs to determine what to eat, and related nonspecific mechanisms such as pinocytosis underpin many major functions of the innate immune system.

Neutrophils are often called the foot soldiers of the immune system and could as well be referred to as cannon fodder. Local activation of the innate immune system mobilizes great numbers of neutrophils to rush out of the blood and into the site of inflammation. The cytoplasm of neutrophils brims with granules of destructive chemicals and lytic enzymes capable of eradicating engulfed pathogens but by spitting out their contents, they can also liquefy connective tissues and, thus, cause considerable damage to the host (Babior *et al.*, 1973; Bretz & Baggiolini, 1974; Damiano *et al.*, 1988; Kjeldsen *et al.*, 1992; Murphy *et al.*, 1977). The extent of collateral damage is restrained by restricting neutrophils' presence in tissues to only when specifically summoned by inflammation, as well as by neutrophils' built-in apoptotic program, which limits their period of activity once they have entered a site of inflammation (Savill *et al.*, 1989). Conversely, DCs are very low in frequency in the blood but always present in tissues, where they continuously sample their surroundings by phagocytosis and macropinocytosis. DCs express highly specialized molecular machinery for internalizing, processing and presenting protein-derived antigens to T cells of the adaptive immune system (Nussenzweig *et al.*, 1980; Rodriguez *et al.*, 1999; Sallusto *et al.*, 1995; Turley *et al.*, 2000). Therefore, they are often called "professional" APCs (antigen-presenting cells), the other two types of immune APCs being innate macrophages and adaptive B cells. Unlike neutrophils that flood tissues to combat pathogens, DC activation induces them to "mature" and migrate away from the site of inflammation to lymph nodes or other secondary lymphoid tissues—strategically placed hotspots of interaction between immune cells—where the DC "presents" fragments of its captured antigen to T cells (Cella *et al.*, 1997; De Smedt *et al.*, 1996; Pierre *et al.*, 1997; Schuler & Steinman, 1985). Antigen presentation, by and large, controls the activation of the adaptive immune response, making

innate APCs an indispensable bridge between the innate and adaptive immune systems (Hawiger *et al.*, 2001; Hawiger *et al.*, 2004; Kretschmer *et al.*, 2005).

The NK cell is another low-frequency but important type of innate immune cell in the blood (Kiessling *et al.*, 1975). They can also be called group 1 innate lymphoid cells (ILCs) after the discovery of the other two significantly less abundant group 2 and 3 ILCs (Bernink *et al.*, 2013; Cella *et al.*, 2009; Cupedo *et al.*, 2009; Mjösberg *et al.*, 2011). Because they are progeny of the common lymphoid progenitor but lack the sophisticated receptors that define B and T cells, ILCs straddle the division of innate and adaptive immunities. It seems that most ILC subsets—apart from, perhaps, the NK cell—have extensive functional overlap with other immune cells and their depletion can, mostly, be substituted by other adaptive and innate immune cells without noticeable hindrance to immune responses. Thus, ILCs have been suggested to be primordial precursors of adaptive T cells that linger as part of our biology, although this hypothesis apparently warrants further verification (Eberl *et al.*, 2015; Kotas & Locksley, 2018). When activated, ILCs secrete cytokine cocktails reminiscent of specific T-cell subsets. The best-studied ILC is the NK cell. Like neutrophils, NK cells enter tissues when they sense inflammation. Unlike other innate immune cells, the function of NK cells does not rely on phagocytosis nor do they make extensive use of PRRs. The factory setting of NK cells is, as their name suggests, to kill and kill again. They can directly execute cells observed as threats, which they determine by the composition of molecules presented on the cell surface. Under normal conditions, other host cells present an abundance of self-molecules that suppress NK-cell activation, but situations that increase cellular stress—such as viral infection or genetic mutations—lead to "missing self," which can switch this balance from suppressive to stimulatory and invite the NK cell to eliminate the afflicted cell (Bauer *et al.*, 1999; Choi & Mitchison, 2013; Correa & Raulet, 1995; Davis *et al.*, 1999; Gasser *et al.*, 2005; Kärre *et al.*, 1986; Litwin *et al.*, 1994; Mandelboim *et al.*, 2001; Moretta *et al.*, 1990a; Moretta *et al.*, 1990b; Pende *et al.*, 1996).

Finally, macrophages, which differentiate from blood-borne monocytes, are essentially important and fascinating innate immune cells that regulate the development and maintenance of the host's bodily functions all the way since before birth until death—they are also the stars of this thesis. Macrophages are true gourmands of the immune system and present in every tissue, where they, as phagocytes, in addition to specialized functions of different tissue-resident macrophages (TRMs) (Gautier *et al.*, 2012), work as general waste management and stand sentry in case pathogens manage to penetrate the protective surface barriers to establish a point of infection. Macrophages are enormously dynamic and notoriously malleable or "plastic." This characteristic plasticity enables macrophages to behave in quite contradictory ways from one moment to the next. Although they are typically the first immune cells to encounter breached pathogens and, subsequently, sound the alarm that

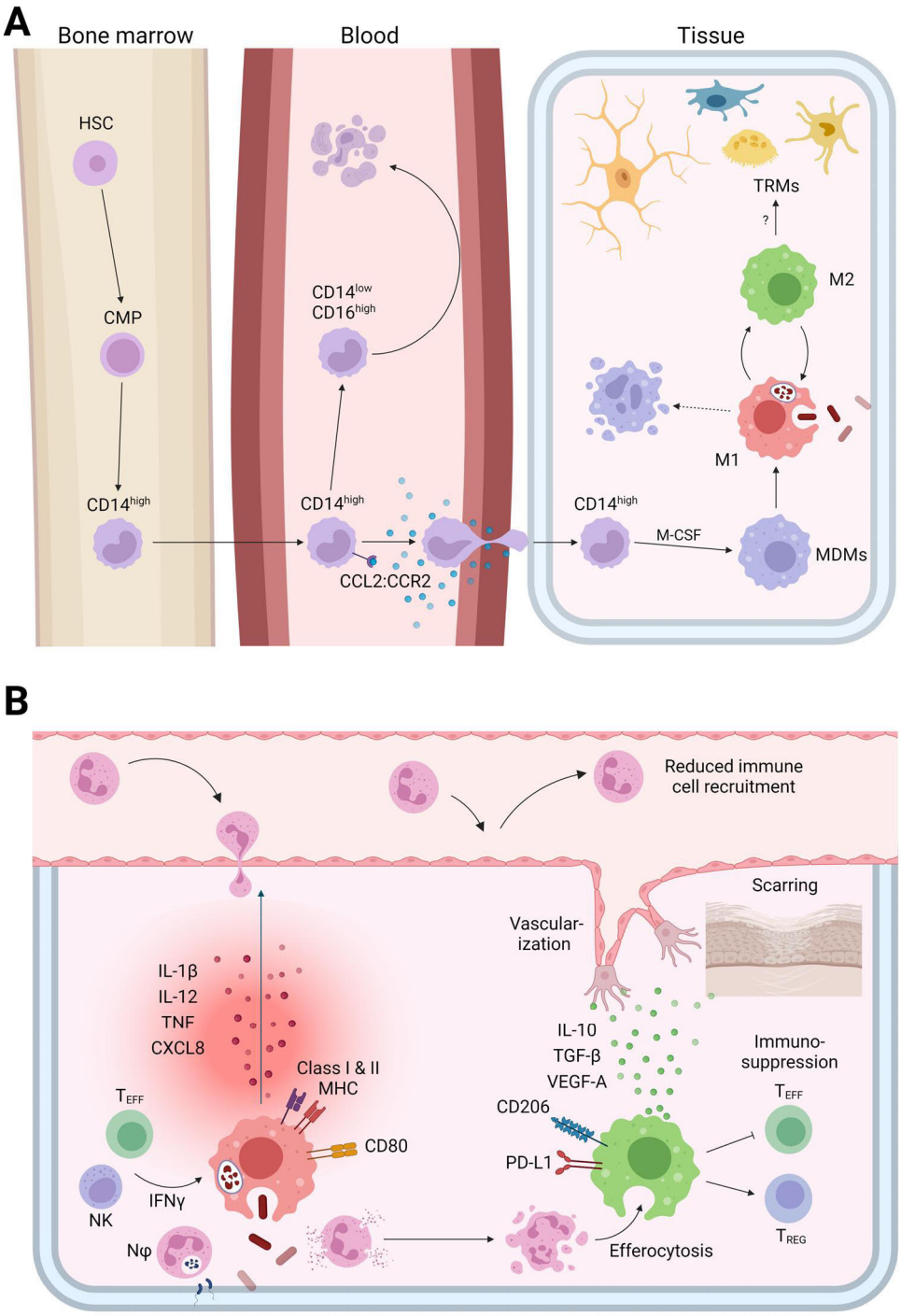
mobilizes the rest of the immune system, macrophages also suppress the immune response and oversee clearing of the battlefield and restoration at the site of inflammation once the source of infection has been dealt with.

2.1.1.2 The Origin of Macrophages

Macrophages colonize tissues already during embryonic development. The first generation is actually produced in the yolk sac—an extraembryonic organ that forms before the placenta—from where these primordial macrophages migrate into the developing embryo when the circulatory system is formed (Palis *et al.*, 1999; Schulz *et al.*, 2012; Takahashi *et al.*, 1989). Later in development, the fetal liver surpasses the yolk sac as the main blood-producing organ. Liver-derived macrophages partially replace earlier macrophages from the yolk sac except in the brain, which becomes largely closed off by the blood–brain barrier (Ginhoux *et al.*, 2010; Gomez Perdiguero *et al.*, 2015; Hoeffel *et al.*, 2015). Thus, cerebral macrophages seem to be the only macrophage population almost exclusively derived from the yolk sac (Sheng *et al.*, 2015). After birth, the liver's duties in blood production are eventually taken up by the bone marrow. The adult bone marrow releases macrophages as precursors called monocytes that can be immunotyped as $CD14^{high}$ $CD16^{low}$ $CCR2^{high}$ to distinguish them from other blood-borne $CD45^{+}$ $CD11b^{+}$ myeloid immune cells (**Figure 1A**) (Kawamura *et al.*, 2017; Passlick *et al.*, 1989; Weber *et al.*, 2000; Wolber *et al.*, 2002; Ziegler-Heitbrock *et al.*, 1988). These so-called "classical" $CD14^{high}$ monocytes travel in the blood for a short time and either migrate into tissues or transition into "nonclassical" $CD14^{low}$ $CD16^{high}$ $CCR2^{low}$ monocytes—through the $CD14^{mid}$ $CD16^{high}$ intermediate state—that inspect and repair the blood vessel lumen before dying by apoptosis (Auffray *et al.*, 2007; Carlin *et al.*, 2013; Cros *et al.*, 2010). Similar monocyte subsets can be identified in mice as $Ly6C^{high}$, $Ly6C^{low}$ and $Ly6C^{mid}$, respectively (Ingersoll *et al.*, 2010; Yona *et al.*, 2013; Ziegler-Heitbrock *et al.*, 1993; Ziegler-Heitbrock *et al.*, 2010). Especially during inflammation, tissues upregulate the secretion of the chemokine (from chemotactic cytokine) CCL2, which pushes $CD14^{high}$ monocytes out of the bone marrow and attracts them into tissues through its receptor, CCR2. Within inflamed tissues, classical monocytes can differentiate into macrophages in the presence of M-CSF (macrophage-colony-stimulating factor), whose receptor, CD115, is expressed mainly by progeny of the common myeloid progenitor (Boring *et al.*, 1997; Byrne *et al.*, 1981; Davies *et al.*, 2013; Lu *et al.*, 1998; Serbina & Pamer, 2006; Strieter *et al.*, 1989; Yoshimura *et al.*, 1989).

Macrophages from the prenatal period initially produce the specialized TRMs present throughout the body, referred to as, for example, microglia in the brain, Kupffer cells in the liver, dust cells or alveolar macrophages in the lungs, Langerhans cells in the skin and red pulp macrophages in the spleen (Ensan *et al.*, 2016; Epelman

Figure 1. The two faces of macrophages regulate both inflammation and its resolution. **A.** The HSC (hematopoietic stem cell) in the adult bone marrow produces CMPs (common myeloid progenitors) that differentiate into $CD14^{high}$ monocytes, which exit the bone marrow and enter circulation. From the blood, $CD14^{high}$ monocytes can be attracted into tissues by inflammatory mediators, for example, the chemokine CCL2, whose receptor, CCR2, is expressed on $CD14^{high}$ monocytes. Alternatively, $CD14^{high}$ monocytes can transform into $CD14^{low}$ $CD16^{high}$ monocytes that patrol and maintain the blood endothelium before, eventually, dying by apoptosis. Within tissues, $CD14^{high}$ monocytes can differentiate into MDMs (monocyte-derived macrophages) in response to, for example, tissue-derived M-CSF (macrophage-colony-stimulating factor). Proinflammatory signals—invading pathogens in particular—promote the polarization of immunostimulatory M1 macrophages that, after clearance of the pathogen, mostly die by apoptosis. Macrophage polarization is highly plastic, and microenvironmental factors may also induce M1 macrophages to repolarize into immunosuppressive M2 macrophages, which can again polarize into M1 macrophages if pathogens re-emerge. Moreover, tissues contain heterogeneous populations of TRMs (tissue-resident macrophages) whose numbers may be replenished by MDMs in a tissue- and context-dependent manner. **B.** During active inflammation, M1 macrophages sound the alarm bells that call to arms other cells of the immune system. M1 macrophages secrete a cocktail of proinflammatory mediators, including the cytokines IL (interleukin)-1β, IL-12 and TNF (tumor necrosis factor) and the chemokine CXCL8, which initiate the innate immune response and recruit Nφ (neutrophils), NK (natural killer) and T_{EFF} (effector T) cells out of the blood and into the site of inflammation. These cytokines also act on the macrophages themselves and, for example, supercharge their phagocytic capacity. Additionally, M1 macrophages express class I and II MHC (major histocompatibility) and costimulatory molecules, for example, CD80 and CD86, that stimulate T_{EFF} cells. NK and T_{EFF} cells, in turn, stimulate M1 macrophages by, for example, secreting IFN (interferon) γ. However, as the tide begins to turn and the pathogen is eradicated, inflammation gradually lessens and M2 macrophages begin to regulate resolution. The efferocytosis of apoptotic neutrophils, in particular, promotes the polarization of M2 macrophages, which secrete anti-inflammatory mediators, including the cytokine IL-10 and growth factors TGFβ (transforming growth factor β) and VEGF-A (vascular endothelial growth factor A), which inhibit the recruitment of other immune cells and induce vascularization and scarring. M2 macrophages also upregulate specific scavenger receptors such as the mannose receptor CD206 and coinhibitory molecules such as PD-L1 (programmed death receptor ligand 1) and suppress T_{EFF} cells but promote the activity of T_{REG} (regulatory T) cells, which also inhibit the effector functions of other immune cells. Created with BioRender.com.


A
Bone marrow
Blood
Tissue
HSC
CMP
CD14high
CD14low
CD16high
CD14high
CCL2:CCR2
CD14high
M-CSF
MDMs
TRMs
?
M2
M1
B
Reduced immune
cell recruitment
Scarring
Vascular-
ization
IL-1β
IL-12
TNF
CXCL8
Class I & II
MHC
CD80
TEFF
IFNγ
NK
Nφ
IL-10
TGF-β
VEGF-A
CD206
PD-L1
Immuno-
suppression
TEFF
TREG
Efferocytosis

et al., 2014; Ginhoux *et al.*, 2010; Gomez Perdiguero *et al.*, 2015; Guilliams *et al.*, 2013; Hoeffel *et al.*, 2012; Schulz *et al.*, 2012; Sheng *et al.*, 2015; Yona *et al.*, 2013). Originally, it was thought that monocyte-derived macrophages (MDMs) replenish TRMs through life as they expire. However, later work on animal models—especially the mouse—strongly suggests that the original TRMs actually persist long after birth by self-renewing independently of the bone marrow similarly to stem cells, whereas MDMs generated during adulthood eventually, for the most part, die out with waning inflammation (Ajami *et al.*, 2011; Ajami *et al.*, 2007; Gautier *et al.*, 2013; Hashimoto *et al.*, 2013; Merad *et al.*, 2002; Soucie *et al.*, 2016; van Furth & Cohn, 1968; Waqas *et al.*, 2017). It remains a matter of ongoing debate if and how adult MDMs replenish TRMs over the host's life and what cell-intrinsic and tissue-derived factors regulate their differentiation—and, importantly, how these findings translate to humans (Bian *et al.*, 2020). Research has previously been hampered by the lack of unambiguous cell-surface markers for sorting TRMs and MDMs from each other, although this limitation has probably finally been bypassed by the arrival of single-cell techniques, namely CyTOF (cytometry by time-of-flight), scRNA-seq (single-cell RNA sequencing), CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing) and INs-seq (intracellular staining and sequencing) (Bendall *et al.*, 2011; Jaitin *et al.*, 2014; Katzenelenbogen *et al.*, 2020; Stoeckius *et al.*, 2017). So far, no cut-and-dried answer has emerged. Rather, every tissue seems to contain different ratios of TRMs and MDMs and some organs—such as the gut—are much more reliant on replenishment from fresh blood-borne monocytes to maintain their baseline macrophage populations, while others—such as the brain—are clearly less so (Bain *et al.*, 2016; Bain *et al.*, 2014; Calderon *et al.*, 2015; Molawi *et al.*, 2014; Tamoutounour *et al.*, 2013). Moreover, it appears that following inflammation, not only can TRMs proliferate to re-establish their numbers but MDMs can also repopulate tissues if the incumbent TRMs have died out—even in the brain—and self-renew (Davies *et al.*, 2013; Davies *et al.*, 2011; Epelman *et al.*, 2014; Ginhoux *et al.*, 2006; Jenkins *et al.*, 2011; Mildner *et al.*, 2007; Scott *et al.*, 2016; van de Laar *et al.*, 2016). Thus, it has been proposed that while tissue-derived factors can instruct the phenomenally plastic MDMs to take over vacant TRM niches, some fundamental differences do exist between TRMs of prenatal and MDMs of adult origin. For example, especially when generated during inflammation, MDMs can be imprinted with transient "memory" of the event, which boosts their responses against subsequent insults (Aegerter *et al.*, 2020; Beattie *et al.*, 2016; Bruttger *et al.*, 2015; Gibbings *et al.*, 2015; Guilliams & Svedberg, 2021; Lavin *et al.*, 2014; Louwe *et al.*, 2021).

2.1.1.3 Macrophages at the Vanguard of Inflammation

Macrophages constitutively express a large repertoire of PRRs to survey their environment for PAMPs and DAMPs. Membrane-bound phagocytic PRRs recognize microbial structures such as carbohydrates (CD14) as well as many self-molecules such as antibodies (CD64), apoptotic bodies (phosphatidylserine receptors), complement proteins (complement receptors), hemoglobin (CD163) and oxidized lipids and lipoproteins (CD36) (Anderson *et al.*, 1990; Ghiran *et al.*, 2000; Greenberg *et al.*, 2006; Kobayashi *et al.*, 2007; Schaer *et al.*, 2006; Schiff *et al.*, 1997). Like neutrophils, macrophages contain granules brimming with destructive chemicals and lytic enzymes—lysosomes—that eradicate material engulfed into intracellular vesicles—phagosomes—through phagolysosomal fusion. Other membrane-bound and cytoplasmic PRRs activate signaling pathways that stimulate chemotaxis, which helps macrophages navigate to the source of stimulus, and the production of effector molecules that activate and reinforce inflammation. The latter is initiated by PRRs positioned throughout cellular compartments as sensors for PAMPs and include TLRs, NLRs, ALRs and RLHs (Toll-, NOD- and AIM-2-like receptors and RIG-I-like helicases, respectively) and the cGAS–STING (cyclic guanosine monophosphate–adenosine monophosphate synthase–stimulator of interferon genes) pathway (Bürckstümmer *et al.*, 2009; Chuang & Ulevitch, 2001; Chuang & Ulevitch, 2000; Inohara *et al.*, 2001; Rock *et al.*, 1998; Takeuchi *et al.*, 1999b; Wu *et al.*, 2013; Yoneyama *et al.*, 2004). These numerous receptors are both overlapping and complementary in that TLRs mostly encounter extracellular pathogens before or after phagocytosis, whereas NLRs, ALRs, RLHs and cGAS–STING mainly sense pathogens that have managed to infiltrate the cytosol, infecting the cell itself.

The responses to signaling PRR stimulation can, through oversimplification, be split in two broad categories: the antiviral and proinflammatory responses. Nucleic acids trigger TLR3, TLR7, TLR8, TLR9, ALRs, RLHs and cGAS–STING, which activate transcription factors of the IFN (interferon) regulatory family that upregulate the expression and secretion of cytokines called type I IFNs, IFNα and IFNβ. These are also called antiviral IFNs, as exposed nucleic acids—often with structures or modifications not encountered in mammals—in phagosomes or the cytosol are typically part of the viral lifecycle (Alexopoulou *et al.*, 2001; Bürckstümmer *et al.*, 2009; Heil *et al.*, 2004; Hemmi *et al.*, 2000; Sato *et al.*, 2000; Sun *et al.*, 2013; Yoneyama *et al.*, 2004). In target cells, type I IFNs, for example, inhibit protein synthesis to block viral dissemination (Metz & Esteban, 1972). Other pathogenic structures trigger TLR1:TLR2 and TLR2:TLR6 heterodimers, TLR4, TLR5 and NLRs, which activate the NF-κB signaling pathway that upregulates the expression and secretion of proinflammatory cytokines, including IL (interleukin)-1β, IL-6, IL-12, TNF (tumor necrosis factor) and the chemokine CXCL8, among many others (**Figure 1B**, *left*) (Hayashi *et al.*, 2001; Inohara *et al.*, 2001; Medzhitov *et al.*, 1997; Pol-

torak *et al.*, 1998; Takeuchi *et al.*, 2002; Takeuchi *et al.*, 2001). These are the macrophage's alarm bells that activate inflammation and mobilize the rest of the immune system. For example, IL-1β and IL-12 activate NK and T cells, TNF activates macrophages themselves as well as other immune cells but induces apoptosis in afflicted nonimmune cells and CXCL8 attracts neutrophils to enter the site of inflammation (Ben Aribia *et al.*, 1987; Carswell *et al.*, 1975; Chan *et al.*, 1991; Muñoz-Fernández *et al.*, 1992; Ostensen *et al.*, 1987; Scheurich *et al.*, 1987; Yoshimura *et al.*, 1987). Conventionally, immunostimulatory macrophages that have been activated by PRR stimulation and proinflammatory cytokines, especially IFNγ—secreted mostly by NK and T cells in response to macrophages' IL-12 and TNF in a positive paracrine feedback loop—and have antimicrobial effector functions are called "classically" activated (Dalton *et al.*, 1993; Nathan *et al.*, 1983). These classically activated macrophages stay, for the most part, at the site of inflammation to directly battle the infectious pathogen and recruit other immune cells to join in on the fight (Randolph *et al.*, 1999).

2.1.1.4 The Soft Side of Macrophages

When the source of inflammation is dealt with, the tissue is eventually cleared of substances that could keep triggering macrophage PRRs. The signaling pathways set off by PRR stimulation concomitantly activate negative autocrine feedback loops that, after a time, tone down the proinflammatory response. For example, both TLR stimulation and TNF upregulate the expression and secretion of anti-inflammatory cytokines such as IL-10, among others, hours to days after the immediate flurry of proinflammatory effector molecules (Huynh *et al.*, 2016). Thus, without continuous PRR stimulation, classically activated macrophages begin to revert back to their docile ground state. Then, without active inflammation, the recruitment of neutrophils and other immune cells diminishes and the built-in apoptotic program of neutrophils that entered the site of inflammation kicks in (**Figure 1B**, *right*). The abundance of apoptotic neutrophils, even in the presence of PRR stimulation, instructs macrophages to clear the tissue by efferocytosis—a quiescent form of apoptotic cell engulfment distinct from classical phagocytosis that does not trigger inflammation—and to upregulate the expression and secretion of anti-inflammatory cytokines, including IL-10 as well as TGFβ (transforming growth factor β) and VEGF-A (vascular endothelial growth factor A), in addition to many others (Byrne & Reen, 2002; Fadok *et al.*, 1998; Golpon *et al.*, 2004; Stern *et al.*, 1996). These immunosuppressive macrophages mediate resolution by, for example, stimulating parenchymal stem cells or tissue-resident progenitors to repair local damage, endothelial cells to reform blood vessels and fibroblasts to scar over what cannot be repaired (Boulter *et al.*, 2012; Heredia *et al.*, 2013; Lucas *et al.*, 2010; Shook *et al.*, 2018).

As a counterpart to classically activated macrophages, immunosuppressive or reparative macrophages are called "alternatively" activated (Gordon, 2003). The first reported stimulus for alternative activation was IL-4, which upregulates the expression of the phagocytic PRR CD206 (also known as macrophage mannose receptor) (Stein *et al.*, 1992). Subsequently, alternative activation states have been achieved through a number of means *in vitro* by, for example, addition of the anti-inflammatory cytokines IL-10 or IL-13, glucocorticoids or immune complexes (Anderson & Mosser, 2002; Doherty *et al.*, 1993; Liu *et al.*, 1999). This "bipolar" concept of macrophage activation was originally described in inbred mouse models and controlled experimental conditions where classical and alternative activation were thought to polarize macrophages to either of two opposing states, mirroring the contemporary paradigm of T-cell differentiation and named proinflammatory M1 and anti-inflammatory M2 macrophages, respectively (Mills *et al.*, 2000). Since then, not only has the universe of T-cell subsets expanded but macrophage plasticity and the reality of macrophage polarization in living organisms have also proven considerably more complex. Especially the arrival of single-cell techniques has truly begun to unravel the diverse activation states of macrophages *in vivo*, which do not seem to fit along a two-dimensional spectrum between two poles, as a single macrophage can even acquire features of both M1 and M2 polarization simultaneously (Muñoz-Rojas *et al.*, 2021). Moreover, the activation state of a macrophage is not fixed but changes over time through autocrine feedback loops and external stimulation. Presently, the terms M1 and M2 are still used as shorthand for describing whether a macrophage is presumed to be more immunostimulatory or -suppressive, respectively, on the basis of its gene signature or an array of cell-surface markers. Such assumptions are sometimes necessary, as it is often not feasible to obtain sufficient primary macrophages to perform functional assays even from experimental animals, let alone from tissue samples of human origin.

Unfortunately, macrophages' remarkable plasticity also renders them susceptible to manipulation that can lead to pathology. Persistent antigens that the immune system is not able to eradicate—present typically in, for example, viral hepatitis, tuberculosis and autoimmune diseases like rheumatoid arthritis—can lead to chronic inflammation, where the usually quite discrete phases of inflammation and its resolution mix. Unlike regular inflammation, which is characterized by the huge but transient infiltration of neutrophils, chronic inflammation is dominated by the buildup of mononuclear cells, that is, lymphocytes and, in particular, monocytes and macrophages (Hurst *et al.*, 2001; Marin *et al.*, 2001). The conflicting signals within a chronically inflamed microenvironment will instruct macrophages both to sustain inflammation, making them inflict more damage and continue the vicious cycle, and to mediate resolution, potentially causing overt fibrosis and eventual loss-of-function in the afflicted tissue. Tumors have long been described as such "wounds that won't

heal" whose chronically inflamed microenvironment disrupts normal macrophage activity, manipulating them to promote tumor development and cancer progression instead (Dvorak, 1986).

2.1.2 The Adaptive Immune System

Lymphocytes of the adaptive immune system make up the remaining 20–45 % of immune cells in the blood. The defining difference between the innate and adaptive immune systems originates from the receptors expressed by them: while the PRRs of innate immunity are hardwired in the germline and change only through evolution, the receptor repertoire of the adaptive immune system is so diverse that at least one receptor can be produced to recognize virtually any organic molecule in existence. Adaptive immunity can also be divided into three complementary layers:

- Humoral (or "soluble") immunity, mediated by B cells.
- Cellular immunity, mediated by T cells.
- Immunological memory, which protects the host from future encounters with the same antigen.

Adaptive immunity is slow to get started but powerful and extremely specific once fully activated. By conservative estimation, the adaptive immune system of humans can theoretically produce at least 10^{15} fully unique receptors (Davis & Bjorkman, 1988). This is quite the feat, considering that humans only have an estimated twenty-five thousand protein-coding genes altogether. Even though they are functionally unalike, the same fascinating mechanism of somatic V(D)J recombination underlies the great diversity of both BCRs and TCRs (B- and T-cell receptors, respectively). Shortly, the genetic regions that contain the instructions for producing BCRs and TCRs are inherited as nonfunctional gene segments from which a functional, protein-coding gene must be cut and pasted together (Brack *et al.*, 1978; Oettinger *et al.*, 1990). These gene segments—called V for variable, D for diversity and J for joining—are alternative pieces of coding sequences stringed one after the other, from which the variable regions of BCRs and TCRs, the parts that bind a certain shape or "epitope" on an antigen, are assembled, seemingly at random, by deleting the unchosen sequence in between. Thus, V(D)J recombination produces multitudes of isogenic B- and T-cell clones that each express their own unique gene for their own unique receptor (Landsteiner, 1945; Nossal & Lederberg, 1958).

2.1.2.1 Humoral Immunity

Humoral immunity is mediated by B lymphocytes—or simply B cells—that, once activated, turn into proliferating antibody factories referred to as plasmablasts. Naïve

B cells mature in the bone marrow and travel through secondary lymphoid organs looking for antigens compatible with their unique BCR, that is, their cognate antigens—an "antigen" being any substance against which antibodies can be generated. The BCR itself resembles a membrane-bound antibody and recognizes antigens in their native conformations, meaning that naïve B cells do not depend on any assistance for the first time they are activated or "primed" (Mizuguchi *et al.*, 1986). The BCR's engagement with its cognate antigen clusters multiple BCRs together on the B cell's surface in the first step of B-cell activation. When sufficiently triggered, the B cell begins to secrete the default class of antibody or Ig (immunoglobulin), IgM. Without additional stimulation, activated B cells produce a burst of antibody and then, for the most part, die by apoptosis. Typically, the full unleashing of humoral immunity requires two-factor authentication, wherein the activated B cell turns into an efficient APC itself and must receive costimulation from its cognate T cell, that is, a T cell that can recognize a TCR-compatible fragment of the same antigen that activated the B cell in the first place (Batista *et al.*, 2001; Lanzavecchia, 1985; Morris *et al.*, 1994; Nossal *et al.*, 1968; Smith *et al.*, 1996). Simply put, a single B cell can produce only so many molecules of antibody. Thus, help from the cognate T cell allows the activated B cell to survive and proliferate to copy itself, thus promoting the B cell's clonal expansion and exponentially increasing the production capacity of the reactive antibody (Alés-Martínez *et al.*, 1991; Tsubata *et al.*, 1993).

With T-cell help, B cells can also differentiate into nonproliferating antibody-secreting plasma cells that live longer than their plasmablast counterparts (Manz *et al.*, 1997). Concurrent with plasma cell development, B cells can undergo heavy-chain class switch recombination, during which the B cell may switch from IgM to one of the other available antibody classes: IgD, IgA, IgE or IgG (Ballieux *et al.*, 1964; Snapper & Paul, 1987). The functionally important antibody classes for the human immune system are IgM, IgA, IgE and IgG. Antibody class or isotype determines the antibody's properties, with each isotype uniquely suited for different purposes: IgM is very efficient at fixing the complement and neutralizing viral particles, IgA is a hardier class of antibody present on mucosal surfaces, IgE fights against parasites and IgG can be a tremendous complement fixer, opsonizer—decorating its targets to appear more delicious to phagocytes—and NK-cell activator (Abramson *et al.*, 1970; Atkinson & Frank, 1974; Bindon *et al.*, 1988; Capron *et al.*, 1984; Perussia *et al.*, 1984; Taylor & Dimmock, 1985; Williams & Gibbons, 1972). In humans, the IgG class is further split into four distinct isotypes—IgG_1, IgG_2, IgG_3 and IgG_4—based on the structure of the antibody's invariant Fc (fragment crystallizable) γ region, which determines their unique effector functions. The best opsonizers and complement fixers are actually IgG_1 and IgG_3, whereas the second-weakest and weakest at both are IgG_2 and IgG_4, respectively (Abramson *et al.*, 1970; Bindon *et al.*, 1988). Typically, humoral immune responses produce all four IgG isotypes.

Their relative abundances have been proposed to regulate the strength of the subsequent innate immune response, with IgG_1 and IgG_3 stimulating and IgG_2 and IgG_4 suppressing the antibody-mediated effector functions of macrophages, NK cells and the complement system (Collins & Jackson, 2013; Urbanek *et al.*, 1980). Moreover, T cells can help activated B cells differentiate into Fo (follicular) B cells that undergo affinity maturation into GC (germinal center) B cells, in which the previously assembled BCR gene of the activated B cells' daughter cells is reshuffled (Sablitzky *et al.*, 1985; Steiner & Eisen, 1967). Of the resulting new B-cell clones, those whose BCR's affinity for the cognate antigen is better than the original's are selected for repeated rounds of clonal expansion and affinity maturation to keep honing the perfect antibody (Allen *et al.*, 2007; Jacob *et al.*, 1991; Phan *et al.*, 2006).

Importantly, not all humoral immune responses require assistance from T cells. While T-cell help is indispensable to generate antibodies against some antigens—so-called TD (thymus-dependent) antigens (Miller & Mitchell, 1967)—B-cell priming itself does not require help in the first place, and B-cell activation actually upregulates the expression of PRRs, which can be directly triggered by pathogenic structures such as bacterial and viral DNA, recognized by TLR9, the presence of which was first sensed by the BCR (Bourke *et al.*, 2003). This can be stimulation enough to induce B-cell activation, proliferation and antibody secretion, as PRR stimulation rather confirms the presence of a pathogen even if T cells have not yet caught up to the fact. These are called TI-1 (type 1 thymus-independent) antigens that, at high concentrations, can induce polyclonal B-cell activation (Coutinho *et al.*, 1974). Furthermore, some antigens with highly repetitive features—so-called TI-2 antigens found in, for example, certain polysaccharides on bacterial surfaces—may cluster enough antigen-specific BCRs together to provide sufficient stimulation to bypass the requirement of T-cell help altogether (Lewis & Goodman, 1977). This is a nice trick, since T cells can only recognize antigens derived from proteins—thymus-independent B-cell activation therefore enormously increases the diversity of antigens against which humoral immunity may be directed to carbohydrates, fatty acids and nucleic acids (Bona *et al.*, 1978; Palinski *et al.*, 1990; Schur & Monroe, 1969). The B cells that prominently respond to TI-2 antigens are the "innate-like" B-cell subsets called B1 cells, located in membrane-enclosed peripheral spaces such as the pleural and peritoneal cavities, and MZ (marginal zone) B cells, located in the MZ between the red and white pulps of the spleen (Martin *et al.*, 2001). B1 and MZB cells constitutively secrete so-called "natural antibodies," that is, the baseline IgM and IgG that are always present in blood (Briles *et al.*, 1981). These natural antibodies are less diverse than those produced by B-cell clones emerging from GCs but quickly available and cross-reactive, meaning that they can recognize many ubiquitous antigens with, for example, carbohydrate structures (Alugupalli *et al.*, 2004; Haas *et al.*, 2005). Hence, B1 and MZB cells are thought of as innate-like first

responders of the humoral immune response: in peripheral body cavities, B1 cells are poised to meet breaching pathogens, whereas MZB cells come into contact with any pathogens borne by the blood, which is continuously sifted through the spleen.

2.1.2.2 Cellular Immunity

T cells, the other type of lymphocyte of the adaptive immune system, are behind cellular immunity. Unlike other immune cells that mature in the bone marrow, the final stages of T-cell maturation occur in the thymus, from where naïve T cells enter the blood (Cooper *et al.*, 1965). The TCR itself is a multimer assembled from the invariant CD3 protein complex and a heterodimer of two TCR chains that undergo somatic recombination (Dong *et al.*, 2019; Hedrick *et al.*, 1984; Yanagi *et al.*, 1984). In humans, approximately 95 % of T cells in the blood are αβ T cells, so called because their TCRs contain a heterodimer of the α and β chains. Most of the rest are γδ T cells, named by similar logic, and even rarer NK T cells (Bank *et al.*, 1986; Brenner *et al.*, 1986; Fowlkes *et al.*, 1987). Like NK cells, both of these "unconventional" T-cell populations are possibly remnants from the evolution of αβ T cells, although both may independently contribute to autoimmune diseases and antitumor immunity and therefore should not be brushed aside despite their very low frequencies (Gentles *et al.*, 2015; Tachibana *et al.*, 2005). αβ T cells are further divided into two main categories based on expression of the TCR coreceptors CD8 and CD4. $CD8^+$ cytotoxic or "killer" T cells—cytotoxic T lymphocytes or CTLs for short—directly kill afflicted cells, whereas $CD4^+$ "helper" T cells or T_H cells secrete cytokines that orchestrate the behavior of other cells and conduct the overall immune response (Zinkernagel & Doherty, 1974a; Zinkernagel & Doherty, 1974b). Unlike B cells that recognize native antigens of any substance, T cells mainly recognize protein antigens—and those only if they have been processed into peptides of specific length and are presented on MHC (major histocompatibility complex) molecules on the surfaces of other cells (Garboczi *et al.*, 1996; Stern *et al.*, 1994). The upside of this is that TCRs have access to epitopes that can normally be hidden by, for example, the folding or localization of the native protein. CTLs recognize antigens presented on class I and T_H cells antigens presented on class II MHC molecules. Their respective CD8 and CD4 coreceptors stabilize the MHC molecule:TCR interaction and potentiate TCR stimulation (Holler & Kranz, 2003; Li *et al.*, 2004). Even though the genes that code both classes of MHC molecules are hardwired in the germline like PRRs, they are highly polymorphic—with altogether tens of thousands of reported alleles in humans—which greatly expands the range of peptides that can be presented to T cells across the human population (Robinson *et al.*, 2020). Class I and II MHC molecules are unevenly distributed across different cells of the host and present

protein antigens derived from distinct sources, explaining the specialized effector functions of CTLs and T_H cells.

2.1.2.3 Antigen Presentation

Class I MHC molecules are expressed on virtually all nucleated cells of the host and advertise peptides derived, for the most part, from endogenous self-antigens of whatever proteins the cell is producing at a given time through the "canonical" cytosolic pathway. Proteasomes in the cytosol continuously degrade old, defective or otherwise redundant proteins into peptides, and the peptides can be further recycled into amino acids. However, some peptides get picked up by the TAP (transporters associated with antigen processing) complex, which hoists them back inside the lumen of the endoplasmic reticulum for loading onto class I MHC molecules (Anderson *et al.*, 1991; Lehnert *et al.*, 2016; van Kaer *et al.*, 1992). The stabilized peptide:class I MHC molecule complex is then transported to the cell surface for antigen presentation. Class I MHC presentation is particularly useful for alerting CTLs to the presence of intracellular pathogens that have overtaken the host cell's translational machinery, because bacterial and viral proteins can be swept up in the same cytosolic pathway (Brunt *et al.*, 1990). Complementary to the cytosolic pathway, specialized APCs—for example, macrophages—can load peptides onto class I MHC molecules also through the TAP-independent vacuolar pathway, in which engulfed exogenous sources of protein antigens are partially degraded and loaded onto class I MHC molecules within phagolysosomes, then transported back to the cell surface (Huang *et al.*, 1994; Pfeifer *et al.*, 1993; Sigal *et al.*, 1999; Townsend *et al.*, 1989). The presentation of exogenous antigens to CTLs is referred to as "cross-presentation." As a third pathway, APCs can even "cross-dress" in peptide-loaded class I MHC molecules taken up from surrounding cells and play hot potato with peptide:class I MHC complexes to pass them between each other (Dolan *et al.*, 2006; Qu *et al.*, 2009; Wakim & Bevan, 2011). In contrast, the expression of class II MHC molecules is much more restricted to specialized APCs, namely macrophages, DCs and B cells (Steimle *et al.*, 1994). Class II MHC molecules focus on presenting peptides derived from exogenous protein antigens that are taken in through phagocytosis (Ramachandra *et al.*, 1999). The class II MHC molecules themselves are synthesized in the endoplasmic reticulum, where they are packed into vesicles that cycle between the cell surface and endolysosomal subcompartments. During this journeying, the vesicles containing class II MHC fuse with phagolysosomes that contain partially degraded peptides, which can be loaded onto the class II MHC molecules and then transported to the cell surface (Castellino & Germain, 1995; Harding & Geuze, 1992; Roche *et al.*, 1993). The nature of the resulting T-cell response is largely dependent on how APCs interpret the agent whose peptides are being presented on class II

MHC molecules. Additionally, there is an exception to antigen presentation on class II MHC molecules, too: lysosomes generated through autophagy—that is, the enclosure of defective proteins and remnants of damaged cell organelles inside vesicles where they are degraded—contain peptides derived from self-antigens and can also fuse with endosomes packed with class II MHC molecules and, thus, deliver endogenous antigens to the cell surface for presentation to T_H cells (Dengjel *et al.*, 2005).

2.1.2.4 T-Cell Activation

To differentiate into fully functional effector T (T_{EFF}) cells, naïve T cells require two signals from APCs: priming of the TCR by its cognate antigen:MHC molecule complex, triggering of stimulatory coreceptors to initiate clonal expansion and, specifically in the case of T_H cells, a definite cocktail of cytokines to set the course of T_H-cell differentiation. This is another example of two-factor authentication before allowing the adaptive immune response to activate, because T cells can acquire both signals only from APCs that have recently been activated themselves. Costimulation in the absence of TCR stimulation has little effect, whereas TCR stimulation in the absence of costimulation induces apoptosis or anergy, that is, inactivation of the T cell, making it unresponsive to later TCR stimulation (Jenkins & Schwartz, 1987; Siefken *et al.*, 1997). Typically, the priming of naïve T cells occurs in secondary lymphoid organs, for example, lymph nodes or the spleen, where mature DCs migrate to present the antigens picked up in inflamed peripheral tissues. Mature DCs are additionally imprinted with information regarding the cause and site of inflammation, which they deconstruct into specific arrays of cell-surface molecules and cytokines that instruct activated T cells where to migrate (Mikhak *et al.*, 2013; Mora *et al.*, 2003; Sigmundsdóttir *et al.*, 2007). Generally, mature DCs also upregulate the expression of both class I and II MHC and costimulatory molecules such as the B7 family members CD80 and CD86, which are ligands of the stimulatory coreceptor CD28 on T cells (Azuma *et al.*, 1992; Hathcock *et al.*, 1993). Receiving both first and second signals instructs T cells to upregulate the expression and secretion of IL-2, which stimulates T cells to undergo clonal expansion (Appleman *et al.*, 2000). IL-2 also provides additional help for CTL activation, which DCs often are not able to fully activate on their own, unless the viral load is particularly high (Ridge *et al.*, 1998; Wu & Liu, 1994). The full activation of CTLs therefore typically requires even three-factor authentication: a mature, recently activated APC and the concomitant priming of T_H cells and CTLs by the same APC.

During subsequent cycles of clonal expansion, the proliferating T-cell clone rewires its transcriptional program and the expanded clone becomes T_{EFF} cells. The third signal instructs T_H cells to differentiate into one functionally distinct subset of T_{EFF} cell, the main subsets being T_H1, T_H2, T_H17 and regulatory T (T_{REG}) cells

(Harrington *et al.*, 2005; Hori *et al.*, 2003; Mosmann *et al.*, 1986). Although other subsets have been reported in the literature—such as follicular T_H cells (T_{FH} cells) responsible for helping FoB cells undergo clonal expansion and affinity maturation in secondary lymphoid tissues (Johnston *et al.*, 2009)—the direct contribution of these subsets for the orchestration of immune responses in peripheral tissues has been most comprehensively described. In the simplest conditions *in vitro*, T_H1 cells—as well as effector CTLs—differentiate in the presence of IL-12, T_H2 cells in the presence of IL-4, T_H17 cells in the presence of IL-6 and TGFβ and T_{REG} cells in the presence of TGFβ alone (Bettelli *et al.*, 2006; Curtsinger *et al.*, 2003; Hsieh *et al.*, 1993; Swain *et al.*, 1990; Veldhoen *et al.*, 2006). Compared to macrophage polarization, T_H-cell differentiation is believed to be much less plastic. The cytokines a T_H cell initially receives trigger positive feedback loops that, at first, inhibit the T_H cell from responding to cytokines specific for other subsets and, finally, establish a subset-specific gene signature through epigenetic reprogramming (Djuretic *et al.*, 2007; Zhou *et al.*, 2008). T-cell differentiation is not necessarily irreversible, however, and significant flexibility between subsets has been reported *in vivo* (Hegazy *et al.*, 2010; Lee *et al.*, 2009). Thus, differentiated T_{EFF} cells seem to some extent retain their ability to adapt to changes in the microenvironment.

Differentiated T_{EFF} cells leave the secondary lymphoid organs and head for the site of inflammation. Probably the most notable difference between naïve and T_{EFF} cells is that T_{EFF} cells no longer require costimulation but can be activated solely by TCR stimulation—although repeated engagement with costimulatory molecules and proinflammatory cytokines does extend their period of activity (Burmeister *et al.*, 2008; Croft *et al.*, 1994; Kedl & Mescher, 1998). This secondary stimulation can be provided by, for example, M1 macrophages at the site of inflammation (Hsieh *et al.*, 1993; Soudja *et al.*, 2012). TCR restimulation instructs effector CTLs to inject target cells with apoptosis-inducing lytic enzymes—for example, granzymes and perforin (Stinchcombe *et al.*, 2001)—and differentiated T_H cells to secrete subset-specific cocktails of cytokines that help and instruct other immune cells to combat different kinds of pathogens. For example, activated T_H1 cells secrete huge amounts of IFNγ, IL-2 and TNF, which help annihilate intracellular pathogens by supercharging macrophages' phagocytic capacity, instructing B cells to class switch to opsonizing IgG and boosting the effector functions of NK cells, CTLs and T_H1 cells themselves (Cherwinski *et al.*, 1987). T_H2 cells secrete IL-4 and IL-13, which help defend against parasites by instructing B cells to class switch to IgE, which activates eosinophils and mast cells, and the digestive tract to produce mucus, respectively (Cherwinski *et al.*, 1987). IL-4 and IL-13 are also canonical cytokines for the polarization of M2 macrophages. T_H17 cells secrete IL-17, which instructs epithelial cells to release CXCL8, the same chemokine macrophages secrete to summon neutrophils into tissues (Fossiez *et al.*, 1996). Thus, T_H17 cells help to fight extracellular patho-

gens such as fungi that CTLs and T_H1 cells are less effective against. T_{REG} cells, on the other hand, differentiate in the absence of proinflammatory cytokines and produce IL-10 and TGF-β—the same cytokines secreted by M2 macrophages—and suppress the activation of other immune cells (Rubtsov *et al.*, 2008).

2.1.2.5 Immunological Memory

Once the immune response has eradicated whatever substance activated it, the partaking immune cells must be efficiently decommissioned to avoid unnecessary collateral damage. As the activation of innate immune cells triggers feedback loops that eventually shut down these cells to limit their period of proinflammatory activity, mechanisms with similar outcomes exist in adaptive immune cells as well (Badovinac *et al.*, 2002). Most B-cell plasmablasts are short-lived and die on their own by apoptosis, while consecutive rounds of restimulation actually make T_{EFF} cells harder to activate, in part through the upregulation of inhibitory coreceptors such as PD-1 (programmed death receptor 1), CTLA4 (cytotoxic T lymphocyte antigen 4) and LAG3 (lymphocyte activation gene 3) (Agata *et al.*, 1996; Alegre *et al.*, 1996; Triebel *et al.*, 1990). These inhibitory coreceptors can bind specific inhibitory molecules or outcompete stimulatory coreceptors over binding to costimulatory molecules. The former is the case for PD-1, whose two ligands, PD-L1 and PD-L2, are expressed on APCs and stromal cells (Freeman *et al.*, 2000; Latchman *et al.*, 2001). CTLA4 and LAG3 work through the latter mechanism. CTLA4 binds the costimulatory molecules CD80 and CD86 with higher affinity than CD28 and transmits a strong inhibitory signal to the T cell, while LAG3 snatches class II MHC molecules away from CD4, thus reducing TCR stimulation (Linsley *et al.*, 1994; Maruhashi *et al.*, 2022; Waterhouse *et al.*, 1995). As a result, T-cell activation is suppressed. Moreover, with overwhelming stimulation, T_{EFF} cells become "exhausted," rendering them functionally anergic and prone to apoptosis (Fuller & Zajac, 2003). However, some effector B and T cells differentiate into subsets of memory cells that persist potentially for several decades, lying in wait in case the antigenic substance emerges again. Unlike the transient "memory" of trained macrophages, adaptive memory is passed down to subsequent generations of daughter cells (Graef *et al.*, 2014). Like effector cells, memory cells require only BCR or TCR stimulation to activate and are, therefore, much easier to deploy than naïve cells (Dubey *et al.*, 1996; Pihlgren *et al.*, 1996). Thus, T-cell activation actually produces at least three kinds of progeny: T_{EFF} cells, T_{CM} (central memory T) cells and T_{EM} (effector memory T) cells, which can be further divided into several different subsets (Sallusto *et al.*, 1999). Both major subsets of memory cells are produced by T_H cells and CTLs, although generation of CTL memory—as well as most B-cell memory—requires T_H-cell help (Janssen *et al.*, 2003; Wang *et al.*, 2017). T_{CM} cells stay in secondary lymphoid organs and have a

stem-cell-like ability to self-renew, while T_{EM} cells reside in tissues as a rapid reaction force against swifter re-encounters. Reactivation of T_{CM} cells induces them to revert back into combat-ready T_{EFF} cells that still seem to have their choice of subsets, while T_{EM} cells are more likely to respond as the subset to which they originally differentiated (Graef *et al.*, 2014; Sallusto *et al.*, 1999). The majority of B-cell memory, on the other hand, originates from GCB cells that emerge from T_{FH}-cell-assisted class switching and affinity maturation and produce self-renewing memory B cells that mostly reside in secondary lymphoid organs (Weisel *et al.*, 2016). Memory B cells maintain the pool of long-lived plasma cells that keep churning out high-affinity antibodies into the bloodstream (Ochsenbein *et al.*, 2000; Slifka *et al.*, 1995). It is these long-lived plasma cells thanks to which many reinfections can go completely unnoticed, since specific pathogens can be proactively neutralized by pre-existing antibodies.

2.2 Clever-1

The other star of this thesis, Clever-1—abbreviated from common lymphatic endothelial and vascular endothelial receptor 1—was originally identified as a cell adhesion molecule on lymphatic endothelial cells (Irjala *et al.*, 2003a). Around the same time, it was reported under the aliases Stabilin-1 and Feel-1 (Adachi & Tsujimoto, 2002; Politz *et al.*, 2002). Based on the sequence of its gene, designated *STAB1*, Clever-1 is structurally a multidomain type I transmembrane glycoprotein 270–300 kilodaltons in size, consisting of a long extracellular portion, a transmembrane helix and a short cytoplasmic tail (Adachi & Tsujimoto, 2002; Politz *et al.*, 2002; Tamura *et al.*, 2003). The extracellular portion of full-length Clever-1 contains four clusters of EGF (endothelial growth factor)-like domains interspersed with altogether seven fasciclin domains, two putative integrin-binding RGD motifs and one X-link domain near the transmembrane helix (Irjala *et al.*, 2003a; Kzhyshkowska *et al.*, 2006c; Politz *et al.*, 2002). Clusters of EGF-like and fasciclin domains reportedly mediate adhesive properties in selectins and fasciclins, respectively, whereas RGD motifs are canonical binding sites for integrins in extracellular matrix molecules such as fibronectin (Hynes, 1999; Kim *et al.*, 2000; Siegelman *et al.*, 1990). The intracellular tail's amino acid sequence has motifs that can directly recruit adaptor proteins—DDSLL and EDDADDD for GGA (Golgi-localized, γ-adaptin-ear-containing, ARF-binding) proteins and NPxF for sorting nexins, respectively—which regulate Clever-1's intracellular localization (Adachi & Tsujimoto, 2010; Kzhyshkowska *et al.*, 2004). Clever-1's gene sequence contains at least 69 exons, which can join into multiple predicted splice variants. Of the possible isoforms, at least two have been verified to be expressed at the protein level (Irjala *et al.*, 2003a; Kzhyshkowska *et al.*, 2006c). The primary structure of Clever-1 contains several cysteine residues

that putatively form disulfide bridges. Additionally, many of Clever-1's asparagine or serine and threonine residues can be posttranscriptionally modified by *N*- and *O*-linked glycosylation, respectively (Irjala *et al.*, 2003a). Functionally, Clever-1 is classified as a scavenger receptor and shares the type H scavenger receptor class only with its homolog, Stabilin-2, with which it has approximately 55 % sequence identity (Falkowski *et al.*, 2003; Harris *et al.*, 2004; Politz *et al.*, 2002). Additionally, the sequence identity between human and mouse Clever-1 is 86 %, which indicates evolutionary conservation of this receptor (Politz *et al.*, 2002).

2.2.1 The Expression & Function of Clever-1

Although Clever-1 protein expression has been reported in organs throughout the body, it appears to be quite restricted to specific types of cells: endothelial cells and innate immune cells of the monocyte–macrophage lineage. Endogenous Clever-1 protein expression was first reported on lymphatic endothelial cells in afferent and efferent lymphatic vessels and high endothelial venules in lymph nodes, but it is also expressed on vascular endothelial cells and sinusoidal endothelial cells in the adrenal cortex, bone marrow, liver, lymph nodes and spleen (Adachi & Tsujimoto, 2002; Goerdt *et al.*, 1991; Hansen *et al.*, 2005; Irjala *et al.*, 2003a; Martens *et al.*, 2006; Prevo *et al.*, 2004; Qian *et al.*, 2009; Salmi *et al.*, 2004). Additionally, Clever-1 is expressed on $CD14^+$ monocytes, M2 macrophages generated *in vitro* as well as TRMs in, for example, the brain, gut, placenta and skin—Kupffer cells in the liver being one Clever-1^- exception (Goerdt *et al.*, 1993; Martens *et al.*, 2006; Palani *et al.*, 2016; Palani *et al.*, 2011; Walsh *et al.*, 1991). The functional profile of Clever-1 suits the "sticky" nature of its protein domains. On endothelial cells, Clever-1 works as an adhesion molecule for immune cells traveling through lymph and blood vessels. For example, on the lumen of normal blood vessels, Clever-1 expression is upregulated in response to inflammation and is required for efficient extravasation of neutrophils and T cells through the vascular endothelium and into the site of inflammation (Irjala *et al.*, 2003a; Karikoski *et al.*, 2009; Patten & Shetty, 2019; Patten *et al.*, 2017; Salmi *et al.*, 2004; Shetty *et al.*, 2011). Similarly, Clever-1 is required on lymph vessels for efficient DC migration into lymph nodes and B-cell and CTL homing to the spleen (Tadayon *et al.*, 2021; Tadayon *et al.*, 2019). Clever-1 appears to control immune cell transmigration both directly by mediating the binding of immune cells to the endothelium and indirectly by regulating the secretion of specific chemokines. For example, Clever-1 knockdown upregulates CXCL13 to attract B cells to the spleen (Tadayon *et al.*, 2019). No immune cells besides monocytes and macrophages express Clever-1 themselves, however, and the counterpart of endothelial Clever-1 on immune cells remains undefined. Still, our group has recently shown that Clever-1 is able to bind B cells, CTLs and T_H cells directly and in a

respectively decreasing capacity, which is consistent with how much Clever-1 disruption affects the migration of these cell populations—suggesting that such a counterpart perhaps does exist (Tadayon *et al.*, 2019). On macrophages and sinusoidal endothelial cells in, for example, the liver, Clever-1 functions also as a phagocytic scavenger receptor that facilitates ground-state waste management. Consistent with the original definition of scavenger receptors, Clever-1 can bind and take in oxidized and acLDL (acetylated low-density lipoprotein) but, additionally, also many other non-self- and self-molecules, including both Gram-positive and -negative bacteria, phosphatidylserine presented on apoptotic cells, SPARC (secreted protein acidic and rich in cysteine), SI-CLP (Stabilin-1-interacting chitinase-like protein) and PL (placental lactogen) (Adachi & Tsujimoto, 2002; Kzhyshkowska *et al.*, 2008; Kzhyshkowska *et al.*, 2006a; Kzhyshkowska *et al.*, 2006b; Park *et al.*, 2009; Tamura *et al.*, 2003). After phagocytosis, Clever-1 directs the sorting of its cargo through distinct intracellular pathways. For example, Clever-1 delivers SPARC and PL swiftly into lysosomes for degradation, from where Clever-1 itself is returned to the cell surface in recycling endosomes (Kzhyshkowska *et al.*, 2008; Kzhyshkowska *et al.*, 2006b). Additionally, Clever-1 delivers newly-synthesized SI-CLP through the Golgi apparatus for packaging into secretory lysosomes (Kzhyshkowska *et al.*, 2006a). Clever-1's ability to regulate the fate of its cargo to either lysosomal degradation or the secretory pathway is particularly interesting in light of accumulating work suggesting that Clever-1 regulates the secretion of certain cytokines from macrophages.

2.2.2 Clever-1 as an Immunosuppressive Molecule

Clever-1 expression on MDMs is strongly correlated with the M2 activation state. On TRMs, Clever-1 participates in maintaining the ground-state immunosuppressive microenvironment when inflammation is not required and would likely be harmful (Goerdt *et al.*, 1993; Goerdt *et al.*, 1991; Irjala *et al.*, 2003a; Kzhyshkowska *et al.*, 2004; Palani *et al.*, 2016; Palani *et al.*, 2011; Politz *et al.*, 2002). For example, this association between Clever-1 and immunosuppression is very apparent in the placenta, where the host's immune system has to be prevented from attacking the developing fetus, which could be recognized as non-self. Normally, practically all macrophages in the placenta are Clever-1^{+}, but during the abnormal and unfortunate inflammation of the placenta known as pre-eclampsia, they rapidly lose Clever-1 expression (Palani *et al.*, 2011). This association suggests some role for Clever-1 in the regulation of immune responses—in fact, previous work in our group has shown that Clever-1 is actually required to restrain proinflammatory overreaction, as disrupting Clever-1 by either RNA or antibody-mediated interference upregulates the secretion of TNF as well as the macrophages' ability to activate and differentiate autologous

T_{EM} cells to the T_H1- but not the T_H2-cell lineage (Palani *et al.*, 2016). Clever-1 disruption has also been shown to upregulate the secretion of, for example, oncostatin M, CCL3 and CXCL13 (Palani *et al.*, 2011; Rantakari *et al.*, 2016; Tadayon *et al.*, 2019). Moreover, in longer-term culture *in vitro*, the expression of Clever-1 on differentiating macrophages can be maintained with M-CSF and increased with M2 polarization by, for example, the addition of IL-4 or glucocorticoids. Conversely, M1 polarization with IFNγ and TLR stimulation—bacterial LPS (lipopolysaccharide), a trigger for TLR4, being a very common stimulus used *in vitro*—rapidly downregulates the expression of Clever-1 (Palani *et al.*, 2016). Thus, Clever-1 is a common marker for immunosuppressive macrophages both *in vitro* and *in vivo*.

On the other hand, there are conditions in which overt suppression of the immune system becomes pathological. One such condition is cancer, which must gain the ability to suppress normal antitumor immune responses in order to survive, develop and progress. Clever-1 expression in cancer was first reported on the intratumoral lymphatic vessels of patients with breast or head and neck squamous cell carcinomas, where it seemed to support metastasis to the lymph nodes. However, multiple types of cancers also contain significant numbers of Clever-1^+ TAMs, and high frequencies of Clever-1^+ TAMs have been associated with negative outcomes for patients with cancers of the bladder, breast and oral cavity (Boström *et al.*, 2015; Irjala *et al.*, 2003b; Kwon *et al.*, 2019; Riabov *et al.*, 2016; Tervahartiala *et al.*, 2017; Timperi *et al.*, 2022; Wang *et al.*, 2020; Ålgars *et al.*, 2012). Cancer cells themselves have not been reported to express Clever-1—with the notable exception of some AMLs (acute myeloid leukemias) that originate from mutations in transitional progenitor cells that normally produce monocytes. Interestingly, these AMLs seem to, to some extent, require Clever-1 for proliferation and drug resistance (Lin *et al.*, 2019). The specific molecular mechanisms macrophage-expressed Clever-1 employs to regulate immune responses have, however, remained unclear. Assessing the contributions of different Clever-1^+ cell populations to effects observed on the systemic level has also been difficult, because the expression of Clever-1 is both broad across different organs and, in some cases, transient. The regulation of Clever-1 expression is not completely similar between human and mouse, either. Due to its complex and sticky structure, it is very possible that all ligands of Clever-1 have not yet been identified. Moreover, it remains to be discovered how direct a hand Clever-1 has in regulating cytokine and chemokine secretion in intracellular secretory pathways or whether these effects are secondary and result from transcriptional changes induced by Clever-1 interference. Nonetheless, it seems quite obvious that the functions of Clever-1 extend beyond the simple mediation of quiescent efferocytosis.

2.3 Cancer & the Immune System

Cancer encompasses an immense and heterogeneous group of diseases that arise when host cells' normal behavior becomes disturbed during the process of carcinogenesis. Cancer is, at its core, a genetic disease. Typically—in humans, at least—carcinogenesis can take even decades, during which time mutations gradually accumulate, at random, in host cells' genomes. This process can be expedited by cellular stress resulting from, for example, exposure to radiation, carcinogens or chronic inflammation, which decreases genetic stability. Eventually, some of these mutations will activate central proto-oncogenes and inactivate tumor suppressor genes, resulting in the stepwise demolishment of the regulatory mechanisms that enable the existence of multicellular life in the first place. Host cells are normally under very strict control with regards to their localization, metabolism and proliferation, and individual cells are very willing to "self-sacrifice," that is, to eliminate themselves by apoptosis to secure the host's survival if cellular equilibrium is disturbed by, for example, infection or mutation. Cells undergoing carcinogenesis become increasingly selfish and refocus their being only on propagating themselves, resulting, at first, in unrestrained proliferation and eventual formation of noncancerous, benign tumors or neoplasms. The final step of carcinogenesis happens when tumor cells undergo malignant transformation and the tumor becomes cancerous, that is, able to invade surrounding tissues and spread throughout the host to form secondary tumors or metastases. Importantly, tumors are not independent clusters of recklessly proliferating polyclonal mutants, but progress in direct contact with surrounding tissues as well as superficially normal immune and stromal cells that infiltrate the developing tumor, where they contribute to forming the TME. Tumors have long been described as chronically inflamed "wounds that won't heal" (Dvorak, 1986): even though the immune system contains several mechanisms of antitumor immunity, chronic inflammation itself promotes carcinogenesis. This contradiction can be explained by the three-tiered framework of cancer immunoediting (Dunn *et al.*, 2004).

2.3.1 Cancer Immunoediting

Although cancer is a genetic disease, cancer immunoediting highlights the immunological component that drives cancer development and progression. It is divided into three separate phases—elimination, equilibrium and escape—to conceptualize how tumor cells can evolve to dodge the otherwise highly efficient antitumor immune response. Antitumor immunity is first triggered when an individual cell begins to exhibit signs of abnormality but, for one reason or another, does not immediately sacrifice itself by apoptosis. During the elimination phase, the immune system picks out and kills these rare tumor cells from among billions of well-behaved siblings, usually so efficiently that nothing that could be observed by any available diagnostic

means has time to develop. Innate immunity has a crucial part in eliminating tumor cells, although no mechanism for specifically detecting tumor cells has, so far, been discovered. Rather, innate immune cells appear to sense metabolic disturbances in tumor cells that are similar to those generated by pathogens and are detected by the same set of PRRs. For example, nucleic acids released from tumor cells are essentially PAMPs and can be sensed by TLR7 and TLR9, ALRs, RLHs or cGAS–STING (Deng *et al.*, 2014). Moreover, cellular stress—consequent of genomic damage, in particular—upregulates the presentation of "eat me" and "kill me" molecules that can be detected by macrophages and NK cells, respectively. For example, calreticulin and HMGB1 (high-mobility group box protein 1) function as DAMPs on the cell surface and bind phagocytic PRRs on macrophages to promote the eating of tumor cells, whereas natural cytotoxicity molecules, such as MICA/B (MHC-class-I-chain-related protein A/B), bind natural cytotoxicity receptors, such as NKG2D, on NK cells to consequently promote the killing of tumor cells (Apetoh *et al.*, 2007; Bauer *et al.*, 1999; Chao *et al.*, 2010). Moreover, cellular stress often downregulates the presentation of class I MHC molecules on the cell surface, which, regardless of presented antigen, transmits a strong suppressive signal to NK cells through KIR (killer cell inhibitory receptor), expressed also by CTLs, to protect normal host cells—removing this suppressive signal further increases NK-cell activation (Wagtmann *et al.*, 1995). Activated NK cells directly dispatch tumor cells and secrete, for example, IFNγ to polarize M1 macrophages that, in turn, secrete proinflammatory cytokines, including IL-12, TNF and CXCL8, to trigger inflammation. M1 macrophages produce cytotoxic ROS and NO (reactive oxygen species and nitric oxide, respectively) as well as TRAIL (TNF-related apoptosis-inducing ligand) and FasL (Fas ligand), both produced also by NK cells, the binding of which to their respective receptors, TRAILR and Fas, on tumor cells—where they are also upregulated by stress—induces apoptosis (Cameron, 1986; Chen *et al.*, 2002; Ho *et al.*, 2011; Lorsbach *et al.*, 1993; Sugita *et al.*, 2002; Takeda *et al.*, 2001; Trauth *et al.*, 1989; Wallin *et al.*, 2003; Wiley *et al.*, 1995). Moreover, tumor cells can express various tumor-associated antigens—neoantigens resulting from mutations in protein-coding genes as well as mis- and overexpressed self-antigens (Chen *et al.*, 1997; Slamon *et al.*, 1987; Wölfel *et al.*, 1995)—that can be delivered to secondary lymphoid organs to prime B and T cells. Proinflammatory cytokines favor T_H1-cell differentiation and CTL activation, which contribute to tumor control in much the same way as to the killing of intracellular pathogens. Additionally, antitumor antibodies secreted by B cells can directly opsonize and fix complement on tumor cells. In the best-case scenario, the adaptive immune response will grant the host long-term immunological memory against previously encountered tumor clones attempting to resurface.

Efficient as it is, immunosurveillance is not always successful at eliminating every single tumor cell that has emerged. In fact, immunosurveillance itself applies

strong pressure on the heterogeneous population of tumor cells that has been produced by randomly accumulating mutations in a perfect example of Darwinian selection. Only the fittest survive, which in this case means clones that are the least immunogenic—or otherwise shielded from antitumor immunity—and often the most genetically unstable, which allows them to acquire additional mutations and adapt to ever-changing circumstances (Koebel *et al.*, 2007). These tumor cells enter the equilibrium phase, which can last for years and years. It is during this period of attrition that tumor cells gain the hallmarks of cancer that allow them to proliferate and spread with impunity. Finally, such tumor-cell clones emerge that wear down antitumor immunity, reach the escape phase and grow into a tumor or progress into a cancer that will eventually threaten the host (Angelova *et al.*, 2018). One representative mechanism of immune escape is the loss of antigen presentation on class I MHC molecules—typically observed in well over half of tumors in many different indications—without which CTLs cannot pinpoint which cells to exterminate. Although this should target the tumor cells for immediate killing by NK cells, tumor cells can, for example, concomitantly upregulate the expression of so-called "nonclassical" class I MHC molecules such as HLA-E, which does not present antigens to CTLs but inhibits CTL and NK-cell activation by binding to the inhibitory receptor NKG2A (Braud *et al.*, 1998; Meissner *et al.*, 2005; Zeestraten *et al.*, 2014). Other central proteins of the cytosolic pathway, such as members of the TAP complex, may also be deleted to prevent antigen presentation to CTLs while preserving, although decreasing, cell-surface class I MHC molecules (Johnsen *et al.*, 1999). Moreover, tumor-associated antigens can themselves become downregulated, modified to become less immunogenic or deleted entirely to remove these distinguishing features (Angelova *et al.*, 2018; Kmieciak *et al.*, 2007; Rosenthal *et al.*, 2019). Tumor cells also often overexpress the checkpoint molecule PD-L1 and the "don't eat me" molecule CD47 that inhibits phagocytosis through signal regulatory protein α (SIRPα) on macrophages (Dong *et al.*, 2002; Majeti *et al.*, 2009; Willingham *et al.*, 2012). Mutations that alter signaling through pleiotropic pathways such as PI3K (phosphoinositide 3-kinase), MAPK (mitogen-activated protein kinase) or Wnt can additionally grant tumor cells the ability to directly exclude T cells. For example, β-catenin stabilization, resulting in constitutive Wnt signaling, can block antitumor CTLs by rejecting specific cross-presenting DCs from tumors so that CTLs cannot be activated (Liu *et al.*, 2013; Peng *et al.*, 2016; Spranger *et al.*, 2015). Mutations that rewire, for example, the TNF and TGFβ signaling pathways can additionally make tumor cells insensitive to these cytokines' antitumor effects. Similarly, mutations in pathways that trigger type I IFNs and normally lead to antiviral responses, for example, cGAS–STING, can promote tumor-cell survival instead of blocking transcription and promoting apoptosis (Biswas *et al.*, 2004; Di Minin *et al.*, 2014; Hong *et al.*, 2022). Central for this thesis, however, there exist several additional mechanisms

of immune escape that rely not so much on tumor cells' intrinsic properties but rather on their incipient capacity to manipulate outwardly normal cells, including immune cells lured into the TME, to promote malignancy.

2.3.2 The Advent of Cancer Immunotherapy

As the immune response shapes the tumor, so the tumor shapes the immune response. Considering the chronic nature of cancer against the typically temporary nature of inflammation, anticancer immunity can be conceptualized as a cyclical process—the "cancer–immune cycle"—wherein a sequence of events must unfold to generate an effective antitumor immune response (Chen & Mellman, 2013). The cancer–immune cycle follows the same basic rules as the generation of any other type of immune response. Each full cycle begins with the release of tumor-associated antigens followed by antigen presentation, T-cell priming and infiltration into the TME and ends with the killing of tumor cells, which, in turn, can release more tumor-associated antigens to begin the next cycle. Thus, the response can become self-propagating—hopefully even self-amplifying—and eventually clear the tumor. Antitumor immune responses obviously fail to reach this positive endpoint in progressing cancers and rather appear to come to a standstill at some stage in the cycle. The stage where this cessation of antitumor activity occurs at depends on the overall balance between factors that activate and suppress antitumor immunity. These factors emerge not only from properties of the cancer but also from external agents that encompass everything from the patient's current medication, ongoing infections and composition of their microbiota to exposure to allergens, microparticles, sunlight and so forth (Lee *et al.*, 2022). Put together, these factors produce an individual's "cancer–immune setpoint" that describes what obstacles must be overcome in order to restart the cancer–immune cycle (Chen & Mellman, 2017).

Conventional pharmacological cancer therapies take advantage of tumor cells' intrinsic properties and typically altogether block cell division or inhibit signal transduction through pathways rendered constitutively active by mutations, such as the PI3K and MAPK pathways. Although they remain effective first-line therapies for many cancers, the targets of these drugs are often not remotely specific to tumor cells and, therefore, the off-target effects of these treatments can be extremely taxing on the patient, as is well known for chemotherapy. Given how fundamentally intertwined cancer becomes with immunity during its development and progression, it is not surprising that the therapeutic potential of reactivating the host's antitumor immune response was acknowledged already well over a century ago (Coley, 1893). Subsequent research in the burgeoning field of cancer immunology finally culminated in the previous decade in the form of immunotherapies that have since revolutionized the treatment of cancer. These are the famous antibodies against the check-

point molecules CTLA4, PD-1 and PD-L1 that block the transduction of inhibitory signals and are called immune checkpoint inhibitors or checkpoint blockers. The first antibodies against these targets to receive FDA (the United States Food and Drugs Administration) approval were, respectively, ipilimumab in 2011 and pembrolizumab in 2014 for treating advanced melanoma and atezolizumab in 2016 for treating urothelial carcinoma (Hodi *et al.*, 2010; Robert *et al.*, 2014; Rosenberg *et al.*, 2016). Many other checkpoint blockers have since joined the fray, for example, nivolumab against PD-1 and durvalumab against PD-L1 (Antonia *et al.*, 2017; Larkin *et al.*, 2015). Although immune-related adverse events are a rather common toxicity of checkpoint blockade, checkpoint blockers have often been at least as effective as chemotherapy with the added benefit of still being, in general, much gentler on patients in comparison. Since the initial approvals, the use of checkpoint blockers has broadened to other cancer indications, and, recently, pembrolizumab was actually the first drug to receive FDA approval for refractory cancers with high tumor mutational burden without a more strictly specified indication (Marcus *et al.*, 2021).

The primary targets of these "first-generation" checkpoint blockers were not tumor cells at all—their objective was to reactivate a patient's pre-existing antitumor T-cell response, which was observed to be superficially active in some patients yet still failed to control tumor growth. It was, therefore, hypothesized that checkpoint engagement locally in the TME froze these T cells in place and kept them from killing tumor cells (Harlin *et al.*, 2006). As inhibitors of inhibitors, checkpoint blockers disrupt the interaction between inhibitory coreceptors and coinhibitory molecules to, in a manner of speaking, disengage the handbrake and allow the antitumor immune response to proceed (Leach *et al.*, 1996). Ultimately, checkpoint blockade has proven that checkpoint engagement is indeed one central mechanism of immune escape in cancer, and checkpoint blockers continue to be remarkably effective in clinical use and yield durable, even indefinite positive responses as monotherapies across a broad range of malignancies. Unfortunately, these positive responses manifest only in the minority of patients, with the best response rates to monotherapy observed, so far, with the PD-1 blocker nivolumab in advanced melanoma, lasting even after 6.5 years in approximately 40 % of patients (Ascierto *et al.*, 2020; Reck *et al.*, 2021; van der Heijden *et al.*, 2021; Wolchok *et al.*, 2021). While combinations of checkpoint blockers—ipilimumab and nivolumab in particular—increase the treatment's efficacy, immune-related adverse events also become commonplace (Wolchok *et al.*, 2021). Thus, it quickly became apparent that blocking these few checkpoint molecules cannot possibly overcome all the means of immunosuppression in cancer's toolbox. Moreover, targeting novel checkpoints presented the possibility of more delicate immunomodulation that could bypass the first checkpoint blockers' common toxicities. Several blockers against other discovered T-cell checkpoints, for example, TIGIT, TIM3 and VISTA (T-cell immunoreceptor with immunoglobulin and

immunoreceptor tyrosine-based inhibitory motif domains, T-cell immunoglobulin domain and mucin domain 3 and V-domain immunoglobulin suppressor of T-cell activation, respectively) have since advanced to clinical trials (for example, NCT04294810, NCT04266301 and NCT02812875, respectively), and relatlimab, an antibody against the LAG3 checkpoint molecule, was recently approved by the FDA for treating advanced melanoma as an enhancer of PD-1 blockade (Tawbi *et al.*, 2022).

2.3.3 Attempts to Circumvent the Limitations of First-Generation Immunotherapies

The effort to understand why only a subset of patients responds to checkpoint blockers has led to the identification of two archetypal immune profiles of cancer. Inflamed or "hot" tumors contain significant amounts of intratumoral T cells in close proximity to tumor cells, whereas noninflamed or "cold" tumors are either immune deserts without an antitumor T-cell response or immune-excluded with peritumoral T-cell infiltrates that are separated from tumor cells (Herbst *et al.*, 2014; Taube *et al.*, 2012). As one would assume, hot tumors are the ones that respond to checkpoint blockade most often, though not always. These tumors resemble a setpoint where an initially productive antitumor T-cell response was halted just before the killing of tumor cells by immunosuppression in the TME. Therefore, the addition of checkpoint blockers can unfreeze these T cells to continue from where they left off. One significant parameter associated with hot tumors is mutational burden, which, theoretically, should be proportional to the amount of available tumor-specific antigens and, consequently, to the fraction of conceivably tumor-reactive T cells, as was the rationale behind the FDA's recent decision regarding pembrolizumab (Marcus *et al.*, 2021; Yarchoan *et al.*, 2017). Often overrepresented among hot tumors are melanomas, non-small-cell lung cancer and cancers of the bladder, colon, head and neck, kidney and liver (Herbst *et al.*, 2014; Yarchoan *et al.*, 2017). Cold tumors, on the other hand, rarely respond to checkpoint blockade at all. Immune deserts do not contain pre-existing antitumor T cells, whereas the antitumor T cells in immune-excluded tumors can still be steered away from tumor cells even if checkpoint blockade reactivates them (Herbst *et al.*, 2014; Salmon *et al.*, 2012). These cold tumors resemble setpoints where antitumor T-cell priming and infiltration are suppressed, respectively. Typically, breast, ovarian, prostatic and pancreatic cancers and glioblastomas are overrepresented among cold tumors (Herbst *et al.*, 2014; Yarchoan *et al.*, 2017).

Although the idea of being able to designate every tumor to one of two categories is enticingly simplistic, the compositions of individual tumors within these groupings are, in reality, extremely varied. Extensive immunogenomic analyses of all

tumor samples in the Cancer Genome Atlas concluded there exist six broad immune subtypes across the thirty-three cancers analyzed, implying that hot and cold tumors can emerge through several distinct mechanisms (Thorsson *et al.*, 2018). Accruing real-world data from the use of checkpoint blockers outside of clinical trials and the development of computational methods will likely continue to improve clinicians' ability to predict an individual patient's response to a particular course of treatment based on their unique cancer–immune setpoint and tumor mutational profile (Liu *et al.*, 2022). Still, regardless of classification method, nearly all tumors contain some infiltrating immune cells, including innate immune cells, and usually exhibit signs of chronic inflammation, for example, the accumulation of monocytes and macrophages. Although the massive success of checkpoint blockade shone the spotlight of cancer immunology on adaptive T cells, it is obvious that T-cell responses do not arise unaided in cancer, either. As with other immune responses, the generation and maintenance of efficient antitumor T-cell responses and the generation of long-lasting memory cells both depend on co-operation with the innate immune system. In particular, innate immune cells are indispensable for efficient antigen presentation as well as T-cell priming and infiltration into the TME (Broz *et al.*, 2014; Spranger *et al.*, 2017)—in addition to having their own effector functions that can result in the killing of tumor cells even without T-cell help, as occurs during the elimination phase of cancer immunoediting.

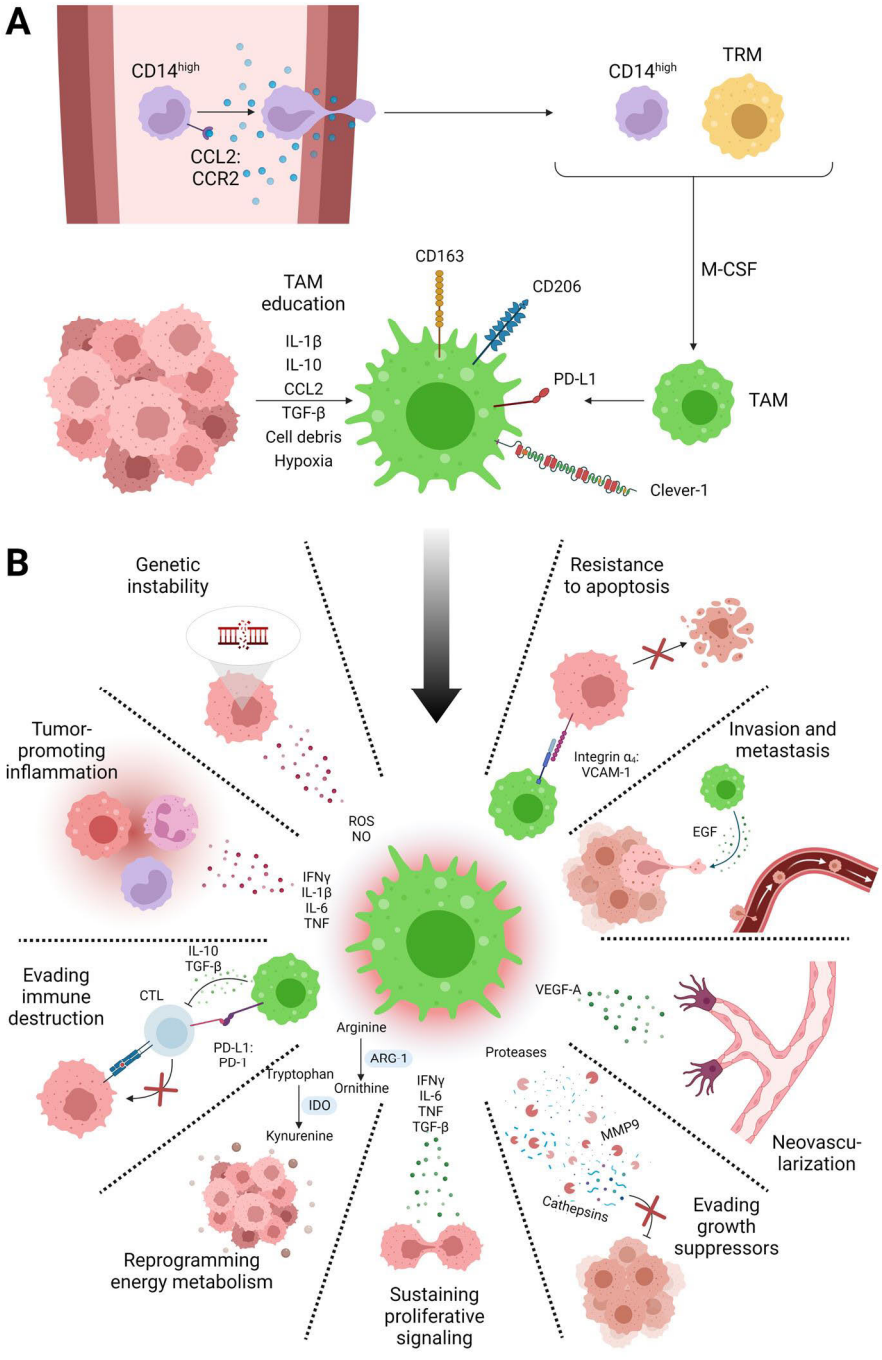
Since both defective T-cell priming and infiltration are features of cold tumors, great interest has risen in the possibility of targeting the innate immune system to restart the cancer–immune cycle or bump the setpoint in order to convert cold tumors hot and susceptible to combinatorial checkpoint blockade. Several approaches have advanced to clinical trials and include, for example, PRR agonists, recombinant growth factors and so-called "broad-spectrum" checkpoint blockers. Small molecules that trigger TLR9 or cGAS–STING to induce DC maturation have indeed shown promise when combined with PD-1 blockade (Cohen *et al.*, 2022; Harrington *et al.*, 2018). However, these synthetic PRR agonists are not tumor-specific and, therefore, their favored route of administration is direct injection into the tumor in order to limit systemic toxicity. Likewise, growth factors such as FLT3L (Fms-like tyrosine kinase 3 ligand) can be injected intratumorally to induce the differentiation of a specific DC population that excels at CTL priming (Hammerich *et al.*, 2019). These vaccine-like treatments—"like" because the injection contains only adjuvant without antigens, which are provided by the tumor itself *in situ*—are being evaluated in phase I and II trials, typically as adjuvants of PD-1 blockade (for example, NCT01042379, NCT04220866 and NCT03789097, respectively). Broad-spectrum checkpoint blockers, on the other hand, target inhibitory receptors with broader expression patterns across innate and adaptive immune cells. For example, monalizumab blocks NK2GA, expressed by both CTLs and NK cells, to re-enable CTL and

NK-cell killing (André *et al.*, 2018). Monalizumab has already advanced to two phase III trials on patients with head and neck squamous cell carcinoma in combination with EGF receptor blockade and on patients with non-small-cell lung cancer in combination with PD-L1 blockade (NCT04590963 and NCT05221840, respectively). As a point of critique, vaccine-like approaches are encumbered by their method of administration, and some tumors are simply not accessible for injection. Also, the aim of many of these treatments is to repopulate the tumor with immune cells that are excluded at the presenting setpoint, as can be the case for specific DC subsets and NK cells. Alternative treatment strategies that target innate immune cells already present in tumors are also under intense research and development—the innate immune cell most frequently observed and most numerous across the majority of tumors being the macrophage.

2.3.4 Tumor-Associated Macrophages

TAMs have emerged as dominating immune cells in the majority of tumors and the central drivers of tumor-promoting inflammation. In fact, multiple meta-analyses have correlated the simple high density of TAMs to negative patient outcomes, although an arguably more significant parameter is their activation state (Cheng *et al.*, 2021; Thorsson *et al.*, 2018; Zhang *et al.*, 2012). It is here that the remarkable plasticity of macrophages is turned against itself: even though the initial antitumor immune response can induce the polarization of antitumor M1 macrophages and while extremely dissimilar TAMs can coexist in the same tumor, the developing TME often skews immunosuppressive on the whole. One reason for this is that inflammation is programmed to be transient and prepares for its own shutdown even as it is turned on. Additionally, tumors often become able to selectively recruit immune cells that are beneficial for them, for example, by overexpressing CCL2 to bait large numbers of monocytes (Ueno *et al.*, 2000). Once recruited, tumor-derived M-CSF supports the differentiation of MDMs—and, possibly, also the proliferation and differentiation of TRMs—into TAMs (**Figure 2A**) (Lin *et al.*, 2001). Tumors also secrete their own concoctions of pro- and anti-inflammatory cytokines and other molecules that can "educate"—or corrupt—TAMs towards a tumor-promoting, M2-like activation state (Hagemann *et al.*, 2006). Moreover, in rapidly growing tumors, the TME often contains debris from damaged and dead cells and poorly oxygenated volumes of shoddy vascularization. These necrotic and hypoxic areas are enriched with DAMPs, such as the "eat me" molecule HMGB1, which attracts macrophages through the phagocytic PRR RAGE (receptor for advanced glycation end products), and also promote M2-like polarization through HIF (hypoxia-inducible factor) transcription factor stabilization, which upregulates a proangiogenic gene signature (Huber *et al.*,

Figure 2. Tumor-associated macrophages promote nearly every hallmark of cancer. A. Tumor typically upregulate the secretion of CCL2, which attracts $CD14^{high}$ $CCR2^{high}$ monocytes to extravasate into the TME (tumor microenvironment). Within the TME, tumor-derived M-CSF (macrophage-colony-stimulating factor) promotes the differentiation of $CD14^{high}$ monocytes, and possibly of tissue-resident macrophages (TRMs), into TAMs (tumor-associated macrophages). Most TAMs resemble M2 macrophages and express many the same surface molecules, for example, the co-inhibitory molecule PD-L1 (programmed death receptor ligand 1) and the scavenger receptors CD206, CD163—and Clever-1. TAMs are educated to acquire their M2-like activation state by tumor-derived factors, including cytokines such as IL (interleukin)-1β and IL-12, chemokines such as CCL2, growth factors such as TGFβ (transforming growth factor β) and debris from dead and dying cells as well as hypoxia in the TME. **B.** Out of ten hallmarks of cancer, TAMs have been shown to directly promote nine. TAMs promote resistance to apoptosis by providing survival signals to nonadherent tumor cells through, for example, the binding of integrin α_4 on TAMs to VCAM-1 (vascular cell adhesion molecule 1) on tumor cells. TAMs promote invasion and metastasis by secreting various growth factors, including EGF (endothelial growth factor), which induces tumor cells to mobilize. Moreover, TAMs promote neovascularization by secreting VEGF-A (vascular endothelial growth factor A). TAMs help tumor cells to evade growth suppressors by secreting proteases such as cathepsins and MMP9 (matrix metalloprotease 9), which degrade inhibitory molecules embedded within the extracellular matrix. TAM-derived cytokines and growth factors, including IFN (interferon) γ, IL-6, TNF (tumor necrosis factor) and TGFβ, sustain proliferative signaling in tumor cells. TAMs force tumor cells to reprogram their energy metabolism by depleting the TME of specific compounds by, for example, metabolizing arginine into ornithine through ARG1 (arginase 1) and tryptophan into kynurenine through IDO (indoleamine 2,3-dioxygenase). The cytokines and coinhibitory molecules expressed by TAMs, for example, IL-10, TGFβ and PD-L1, suppress effector T cells, including CTLs (cytotoxic T lymphocytes), and help tumor cells to evade immune destruction. The cytokines secreted by TAMs, including IFNγ, IL-1β, IL-6 and TNF, also sustain tumor-promoting inflammation, while TAM-derived ROS (reactive oxygen species) and NO (nitric oxide) promote the genetic instability of tumor cells. Created with BioRender.com.


A
CD14high
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TRM
M-CSF
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IL-1β
IL-10
CCL2
TGF-β
Cell debris
Hypoxia
CD163
CD206
PD-L1
TAM
Clever-1
B
Genetic
instability
Resistance
to apoptosis
Tumor-
promoting
inflammation
Invasion and
metastasis
Integrin α4:
VCAM-1
ROS
NO
EGF
IFNγ
IL-1β
IL-6
TNF
IL-10
TGF-β
Evading
immune
destruction
CTL
PD-L1:
PD-1
Arginine
ARG-1
Tryptophan
Ornithine
IDO
Kynurenine
VEGF-A
Proteases
MMP9
Cathepsins
Neovascu-
larization
Evading
growth
suppressors
IFNγ
IL-6
TNF
TGF-β
Reprogramming
energy metabolism
Sustaining
proliferative
signaling

2016; Laoui *et al.*, 2014; Vaupel *et al.*, 1991). These M2-like TAMs are usually so called because they express many of the same marker genes as M2 macrophages *in vitro*, including CD163, CD206, MARCO, PD-L1—and Clever-1 (Bergamaschi *et al.*, 2008; Hu *et al.*, 2015; Komohara *et al.*, 2014; Kuang *et al.*, 2009; Schledzewski *et al.*, 2006).

2.3.4.1 TAMs Cultivate the TME to Support the Hallmarks of Cancer

Reciprocally, corrupted TAMs directly assist tumor cells in acquiring all but one hallmark of cancer (**Figure 2B**). Importantly, macrophages' complicity with cancer does not end at tumor development and progression: it has been demonstrated that TAMs can also endow cancers with therapeutic resistance against both conventional therapies and checkpoint blockade. Of the ten hallmarks of cancer, only replicative immortality—that is, tumor cells' characteristic ability to stabilize their telomeres over boundless cell divisions in a manner similar to stem cells—is difficult to directly attribute to any external factor (Hanahan & Coussens, 2012). While it is conceptually obvious how M2-like TAMs, similar to anti-inflammatory M2 macrophages brought on by resolution as they are, would benefit cancer development and progression, both M1 and M2-like TAMs can, in fact, be tumor-promoting. Notably, M1-like TAMs can sustain "smoldering" pockets of tumor-promoting inflammation by producing proinflammatory cytokines, ROS and NO, which can also decrease the genetic stability of tumor cells by directly damaging the genome (Gasche *et al.*, 2001; Greten *et al.*, 2004; Heinecke *et al.*, 2014; Zhuang *et al.*, 1998). Meanwhile, M2-like TAMs can create the overall immunosuppressive TME that helps tumor cells evade immune destruction through efferocytosis—effectively sweeping tumor-associated antigens "under the rug," that is, into phagolysosomes for degradation—as well as the secretion of IL-10 and TGFβ and expression of PD-L1, among other molecules, which suppress DC maturation and NK-cell and CTL activation (Cook *et al.*, 2013; Kuang *et al.*, 2009; Peng *et al.*, 2017; Ruffell *et al.*, 2014; Thomas & Massagué, 2005). These mechanisms can also render checkpoint blockade ineffective both directly by suppressing infiltrating antitumor CTLs and indirectly by fostering a cold TME (Arlauckas *et al.*, 2017; Peranzoni *et al.*, 2018). Moreover, TAMs express multiple enzymes that produce immunomodulatory metabolites. For example, the fatty acid metabolite PGE_2 (prostaglandin E_2), produced downstream of COX2 (cyclo-oxygenase 2), upregulates the secretion of CXCL12, which attracts monocytes and so-called M-MDSCs (monocytic myeloid-derived suppressor cells) through its receptor, CXCR4, to enter the tumor (Le *et al.*, 2016; Obermajer *et al.*, 2011). M-MDSCs are heterogeneous, strongly immunosuppressive cells that resemble immature monocytes, whose numbers increase during inflammation and cancer (Movahedi *et al.*, 2008; Youn *et al.*, 2008). Notably, CXCR4 is expressed also on granulocytes and

PMN (polymorphonuclear)-MDSCs, granulocytes' equivalent to M-MDSCs (Seubert *et al.*, 2015). TAMs and M-MDSCs also express the enzymes NOS2 (nitric oxide synthase 2), ARG1 (arginase 1) and IDO (indoleamine 2,3-dioxygenase). Downstream, NOS2 produces NO while ARG1 produces ornithine, which promotes tissue regeneration, and IDO produces kynurenine, which promotes M2 polarization and T_{REG}-cell differentiation (Chang *et al.*, 2001; Kwak *et al.*, 2020; Munn *et al.*, 2005; Nagaraj *et al.*, 2007; Yu *et al.*, 2013). Concomitantly, TAMs and MDSCs drain the TME of specific nutrients—for example, NOS2 and ARG1 deplete arginine, IDO depletes tryptophan and the cystine–glutamate antiporter depletes cystine and cysteine from the extracellular space—which can both inhibit T_{EFF}-cell proliferation and force tumor cells to reprogram their energy metabolism to maximize growth in an increasingly unfavorable microenvironment (Munn *et al.*, 2005; Rodriguez *et al.*, 2004; Srivastava *et al.*, 2010).

2.3.4.2 TAMs Foster Tumor Growth & Vascularization

Although both pro- and anti-inflammatory cytokines such as TNF and TGFβ, respectively, promote apoptosis and thus inhibit the proliferation of epithelial cells in particular, many tumors repurpose the immune system's own set of tools and appropriate these cytokines as growth factors instead to sustain proliferative signaling. Especially TGFβ inhibits the proliferation of normal endothelium, but elevated TGFβ in tumors is actually correlated with more advanced disease and negative outcomes (Li *et al.*, 2019). Tumor cells usually become insensitive to TGFβ, thus emphasizing its other functions—including immunosuppression (Mariathasan *et al.*, 2018). Tumor cells can similarly co-opt proinflammatory cytokines, for example, IFNγ and IL-6 (Bent *et al.*, 2021; Shin *et al.*, 2017). Additionally, many growth factors and growth suppressors are embedded within the extracellular matrix, from where proteases secreted by TAMs can either free them to sustain proliferative signaling or digest them to help tumor cells evade growth suppressors. For example, MMP9 (matrix metalloprotease 9), itself upregulated by TGFβ, can process latent TGFβ to its bioactive form, whereas cathepsin S cuts up antiangiogenic peptides released from degraded collagen (Gocheva *et al.*, 2010; Wang *et al.*, 2006; Yu & Stamenkovic, 2000; Zhou & Qi, 2015). TAM-derived cathepsins can also promote resistance against chemotherapy, possibly through the upregulation of TNF secretion (Shree *et al.*, 2011). TAMs also respond to hypoxia by increasing the production of VEGF-A, either by secreting it directly, processing latent VEGF-A through MMP9 or upregulating VEGF-A secretion from endothelial cells (Barbera-Guillem *et al.*, 2002; Carmi *et al.*, 2013; Giraudo *et al.*, 2004). Subsequently, active VEGF-A coerces endothelial cells to proliferate and assemble into blood vessels (Lin *et al.*, 2006). Proteolytic remodeling of the TME thus also promotes neovascularization, which is essential for

tumor progression, because poor nutrient delivery, hypoxia and accumulating waste quickly limit proliferation. Moreover, the buildup of specific metabolites, such as lactic acid, a byproduct from the glycolysis of glucose, promotes T_{REG}-cell differentiation and M2-like TAM polarization (Colegio *et al.*, 2014; Watson *et al.*, 2021). A subset of perivascular $TIE2^{+}$ (tyrosine kinase with Ig and EGF homology domains 2) TAMs, in particular, has been associated with neovascularization (De Palma *et al.*, 2005). However, TAMs appear to be bad foremen, as the newly constructed vessels are usually of poor quality and rather deteriorate oxygenation and waste disposal, thus creating a positive feedback loop between TAMs and neovascularization (Wenes *et al.*, 2016). The abnormal tumor vasculature additionally limits the efficacy of therapies, because poor perfusion and leaky vessels can obstruct the distribution of drug molecules into the tumor parenchyma (Tannock *et al.*, 2002).

2.3.4.3 TAMs Promote Cancer Metastasis

TGFβ can also promote epithelial–mesenchymal transition—the switch from stationary to mobile—in tumor cells (Giampieri *et al.*, 2009). Surface molecules on TAMs can provide these detached tumor cells the support they normally receive from neighboring cells and extracellular matrix and, thus, enable them to resist apoptosis. For example, the binding of integrin α_4 on TAMs to VCAM-1 (vascular cell adhesion molecule 1) on tumor cells promotes tumor-cell survival (Chen *et al.*, 2011). TAMs also secrete many other growth factors, including EGF, in response to M-CSF provided by tumor cells (Wyckoff *et al.*, 2004). Several cancers overexpress EGF receptors, the famous one being HER2, whose overexpression in a subset of breast cancers is heavily correlated with increased recurrence and reduced survival (Press *et al.*, 1993; Seshadri *et al.*, 1993; Slamon *et al.*, 1987). EGF promotes not only proliferation but also cell migration. This results in a waltz between tumor cells producing M-CSF and TAMs producing EGF that promotes tumor-cell invasion through surrounding tissues along tracks of fibrous extracellular matrix deposited by TAMs (Afik *et al.*, 2016; Goswami *et al.*, 2005). Moreover, the proteases secreted by TAMs can digest the basal lamina around blood vessels, thus exposing the endothelial cells to tumor cells (Gocheva *et al.*, 2010; Vasiljeva *et al.*, 2006). The leaky vascular wall is also easier to pass through, which makes it possible for tumor cells to seed the blood and metastasize to other organs. Additionally to regulating neovascularization, the cytokines, enzymes and growth factors secreted by TAMs oversee the construction of new lymphatic vessels inside the tumor that, like the tumor vasculature, can be similarly dysfunctional and leaky (Jeon *et al.*, 2008; Schoppmann *et al.*, 2002). Cancers with highly metastatic tumors, such as breast cancer and melanoma, usually have abundant lymphatic vasculature, which facilitates the migration of tumor cells into sentinel lymph nodes. For example, in breast cancer, the axillary lymph nodes

located in the armpits are typically tumor cells' first stop before spreading further (Schoppmann *et al.*, 2006). Moreover, tumor-derived factors can travel through the blood to manipulate macrophages even in distant organs and instruct them to prepare a warm welcome for metastasizing tumor cells. Such factors include, for example, TNF, TGFβ, MMP9 and VEGF-A (Hiratsuka *et al.*, 2002; Hiratsuka *et al.*, 2006; Kaplan *et al.*, 2005). These soluble intermediaries can, for example, instruct macrophages in distant tissues to secrete CXCL12, which recruits more monocytes or M-MDSCs, and produce MMP9, which modifies the local tissue microenvironment to form a premetastatic niche around which the endothelium permeabilizes and upregulates adhesion molecules such as VCAM-1 that circulating tumor cells can grab hold of to ease intravasation (Chen *et al.*, 2011; Kaplan *et al.*, 2005).

2.3.4.4 TAM Heterogeneity Transcends the M1–M2 Polarization Model

The arrival of single-cell techniques has recently begun to unravel the true complexity of macrophage activation *in vivo*, especially that of TAMs in numerous human tumors (Azizi *et al.*, 2018; Chan *et al.*, 2021; Cheng *et al.*, 2021; Mujal *et al.*, 2022; Pelka *et al.*, 2021; Zhang *et al.*, 2019; Zilionis *et al.*, 2019). For example, scRNA-seq of tumor samples has revealed that single TAMs can express genes indicative of both M1 and M2 polarization simultaneously *in vivo* (Cheng *et al.*, 2021; Mujal *et al.*, 2022). Moreover, the identified TAM subclusters do not necessarily arrange themselves into discrete populations, but the subclusters rather display overlapping gene signatures in which single TAMs settle along gradients of gene expression (Zilionis *et al.*, 2019). Importantly, single-cell analyses have identified TAM subclusters associated with positive and, so far, especially with negative outcomes. For example, somewhat vindicating for the conventional classification, *CXCL10*$^+$ M1 TAMs and TAMs expressing an IFN-stimulated gene signature correlated most with positive prognoses and responses to checkpoint blockade, whereas *SPP1*$^+$ and *VCAN*$^+$ M2-like TAMs associated with poorer survival and lack of treatment response, respectively (Bassez *et al.*, 2021; Cheng *et al.*, 2021; Zhang *et al.*, 2021). Significantly, these analyses also identified "nonconforming" TAM subclusters that actually had the strongest associations with negative outcomes but could not be categorized as either M1 or M2-like. For example, *MMP12*$^+$, *FABP5*$^+$ and *FN1*$^+$ TAMs correlate with negative prognoses, whereas *CX3CR1*$^+$ TAMs predict ineffective PD-1 blockade (Bassez *et al.*, 2021; Cheng *et al.*, 2021; Wu *et al.*, 2021; Zilionis *et al.*, 2019). Notably, it was also discovered that some mutually exclusive TAM subclusters can have overlapping functions, such as *SPP1*$^+$ TAMs, which expressed a hypoxia-regulated proangiogenic gene signature and were enriched in lung, colorectal, ovarian and pancreatic cancers but were absent from skin and renal cancers, where a similar proangiogenic gene signature was expressed by *VCAN*$^+$ and *FN1*$^+$

TAMs, respectively (Cheng *et al.*, 2021; Zhang *et al.*, 2020). Thus, even though scRNA-seq might not find the same subclusters in different tumors, they may still contain TAMs with equivalent functionality. It was also recently discovered that out of all tumor-infiltrating myeloid cells, TAM subclusters are apparently the least conserved between human tumors from different tissues of origin as well as between human and mouse (Cheng *et al.*, 2021; Zilionis *et al.*, 2019). Interestingly, these analyses have also identified several "bystander" TAM subclusters that associate with neither positive nor negative outcomes. These include, for example, *EGR1*$^+$, *SIGLEC1*$^+$ and *LYVE1*$^+$ M2-like and *APOE*$^+$ nonconforming TAMs (Bassez *et al.*, 2021; Wu *et al.*, 2021).

Although recent scRNA-seq analyses seem to suggest that TAM activation states might be conserved between patients whose tumors originate from the same tissue (Cheng *et al.*, 2021)—providing some consistency among the emerging complexity—it remains to be answered how well this method can predict a cell's actual functional capabilities, which are determined by the amount of translated proteins and posttranslational regulation of protein activity, neither of which necessarily map one-to-one to the number of gene transcripts (Yang *et al.*, 2020). Other outstanding questions regarding TAM biology include:

- How many different activation states are TAMs actually able to acquire?
- What are the drivers behind these complex activation states?
- What is the functional significance of these activation states?
- How much do the functions of different activation states overlap?
- What markers could unambiguously distinguish tumor-promoting TAMs from those that are intrinsically antitumor, predictive of positive outcomes to specific treatments or would best respond to TAM-targeted treatments, including immunotherapeutic "re-education?"

2.3.5 TAMs as Immunotherapeutic Targets

The research and development of cancer immunotherapies has become an incredibly active field both academically and in the private sector. Unfortunately, no novel immunotherapy has yet significantly improved upon the clinical efficacy of first-generation checkpoint blockers, which, although revolutionary, is still limited. The idea of a silver bullet that could cure any cancer has largely been abandoned in lieu of precision medicine, according to which therapies are selected on the basis of the patient's individual characteristics, that is, their cancer–immune setpoint. Therefore, the discovery of biomarkers that could predict which patients would benefit from which treatments has emerged as an important parallel line of research. Moreover,

although curing cancer is still the main objective, treatments that stabilize the disease would also be incredibly beneficial for patients with progressing malignancies that are refractory to current therapies, since these diseases could thus be rendered chronic instead of fatal. Nevertheless, research in cancer immunology advances every day and new candidates are constantly coming up the drug development pipeline. Given their high abundance in most tumors and significant contributions to the development, progression and therapeutic resistance of virtually all cancers, TAMs have attracted significant interest as immunotherapeutic drug targets. My intention for the following chapter is not to provide an exhaustive catalog of all drugs and drug candidates targeted at TAMs that are currently in use or development, but rather a review of the principal concepts of TAM targeting, with some relevant examples of each approach and, if applicable, the current stage of their clinical development. Many excellent and recent reviews can provide a detailed assessment of the whole landscape (Goswami *et al.*, 2022; Jahchan *et al.*, 2019; Kowal *et al.*, 2019; Pittet *et al.*, 2022). Moreover, I will focus the discussion on pharmacological interventions and mainly on solid tumors, even though the same therapeutic strategies can and have been used to treat hematological malignancies as well, in some cases with great success. An additional barrier to the successful treatment of solid tumors is typically the poor infiltration of activated effector cells into the tumor itself, which obviously blocks their access to the tumor cells they are supposed to kill. The immunotherapies targeted at TAMs that have been innovated so far can be roughly divided into three overarching categories: depletory, activating and re-educational.

2.3.5.1 TAM Depletion

Inhibition of Monocyte Infiltration

Since the presence of TAMs is in itself so strongly associated with negative outcomes due their numerous tumor-promoting activities, an obvious first choice would be to stop them from infiltrating tumors in the first place. To this end, several pharmacological agents that inhibit, for example, the CCL2:CCR2 and CXCL12:CXCR4 signaling axes have been developed and already tested in clinical trials. The rationale behind CCL2:CCR2 disruption is to inhibit $CD14^+$ monocyte infiltration into the TME, thereby depleting TAMs by blocking the tumor's access to these blood-borne precursors that maintain its macrophage pool. In preclinical models, inhibiting CCL2:CCR2 signaling did indeed prevent monocyte recruitment, improve tumor control and reduce metastasis. Separate phase I trials with the CCL2 blocker carlumab (NCT01204996) and two small-molecule antagonists against CCR2 (NCT01413022, NCT02345408 and NCT02732938), all combined with standard-of-care, showed some modest improvement of tumor control and TAM reduction in

patient subsets, all of whom had pretreated, refractory cancers (Linehan *et al.*, 2018; Noel *et al.*, 2020; Nywening *et al.*, 2016; Sandhu *et al.*, 2013)—but further clinical development of all three drug candidates seems to have been terminated since. Even though carlumab did initially decrease the serum levels of CCL2, these levels actually increased with longer-term treatment, which could suggest the emergence of some compensatory feedback mechanism that renders long-term CCL2:CCR2 disruption ineffective (Sandhu *et al.*, 2013). The fact that withdrawing CCL2 blockade actually accelerated monocyte infiltration and tumor growth points to the same conclusion (Bonapace *et al.*, 2014). The inhibition of CXCL12:CXCR4 signaling works under a similar premise and had comparable effects preclinically (Hughes *et al.*, 2015)—antagonists against CXCR4 have, however, yielded somewhat more promising results in clinical trials with patients whose cancers are equally challenging. For example, in recent phase II trials on patients with pancreatic ductal adenocarcinoma (NCT02826486 and NCT02826486), the CXCR4 peptide antagonist motixafortide combined with PD-1 blockade promoted cold-to-hot tumor conversion and synergized with combinatorial chemotherapy (Bockorny *et al.*, 2021; Bockorny *et al.*, 2020). Currently, motixafortide is under evaluation in other combinations and cancer indications in phase II trials (NCT01838395 and NCT04543071). Another CXCR4 antagonist, mavorixafor, has also recently concluded phase II trials (NCT02923531 and NCT02823405) with promising preliminary results on patients with renal cell carcinoma (Choueiri *et al.*, 2021).

Nonspecific Macrophage Depletion

The wholesale depletion of TAMs through the inhibition of M-CSF:CD115 signaling appeared, at first, very promising based on data from many preclinical studies. However, results from clinical trials testing various types of CD115 blockers on patients with solid tumors have been less than overwhelming as monotherapies (Butowski *et al.*, 2016; Dowlati *et al.*, 2021; Gomez-Roca *et al.*, 2015; Papadopoulos *et al.*, 2017; Ries *et al.*, 2014)—apart from the very specific exception of diffuse tenosyvial giant-cell sarcoma, which actually arises from $CD115^+$ cells, overexpresses M-CSF and responds well to, for example, the CD115 blocker emactuzumab even as a single agent (Cassier *et al.*, 2020; Cassier *et al.*, 2015). Later research indicates that M-CSF:CD115 disruption is actually quite prone to induce therapeutic resistance through several possible mechanisms that compensate for the loss of M-CSF-dependent TAMs. For example, in multiple tumor models, when signaling through CD115 was inhibited, a subset of stromal cells that were, surprisingly, also $CD115^+$ upregulated CXCL1—or CXCL8 in humans—which recruited granulocytes and PMN-MDSCs into the TME through its receptor, CXCR2 (Kumar *et al.*, 2017). PMN-MDSCs can, in many regards, stand in for TAMs and promote, for example,

T-cell suppression, neovascularization, invasion and metastasis (Movahedi *et al.*, 2008; Youn *et al.*, 2008). Moreover, other growth factors can substitute for M-CSF. For example, granulocyte-colony-stimulating factor (G-CSF) could differentiate a subset of $CD14^+$ monocytes that expressed its receptor, CD114, into M2-like TAMs that promoted breast cancer metastasis (Hollmén *et al.*, 2016). Likewise, in brain metastases of breast cancer, stromal granulocyte–macrophage-colony-stimulating factor (GM-CSF) supported the survival and differentiation of tumor-promoting TAMs through its receptor, CD131 (Klemm *et al.*, 2021). Another significant finding from preclinical tumor models is that M-CSF:CD115 disruption is actually unable to deplete all macrophages but will instead force adaptive changes on the TME that leave pockets of TAMs with which tumor cells can nestle. For example, in a glioblastoma model, CD115 inhibition induced macrophages to secrete IGF-1 (insulin-like growth factor 1), which stimulated PI3K signaling in tumor cells through the receptor IGF-1R to promote survival and proliferation (Quail *et al.*, 2016). Notably, combining M-CSF:CD115 disruption with CXCR2, CD114, CD131 or IGF-1R inhibition, respectively, overcame therapy-induced resistance in all four cases. This suggests that if M-CSF:CD115 disruption were used to actually treat patients with cancer, it would require constant monitoring and treatment of—and available means of treating—various emerging mechanisms of adaptive resistance.

Depletion of Specific TAM Subsets

Strategies for TAM depletion that specifically target immunosuppressive TAM subsets have also been developed. One such approach targets CD163, whose expression on macrophages follows a pattern similar to Clever-1. Moreover, $CD163^+$ TAMs are strongly associated with negative outcomes in nearly all studied cancer cohorts—only colorectal adenocarcinoma and osteosarcoma were exceptions to this rule (Komohara *et al.*, 2014). Functionally, CD163 scavenges defunct hemoglobin from the extracellular space, which is transformed into anti-inflammatory metabolites inside macrophages (Kristiansen *et al.*, 2001). A standard method of nonspecific macrophage depletion *in vivo* is the intravenous injection of lipid nanoparticles, which phagocytes voraciously eat up. Loading these lipid nanoparticles with cytostatic agents, usually with clodronate, kills the cells that ingest them (van Rooijen & Hendrikx, 2010). In a preclinical model of melanoma that is refractory to checkpoint blockade, lipid nanoparticles that were loaded with the chemotherapeutic agent doxorubicin and coated with an antibody against CD163 specifically depleted the $CD163^+$ TAM subset, increased the relative amount of M1 TAMs and improved tumor control (Etzerodt *et al.*, 2019). The translatability of such an approach remains to be investigated clinically but is nonetheless an intriguing idea. Additionally, many targeted approaches against $TIE2^+$ TAMs that accumulate around blood vessels have

been attempted—this subset is strongly associated with neovascularization as well as metastasis by loosening the integrity of the vascular wall (De Palma *et al.*, 2005; Harney *et al.*, 2015). Activity of the TIE2 receptor, expressed on endothelial cells, in particular, as well as pericytes in addition to the $TIE2^+$ TAM subset, is regulated by its two main ligands, ANG (angiopoietin) 1 and ANG2 (De Palma *et al.*, 2005; Dumont *et al.*, 1992; Teichert *et al.*, 2017). The constitutively expressed ANG1, a potent agonist of TIE2, limits the generation of aberrant blood vessels in response to tissue injury, while ANG2, whose expression and secretion from endothelial cells is upregulated by inflammation, is a competitive antagonist of ANG1 and a partial agonist of TIE2 (Davis *et al.*, 1996; Yuan *et al.*, 2009). Consequently, ANG2 sensitizes blood vessels to VEGF-A by blocking ANG1 and activates $TIE2^+$ TAMs (Coffelt *et al.*, 2010; Lobov *et al.*, 2002). Thus, the inhibition of ANG2:TIE2 signaling should disrupt the positive feedback loop between TAMs and neovascularization. In preclinical tumor models, inhibiting ANG2 binding to TIE2 could indeed block the perivascular accumulation of $TIE2^+$ TAMs, improve tumor control and reduce metastasis (Brown *et al.*, 2010; Harney *et al.*, 2017; Oliner *et al.*, 2004). However, these promising preclinical results have not been reproduced in clinical trials, despite extensive efforts in several cancer indications with different drug candidates against ANG2 and different combinations of treatments (Diéras *et al.*, 2015; Eatock *et al.*, 2013; Lee *et al.*, 2020; Marth *et al.*, 2017; Monk *et al.*, 2016; Peeters *et al.*, 2013; Rini *et al.*, 2012; Vergote *et al.*, 2019). It seems that the clinical development of many drug candidates targeting ANG2 has since been terminated, although some are still ongoing (for example, NCT01042379 and NCT03239145). TIE2 inhibitors have fared somewhat better so far. Two small-molecule inhibitors, regorafenib and ripretinib, have received FDA approval as last-line salvage therapies for metastatic colorectal cancer, hepatocellular carcinoma and gastrointestinal stromal tumors, and rebastinib was approaching the planned end of phase II trials on patients with locally advanced or metastatic solid tumors in combination with chemotherapy before being discontinued due to company restructuring (NCT03601897, NCT03717415) (Blay *et al.*, 2020; Grothey *et al.*, 2013). However, these inhibitors are not exactly specific for TIE2 but each inhibit an array of receptors based on their individual affinities. For example, rebastinib has lesser affinity for, at least, VEGFR2 and FLT3 and other intracellular signaling molecules (Harney *et al.*, 2017). Altogether, it seems somewhat specious whether these TIE2 inhibitors' clinical benefits are consequent of any effects on $TIE2^+$ TAMs, specifically. The same could be said of ANG2 blockers' lack thereof, which could also result from endothelial adaptation against this mode of treatment (Jakab *et al.*, 2022).

2.3.5.2 Antitumor TAM Activation

Phagocytosis Enhancers

Because TAMs are already present in most tumors and usually at quite high frequencies, approaches that boost their intrinsic antitumor activity have also been developed. These are mostly pharmacological agents that induce the phagocytosis of tumor cells. Tumor cells often overexpress “don’t eat me” molecules such as CD47, which suppresses phagocytosis by binding to its receptor, SIRPα, on macrophages. Blocking the interaction between CD47 and SIRPα leaves “eat me” molecules on tumor cells available for recognition, which stimulates TAMs to increase tumor-cell clearance as well as T-cell activation *in vitro* and *in vivo* (Majeti *et al.*, 2009; Oldenborg *et al.*, 2000; Willingham *et al.*, 2012). Consequently, several CD47 blockers have been developed and tested in clinical trials. For example, after very promising results from a phase I testing the efficacy of magrolimab, an antibody that blocks CD47, on patients with non-Hodgkin lymphoma in combination with standard-of-care rituximab (NCT02953509), several trials testing magrolimab on solid tumors in combination with, for example, PD-1 blockade and chemotherapy have since advanced to phase II (Advani *et al.*, 2018). The novel recombinant protein evorpacept, which fuses the high-affinity CD47-binding region of SIRPα with a mutated, inactive antibody Fcγ region, also yielded positive results in phase I in combination with PD-1 blockade and has advanced to a combined phase II and III on patients with gastric cancer (NCT05002127), with more phase I and II trials also ongoing (Kauder *et al.*, 2018; Lakhani *et al.*, 2021). A more recent candidate for immunotherapeutic targeting is the “don’t eat me” molecule CD24, whose binding to Siglec-10 on macrophages similarly inhibits phagocytosis (Barkal *et al.*, 2019). The overexpression of CD24 could therefore provide resistance against CD47 blockade. Stimulation of CD40, a receptor expressed on most APCs, including M1 macrophages, is another means of antitumor TAM activation. The CD40 ligand, expressed on, for example, recently activated T_H cells, increases phagocytosis but also induces IL-12 secretion from macrophages and, consequently, promotes T_H1-cell differentiation—thereby promoting cold-to-hot tumor conversion (Beatty *et al.*, 2011; Imaizumi *et al.*, 1999). Such an effect has been reported in many preclinical models and was actually well demonstrated recently in three clinical trials on patients with advanced solid tumors: two phase I trials for selicrelumab and phase II for sotigalimab, both CD40 agonists. In addition to cold-to-hot tumor conversion, selicrelumab in combination with emactuzumab could at least block disease progression in a significant portion of patients, although the larger phase II could not demonstrate any additional benefit from sotigalimab on top of PD-1 blockade and chemotherapy (Byrne *et al.*, 2021; Machiels *et al.*, 2020; Padrón *et al.*, 2022).

Phagocytosis-Enhancing Activity of Existing Cancer Drugs

Moreover, many antibody-based therapies targeted at tumor cells can also be categorized as boosters of macrophage antitumor activity, a mechanism-of-action that, intentional or not, arises from the therapeutic antibody's Fcγ region. For example, rituximab, whose target, CD20, is expressed on B cells as well as leukemic cells derived from the B-cell lineage, in practice opsonizes its target cells and induces antibody-dependent cellular phagocytosis (ADCP) directly through macrophages' activating FcγRs (Fcγ receptors) or indirectly through the complement protein C1q (van der Meid *et al.*, 2018). Another example is trastuzumab, which targets HER2, the EGF receptor famously overexpressed in a subset of breast cancers, in particular. Trastuzumab's primary mechanism-of-action is to block HER2 signaling, but it can also induce the ADCP of tumor cells (Shi *et al.*, 2015). ADCP is most efficiently elicited by the human IgG isotypes IgG_1 and IgG_3, whose Fcγ regions have the strongest affinities for FcγRI—also known as CD64—and also activate the complement system through C1q (Abramson *et al.*, 1970; Bindon *et al.*, 1988). The actual immunological activity of different antibody isotypes is therefore an important parameter to consider in the development of therapeutic antibodies. For example, magrolimab is a human antibody of the IgG_4 isotype, whose affinity for FcγRI is low and for C1q virtually nonexistent, which could explain its relatively low toxicity, considering that its target is constitutively expressed in every tissue (Advani *et al.*, 2018). This consideration was probably also behind the development of evorpacept. I must additionally note that antibody-dependent cellular cytotoxicity (ADCC), mediated by, for example, the binding of IgG_1 and IgG_3 to CD16 on NK cells, is also an important mechanism of antibody-dependent clearance of target cells.

2.3.5.3 TAM Re-education

The Concept of TAM Re-education

Following the unexpected finding that M-CSF:CD115 disruption actually failed to completely deplete TAMs even in preclinical tumor models where CD115 inhibition resulted in significantly improved tumor control, it was additionally discovered that the activation state of the remaining TAMs actually differed significantly from TAMs in the control group. This was observed, for example, in models of brain, breast, colon, lung and pancreatic tumors and melanoma, where inhibition of tumor growth in response to CD115 inhibition was actually not mediated by TAM depletion of itself but rather by shifting the activation state of surviving TAMs from pretreatment M2-like dominance to favor antitumor M1 TAMs, suggesting that macrophages can retain their plasticity even in thrall of cancer (Hoves *et al.*, 2018; Perry

et al., 2018; Pfirschke *et al.*, 2022; Pyonteck *et al.*, 2013; Wiehagen *et al.*, 2017; Zhu *et al.*, 2014). These remaining TAMs were actually indispensable for improved tumor control—supporting the notion of TAM "re-education" from tumor-promoting to antitumor as a means of cancer therapy. Many treatment strategies to this end are under development, some of which have now advanced to clinical trials. PRR agonists can also be considered one method of TAM re-education, albeit an unspecific one. In addition to inducing the maturation of DCs, they should also induce the M1 polarization of TAMs when locally administered. For example, a phase III investigating intratumorally injected TLR9 agonist vidutolimod as a PD-1 blockade adjuvant in metastatic melanoma is currently ongoing (NCT04695977) after positive results from phase II, while the topically administered TLR7 agonist imiquimod received FDA approval for the treatment of superficial basal-cell carcinoma already in 2004 (Davar *et al.*, 2020; Geisse *et al.*, 2004). One could claim that TAM re-education therefore actually predates checkpoint blockade as a clinically adopted immunotherapy (Dummer *et al.*, 2003). At the moment, agonists for every single TLR as well as cGAS–STING seem to be in the drug development pipeline for various cancer indications. Moreover, the antitumor effects of signaling pathway inhibitors—primarily meant to stifle hyperactive signaling pathways in tumor cells—can partially result from TAM re-education. For example, in preclinical models, active PI3K signaling promoted the polarization of M2-like TAMs, which could be overcome with pharmacological PI3K inhibition (Kaneda *et al.*, 2016)—three phase II trials are currently investigating eganelisib, a selective inhibitor of the immune-cell-enriched PI3K isoform γ, in combination with PD-1 blockade on patients with head and neck squamous, renal cell or urothelial carcinoma or breast cancer (NCT03980041, NCT03795610 and NCT03961698, respectively). Some PI3K inhibitors in clinical use, mostly for the treatment of hematological malignancies, also target the γ isoform, meaning their effects could, in part, result from TAM re-education (Horwitz *et al.*, 2018). Likewise, the antitumor effects of PD-1 blockade can be partially mediated by a subset of PD-1^{+} M2-like TAMs, whose phagocytic response to CD47 blockade was further increased by concomitant PD-1 blockade (Gordon *et al.*, 2017). Therefore, combining checkpoint blockade with experimental treatments, the typical setup in most clinical trials currently, could actually potentiate the macrophage-targeted therapy in addition to promoting an antitumor T-cell response.

Targeted Re-education of Specific TAM Subsets

Alternative means of more specific TAM re-education have also been developed recently. These experimental therapies are still mostly in the preclinical phase but have provided proof-of-concept for re-educating tumor-promoting TAMs by targeting cell-surface molecules enriched on specific subsets. For example, expression of the

scavenger receptor MARCO (macrophage receptor with collagenous structure) is exclusive to APCs, including macrophages, on which it is upregulated by M2 and downregulated by M1 polarization. Functionally, MARCO is a phagocytic PRR for bacterial structures, which, inside phagosomes, may trigger signaling PRRs to initiate inflammation (Arredouani *et al.*, 2005; Elomaa *et al.*, 1995; Gratchev *et al.*, 2005). MARCO is also overexpressed on a subset of M2-like TAMs that accumulate around blood vessels similarly to $TIE2^+$ TAMs (Eisinger *et al.*, 2020; Georgoudaki *et al.*, 2016). Moreover, $MARCO^+$ TAMs associate with negative outcomes in, at least, patients with breast and pancreatic cancer (Bergamaschi *et al.*, 2008; Shi *et al.*, 2021). In preclinical models of breast, colon and non-small-cell lung cancer and melanoma, treatment with a specific antibody against MARCO improved tumor control and reduced metastasis by increasing the frequency of M1 at the expense of M2-like TAMs, which made the tumors susceptible to checkpoint blockade (Eisinger *et al.*, 2020; Georgoudaki *et al.*, 2016). Interestingly, TAM re-education depended on the antibody's concomitant binding to MARCO and the inhibitory Fcγ receptor FcγRIIB, which triggered MARCO's internalization and upregulated glycolysis, typically associated with M1 polarization (Eisinger *et al.*, 2020; Georgoudaki *et al.*, 2016). Moreover, the subsequent cold-to-hot tumor conversion and antitumor capacity of CTLs depended on NK-cell activation through increased IL-15 and TRAIL, which, like FasL, is also expressed on NK cells in addition to M1 macrophages (Eisinger *et al.*, 2020; La Fleur *et al.*, 2021). A blocking antibody against human MARCO has been developed but no clinical trials appear to be recruiting at the time of writing (Eisinger *et al.*, 2020).

TREM2 (triggering receptor expressed on myeloid cells 2) is another recently identified target for TAM re-education. TREM2 is expressed on, as its name suggests, myeloid cells, including monocytes and various TRMs such as microglia and adipose-tissue, liver and lung macrophages on which it functions as a scavenger receptor that binds various fatty acid structures, including lipoproteins, lipid nanoparticles, bacteria and cells and promotes myeloid cell survival and inhibits M1 polarization by activating PI3K and β-catenin signaling (Daws *et al.*, 2003; Jaitin *et al.*, 2019; Peng *et al.*, 2010; Ramachandran *et al.*, 2019; Schmid *et al.*, 2002; Wu *et al.*, 2015). As such, TREM2 probably also promotes efferocytosis. Additionally, TREM2 was identified as a cell-surface marker of M2-like TAMs in preclinical models of breast and colon cancer and sarcoma as well as a marker of M2-like TAMs in human tumors (Katzenelenbogen *et al.*, 2020; Lavin *et al.*, 2017; Molgora *et al.*, 2020; Song *et al.*, 2019; Timperi *et al.*, 2022). $TREM2^+$ TAMs also associate with negative outcomes in gastric cancer and renal cell carcinoma (Zhang *et al.*, 2016; Zhang *et al.*, 2018). In preclinical tumor models, blocking TREM2 with a specific antibody improved tumor control but this effect was not mediated by the depletion of $TREM2^+$ TAMs, as the used mouse IgG_{2a} antibody was modified in its Fcγ region

to abolish all binding to FcγRs and C1q (Molgora *et al.*, 2020). Rather, TREM2 blockade worked by inhibiting signaling through TREM2, which did not change the overall frequency of TAMs but decreased the proportion of M2-like TAMs and rendered tumors susceptible to PD-1 blockade (Molgora *et al.*, 2020). Interestingly, TREM2 expression is not necessarily increased in all tumors. In fact, it reportedly decreased with disease progression in cohorts of patients with colorectal and hepatocellular carcinoma and lung cancer, where the loss of TREM2 on tumor cells themselves actually associated with negative outcomes (Kim *et al.*, 2019; Tang *et al.*, 2019; Yao *et al.*, 2016). Mechanistically, in tumor cells, TREM2 reduced proliferation and motility by inhibiting PI3K and β-catenin signaling (Tang *et al.*, 2019)—which is totally opposite to its reported effect on myeloid cells. This could be explained by the different wiring of signaling pathways in myeloid and tumor cells but nevertheless raises the possibility of therapy-induced disease progression if some patients with cancer in which the tumor cells themselves are $TREM2^+$ were treated with TREM2 blockade. Interestingly, an activating antibody against TREM2 has advanced to phase II on patients with early Alzheimer's disease (NCT04592874) (Wang *et al.*, 2020), so given acceptable safety and tolerability it could, possibly, also be tested on patients with $TREM2^+$ tumors. Another TREM2 antibody recently begun phase I on patients with metastatic solid tumors in combination with PD-1 blockade (NCT04691375), but its mechanism-of-action is actually depletory, by eliciting ADCP and ADCC, rather than re-educational (Patnaik *et al.*, 2022).

3 Aims

Clever-1 has long been an established marker of so-called alternatively activated, anti-inflammatory or immunosuppressive M2 macrophages. Work published over the years by us and others, in addition to at-the-time unpublished observations in our group, propose Clever-1 to be a central immunosuppressive molecule at the interface of innate and adaptive immunities in homeostasis, infection and cancer. As ever, each observation raised a plethora of new questions regarding *how* exactly does Clever-1 exert its effects on the immune system, which Clever-1^+ cell type—macrophage or endothelial cell—mediates these effects and could the responsible Clever-1^+ cells be manipulated for therapeutic purposes. Therefore, the aims of this thesis were to:

- Investigate the role of Clever-1 in the regulation of humoral and cellular adaptive immune responses.
- Evaluate the suitability of Clever-1 as an immunotherapeutic cancer drug target in preclinical models.
- Elucidate the molecular mechanisms by which Clever-1 regulates the suppressive activation state and function of macrophages.

4 Materials & Methods

The methods that were used in the original publications **I**–**III** to generate the results I present in this thesis are listed in **Table 1**. I describe select methods in more detail in this chapter. More detailed descriptions of all used materials and methods can be found from the original publications.

Table 1. Methods used in the original publications.

METHOD	PURPOSE	PUBLICATION
Animal husbandry	Maintenance of experimental mouse strains	I, II
Antibody uptake	*Ex vivo* analysis of antigen binding	I, III
Antibody-mediated cell depletion	Removal of specific subtypes of cells for functional *in vivo* studies	II
Cell culture	Maintenance of primary cells and cell lines	I, II, III
Coimmunoprecipitation	Antibody-mediated pulldown of proteins and protein complexes	III
Cytometry by time-of-flight	Characterization and quantification of cell suspensions based on labeling with metal-conjugated probes and mass cytometry	III
ELISA & Multiplex assays	Quantification of analytes in blood plasma or other biological fluids	I, II, III
Ex vivo bioluminescence imaging	Analysis of tumor and metastatic burden of luciferase-expressing cell lines	II
Flow cytometry	Characterization and quantification of cell suspensions based on labeling with fluorescent probes	I, II, III
Hematopoietic chimeras	Creation of hematopoietically chimeric mice	II
Immunization	Induction of a humoral immune response	I
Fluorescent confocal microscopy	Visualization of molecules and their subcellular localization within cells with fluorescent probes	I, II
Immunohistochemistry	Visualization of molecules and their localization in tissue sections	I, II

METHOD	PURPOSE	PUBLICATION
In vitro coculture experiments	Analysis of cellular interactions in a simplified, controlled environment	I, II, III
In vivo antigen capture	Analysis of cells' phagocytic activity *in vivo*	I
Leukocyte enumeration	Quantification of white blood cell populations	I
Magnet-assisted cell sorting	Enrichment of specific cell types with antibody-coated magnetic beads for *in vitro* assays	I, II, III
Mass spectrometry	Very-high-throughput measurement of molecular mass-to-charge ratios	III
Quantitative PCR	Low- to medium-throughput analysis of gene expression, validation of RNA sequencing results	I, II
RNA interference	siRNA-mediated knockdown of gene expression by induced mRNA degradation	III
RNA sequencing	Global analysis of gene expression	I
Seahorse assays	Analysis of cellular metabolism	II
Splenectomy	Surgical removal of the spleen	I
Statistical analysis	Determination of statistical significance in differences between experimental groups	I, II, III
Tumor models and antibody treatments	Preclinical models for testing the efficacies of immunotherapies *in vivo*	II
Western blotting	Semiquantitative analysis of protein expression or phosphorylation (or other post-translational modification)	II, III

4.1 Experimental Animals

Animal work was conducted in the Central Animal Laboratory, University of Turku, Turku, Finland. All experimental animals used in the studies I present here were mice. The full, macrophage-specific and blood endothelial Clever-1 knockout strains Clever-$1^{-/-}$, *Lyz2-Cre*/Clever-$1^{fl/fl}$ and *Tie2-Cre*/Clever-$1^{fl/fl}$, respectively, and their wildtype controls were all derived from the C57BL/6N;129SvJ mixed background and were generated as described by Karikoski *et al.*, 2014. To create $DsRed^{+}$ wildtype and Clever-$1^{-/-}$ reporter mice, Tg(CAG-DsRed*MST)1Nagy/J mice were purchased from the Jackson Laboratories and crossbred with Clever-1 knockout mice. The mice were housed in a specific-pathogen-free environment with a 12-hour light/dark cycle and stable 22 °C temperature. The mice had access to chow and

water *ad libitum*. All animal experiments were reviewed and approved by the local Ethical Committee for Animal Experimentation. All experiments were carried out in adherence to the Finnish Act on Animal Experimentation (497/2013) and the 3Rs principles under the animal license numbers 5587/04.10.07/2014 and 5762/04.10.07/2017. For experiments, mice were used at 2–4 months of age. The experimental groups were matched for age and sex.

4.2 Animal Models

4.2.1 Tumor Models

To generate LLC1, EL4 and CT26.WT tumors, 0.5×10^6 cells in 200 µl of phosphate-buffered saline (PBS) (806552; Sigma) were injected subcutaneously into the flanks. To generate orthotopic E0771 or 4T1 tumors, 0.1×10^6 cells in 50 µl of PBS were injected subcutaneously into the fourth mammary fat pads. Tumor outgrowth was monitored with digital calipers. The humane endpoint for tumor diameter was 15 mm. Mice were immediately euthanized in cases of clearly worsened general condition or tumor rupture. Tumor volumes were calculated with the following formula: longer diameter × shorter diameter^2 ÷ 2.

4.2.2 Hematopoietic Chimeras

To generate hematopoietic chimeras, wildtype recipient mice were irradiated twice with 5 Gy with a three-hour interval and injected intravenously with 10×10^6 bone marrow cells collected from DsRed$^+$ wildtype or Clever-1$^{-/-}$ reporter mice in PBS. The mice were left to reconstitute for two months before they were used for experiments. Successful chimerism was determined by measuring the frequency of DsRed$^+$ cells in the blood at experimental endpoints, which was consistently >90 %.

4.2.3 Antibody-Mediated Cell Depletion

To deplete mice of macrophages or CD8$^+$ T cells, the mice were injected intraperitoneally with 200 µg of CD115 antibody (clone AFS98; BioXCell) every other day or 100 µg of CD8β antibody (clone 53-5.8; BioXCell) once weekly in PBS, respectively. For a combined control group, mice were injected intraperitoneally with a combination of equivalent amounts of isotype-matched irrelevant antibodies (clones 2A3 and HRPN, respectively; BioXCell). For cell-depletion experiments, the injections of depleting antibodies were started eight days before tumor induction and were continued until the experimental endpoint.

4.2.4 Immunotherapies

As immunotherapeutic treatments, tumor-bearing mice were injected intraperitoneally with 200 µg of Clever-1 antibody (clone mStab1-1.26; InVivo Biotech), 200 µg of anti-PD-1 (clone RMPI-14; BioXCell), a combination of Clever-1 and PD-1 antibodies or a combination of equivalent amounts of isotype-matched irrelevant antibodies (clones MOPC-21 and 2A3, respectively; BioXCell). Antibody injections were performed on days 3, 6, 9 and 12 after tumor induction and the mice were sacrificed on day 15.

4.3 Cell Lines

The cell lines LLC1 Lewis lung carcinoma and E0771 medullary mammary adenocarcinoma were cultured in high-glucose Dulbecco's modified Eagle's medium (D6429; Sigma) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (F7524; Merck) and penicillin/streptomycin (15140-122; Thermo Fisher Scientific). The luciferase-expressing 4T1-luc2 mammary gland carcinoma, CT26.WT colon carcinoma, EL4 lymphoma and its ovalbumin-expressing derivative EG.7 cell lines were cultured in RPMI-1640 medium (R5886; Merck) supplemented with 10 % FBS, 2 mM L-glutamine (35050-038; Thermo Fisher Scientific), 1 mM sodium pyruvate (11360-039; Thermo Fisher Scientific) and penicillin/streptomycin with 5 µM β-mercaptoethanol for EL4 and EG.7 cells and 0.5 mg/ml G418 instead of penicillin/streptomycin for EG.7 cells. The KG-1 acute myelogenous leukemia cell line was cultured in Iscove's modified Dulbecco's medium (IMDM) (Thermo Fisher Scientific; 31980022) supplemented with 20 % FBS and penicillin/streptomycin. The LLC1, EL4, EG.7, CT26.WT and KG-1 cell lines were obtained from the American Type Culture Collection (CRL-1642, TIB-39, CRL-2113, CRL-2638 and CCL-246, respectively). The E0771 cell line was a kind gift from Professor Burkhard Becher, University of Zurich, Zurich, Switzerland. The cell lines were routinely tested for mycoplasma but were not routinely verified.

4.4 RNA Interference & KG-1 Macrophage Differentiation

RNA interference with synthetic small interfering RNAs (siRNAs) was used to transiently knock down Clever-1 gene expression from the Clever-1^{high} KG-1 human myelogenous leukemia cell line for functional studies. The siRNAs were introduced into KG-1 cells by electroporation, which resulted in robust downregulation of Clever-1 protein expression. On the day before transfection, KG-1 cells were split 1:1 in fresh culture medium. On the day of transfection, KG-1 cells were collected, washed, counted and resuspended in OPTI-MEM reduced serum medium

(51985026; Thermo Fisher Scientific) at 20×10^6 cells/ml. Stock siRNAs reconstituted in sterile ddH_2O were added to the cell suspensions to 2 µM. For electroporation, 100 µl of the cell suspension and siRNA mix was transferred to an electroporation cuvette (P45050; Thermo Fisher Scientific) and electroporated using the program U-001 on the Nucleofector IIa device (Amaxa Biosystems), then plated in 2 ml of fresh culture medium per electroporation. The siRNAs used were the non-targeting ON-TARGETplus Control Pool (D001810-10-20) and two single siRNAs targeting the human Clever-1 mRNA, siR1 (AUGAUGAGCUCACGUAUAA) and siR2 (UCAAGUCGCUGCCUGCAUA) (J-014103-05-0020 and J-014103-08-0020, respectively; all three from Dharmacon). After electroporation, the KG-1 cells were left to recuperate in fresh culture medium overnight. KG-1 macrophage differentiation was induced by adding phorbol 12-myristate 13-acetate (P8139; Merck) to 300 nM and incubating the cells for another three days. Adherent cells were collected for experiments.

4.5 Primary Macrophage Differentiation & Polarization

To generate primary human macrophages, first, peripheral blood mononuclear cells were collected from buffy coats provided by the Finnish Red Cross by Ficoll-Paque PLUS density gradient centrifugation. Next, $CD14^+$ monocytes were isolated from collected peripheral blood mononuclear cells with CD14 Microbeads (130-050-201; Miltenyi Biotec). Their density was adjusted to 1.0×10^6 cells/ml in macrophage differentiation medium (IMDM supplemented with 10 % FBS, penicillin/streptomycin and 50 ng/ml recombinant human M-CSF [574806; BioLegend]), of which 10 ml was added per T-75 flask. The monocytes were differentiated into macrophages for seven days with one medium change on the third or fourth day. For M2 polarization, the differentiated macrophages were incubated for another two days in IMDM supplemented with 10 % FBS, penicillin/streptomycin, 100 nM dexamethasone (D-2915; Merck) and 20 ng/ml recombinant human IL-4 (200-04; Peprotech).

4.6 Antibodies

I have compiled the antibody clones used in the original publications, the methods they were used for and their providers into **Table 2**.

Table 2. Antibody clones and their providers.

REACTIVITY	CLONE	COMPANY	METHOD
ATP6V0A1	NBP1-59949	Novus Biologicals	Fluorescence microscopy, western blotting
ATP6V1A	PA5-29191	Invitrogen	Western blotting
B220/CD45R	RA3-6B2	BD	Flow cytometry
CD11b	M1/70	BD	Flow cytometry
CD138	281-2	BD	Flow cytometry
CD19	1D3	BD	Flow cytometry
CD206	C068C2	BioLegend	Flow cytometry
CD21/CD35	7G6	BD	Flow cytometry
CD23	3C7	BD	Flow cytometry
CD24	M1/69	BD	Flow cytometry
CD3	17A2	BD	Flow cytometry
CD4	GK1.5	BD	Flow cytometry
CD43	S7	BD	Flow cytometry
CD44	IM7	BD	Flow cytometry
CD45	30-F11	BD	Flow cytometry
CD5	53-7.3	BD	Flow cytometry
CD62L	MEL-14	BD	Flow cytometry
CD8α	53-6.7	BD	Flow cytometry, fluorescence microscopy
Clever-1	3-372	InVivo Biotech	Flow cytometry, fluorescence microscopy, western blotting
Clever-1	9-11	InVivo Biotech	Flow cytometry, fluorescence microscopy, immunoprecipitation
Clever-1	CP12 (FP-1305)	Abzena	Flow cytometry, fluorescence microscopy, immunoprecipitation
Fas	Jo2	BD	Flow cytometry
Fc Block (anti-CD16/anti-CD32)	2.4G2	BD	Flow cytometry
FoxP3	FJK-16s	Invitrogen	Flow cytometry
GAPDH	5G4	Hytest Ltd.	Western blotting
Goat anti-mouse IgG	Polyclonal	Invitrogen	Flow cytometry

REACTIVITY	CLONE	COMPANY	METHOD
Granzyme B	PA1-26616	Invitrogen	Immunofluorescence microscopy
Human IgG_4 isotype control	QA16A15	BioLegend	Immunoprecipitation
IgD	11-26c.2a	BD	Flow cytometry
IgM	II/41	BD	Flow cytometry
KI67	SolA15	Invitrogen	Flow cytometry
Lag3	C9B7W	Invitrogen	Flow cytometry
LAMP-1	D2D11	Cell Signaling Technology	Fluorescence microscopy
Ly6C	AL-21	Invitrogen	Flow cytometry
Ly6G	1A8	BD	Flow cytometry
Mouse IgG control	Polyclonal	Rockland	Fluorescence microscopy
Nos2	CXNFT	Invitrogen	Flow cytometry
PD-1	J43	Invitrogen	Flow cytometry
Phospho-mTOR (S2248)	EPR426(2)	Abcam	Western blotting
Phospho-NF-κB (S536)	93H1	Cell Signaling Technology	Western blotting
Rabbit IgG Control	Polyclonal	BioXCell	Fluorescence microscopy
Rat IgG_{2a} isotype control	eBR2a	Invitrogen	Flow cytometry, fluorescence microscopy, immunoprecipitation
Rat IgG_{2a} isotype control	2A3	BioXCell	Flow cytometry, fluorescence microscopy
TCIRG1/ATP6V0A3	PA5-90425	Invitrogen	Western blotting

4.7 Flow Cytometry

4.7.1 Preparation of Single-Cell Suspensions

Mice were sacrificed by CO_2 asphyxiation. Blood was collected in EDTA tubes (365974; BD) and red blood cells were lysed with 1× RBC Lysis Buffer (00-4300-54; Invitrogen) using manufacturer's instructions. Single-cell suspensions from the spleen and bone marrow were isolated by mechanical teasing as described by

Rantakari *et al.*, 2015. Lymph nodes were cleaned of excess fat tissue and dissociated mechanically. Tumors were mechanically and enzymatically processed into single-cell suspensions with the Mouse Tumor Dissociation Kit (130-096-730; Miltenyi Biotec) using the manufacturer's instructions and passed through 70 µm preseparation filters (130-095-823; Miltenyi Biotec). Myeloid cells and T cells were sequentially enriched from the bulk tumor single-cell suspension with CD11b (130-049-601; Miltenyi Biotec) and CD90.2 Microbeads (130-049-101; Miltenyi Biotec), respectively, on MS Columns (130-042-201; Miltenyi Biotec) using the manufacturer's instructions. Before staining for flow cytometry, the cell suspensions were passed through 30 µm preseparation filters (130-041-407; Miltenyi Biotec). Macrophages cultured *in vitro* were detached at room temperature with 5–10 mM EDTA in PBS and gentle scratching and passed through 30 µm preseparation filters before staining.

4.7.2 Cell Staining for Flow Cytometry

Before staining single-cell suspensions with fluorochrome-conjugated antibodies, the unspecific binding of staining antibodies to Fcγ receptors was blocked by preincubating single-cell suspensions with Fc Block (553142; BD) or normal human IgG cocktail (Kiovig). When surface-stained single-cell suspensions were analyzed without fixation, 7-AAD (00-6993-50; Invitrogen) was used as the viability dye. To detect intracellular antigens, surface-stained single-cell suspensions were fixed with 4 % paraformaldehyde (sc-281692; Santa Cruz) and stained in 1× Permeabilization Buffer (00-8333-56; Thermo Fisher Scientific). To simultaneously detect cell-surface and intranuclear antigens, the Transcription Factor Staining Buffer Set (00-5523-00; Invitrogen) was used with manufacturer's instructions. If cells were fixed, they were first labelled with a fixable viability dye (Invitrogen). Samples were acquired with the LSRII or LSRFortessa flow cytometers (both from BD) and analyzed with the FlowJo 10 software (Treestar). For flow cytometric stainings, the mouse Clever-1 antibody mStab1-1.26 (InVivo Biotech) and its isotype-matched irrelevant antibody control MOPC-21 (BioXCell) were conjugated with the Alexa Fluor 647 Protein Labelling Kit (A20173; Thermo Fisher Scientific) in-house using manufacturer's instructions.

4.8 Coimmunoprecipitation, Mass Spectrometry Sample Preparation & Data Analysis

Differentiated and polarized primary human M2 macrophages were detached with 5 mM EDTA in PBS and gentle scraping. The collected macrophages were resuspended in immunoprecipitation lysis buffer (20 mM Tris-HCl [pH 8.0], 137 mM

NaCl, 1 % Triton X-100, 2 mM EDTA) supplemented with 2× cOmplete Protease Inhibitor Cocktail (Merck). The cells were lysed at +4 °C with mixing for 30 min and centrifuged at 14,000 *g* and +4 °C for 10 min. The supernatant was collected and the total protein concentration adjusted to 1 mg/ml with immunoprecipitation lysis buffer. For each immunoprecipitation, 1 mg of total protein was combined with 10 µg of 9-11 (InVivo Biotech), FP-1305 (clone CP12; Abzena) or rat IgG_{2a} (clone eBR2a; Thermo Fisher Scientific) or human IgG_4 isotype-matched irrelevant control antibody (clone QA16A15; BioLegend), respectively, and incubated at +4 °C with mixing overnight. Immune complexes were precipitated with 100 µl of Dynabeads Protein G beads (10003D; Thermo Fisher Scientific) at +4 °C with mixing for one hour. The beads were washed with immunoprecipitation lysis buffer at room temperature with mixing for 3×10 min. Remaining proteins were eluted from the beads in 50 µl of 2× SDS sample buffer (120 mM Tris-HCl [pH 6.8], 20 % glycerol, 4 % SDS, 50 mM DTT) at +95 °C for 5 min. The samples were run approximately 1.5 cm into 10 % resolving Tris-glycine SDS-PAGE gels. The gels were stained with Coomassie Brilliant Blue R-250 and the protein lanes were cut and submitted for mass spectrometry at the Turku Proteomics Facility.

The following criteria were used to include a protein as a specific hit for 9-11 or FP-1305:

1. The protein was present in at least two out of three biological replicates.
2. The protein was identified by at least three unique peptides.
3. The protein was either not immunoprecipitated by the corresponding isotype-matched irrelevant control antibody or its enrichment by unique peptides was at least three-fold greater with the specific Clever-1 antibody.

To prune likely contaminants from the protein interactomes, the spectral count data were uploaded into CRAPome and compared against 16 controls (cell type HEK293, total cell lysate, Dynabeads magnetic affinity support) with default settings (Mellacheruvu *et al.*, 2013). FC-A > 4 was used as the cutoff value. The remaining proteins were mapped in Cytoscape 3.7.2 based on high-confidence (>0.7) interactions downloaded from the STRING database. Protein clusters were identified with the Markov clustering algorithm of the clusterMaker app with default settings. The STRING Enrichment app was used to identify significantly enriched GO Biological Process terms in the five largest clusters (redundancy cutoff >0.7). Functional enrichment among differently precipitated proteins was determined by Ingenuity Pathway Analysis (Qiagen).

5 Results

5.1 Macrophage Clever-1 Suppresses Humoral Immunity (I)

5.1.1 Clever-1 Deficiency Elevates Antibody Levels at Baseline & After Immunization

Initially, we measured the plasma concentrations of total IgM and IgG in wildtype and Clever-$1^{-/-}$ mice at baseline and after immunization with classical TD and TI-2 model antigens NP-KLH (4-hydroxy-3-nitrophenylacetic acid–keyhole limpet hemocyanin) and NP-Ficoll, respectively, by ELISA. We observed a significant increase in baseline IgM and IgG concentrations in Clever-$1^{-/-}$ mice (**I: Fig. 1A, B**). Additionally, kinetic measurements of plasma antibody concentrations following immunization with NP-KLH or NP-Ficoll showed enhanced antigen-specific humoral responses in Clever-$1^{-/-}$ mice, especially in the latter thymus-independent case (**I: Fig. 1D–H**).

5.1.2 Clever-$1^{-/-}$ Mice Have Enlarged Spleens & Altered Splenic B-Cell Populations

Because the spleen is a major reservoir for antibody-producing B cells, we analyzed the composition of splenic B cells in wildtype and Clever-$1^{-/-}$ mice by flow cytometry in an effort to understand what was behind the enhanced antibody production in the absence of Clever-1. However, we observed no significant differences in the frequencies of total $B220^+$ B cells among $CD45^+$ immune cells between wildtype and Clever-$1^{-/-}$ mouse spleens (**I: Fig. 2A, B**), although the spleens from Clever-$1^{-/-}$ mice were significantly larger and consequently contained higher absolute numbers of B cells (**I: Fig. 2C–E**). More detailed phenotyping of splenic B-cell populations allowed us to see a significant decrease in the frequency of MZB cells in Clever-$1^{-/-}$ mice at baseline and after NP-KLH but not NP-Ficoll immunization, while the frequency of follicular B cells was unaffected at baseline and after immunization with either NP-KLH or NP-Ficoll (**I: Fig. 2F–H**). Additionally, immunizing Clever-$1^{-/-}$ mice with NP-KLH had no effect on the frequencies of GCB cells or plasma cells,

but immunizing Clever-1$^{-/-}$ mice with NP-Ficoll robustly increased the frequencies of both GCB cells and plasma cells when compared to wildtype mice (**I: Fig. 2I, J**).

5.1.3 The Elevated Antibody Levels in Clever-1$^{-/-}$ Mice Are Produced by Factors Outside of the Spleen

Immunofluorescence imaging of wildtype mouse spleen sections showed the expression of Clever-1 to be exclusive to the VAP (vascular adhesion protein)-1^{+} vascular endothelial cells of the red pulp, while MZ macrophages, metallophilic macrophages and red pulp macrophages were all negative for Clever-1 (**I: Fig. 3A**). We observed no differences in the scavenging abilities of MZ macrophages between wildtype and Clever-1$^{-/-}$ mice at short- or long-term timepoints, measured as the accumulation of intravenously injected fluorescent NP-Ficoll (**I: Fig. 3B, C**). To understand if the spleen was at all required for generating the elevated humoral immune response in Clever-1$^{-/-}$ mice, we performed immunization experiments with NP-Ficoll on wildtype and Clever-1$^{-/-}$ mice after sham surgery or splenectomy (**I: Fig. 3D**). Although splenectomy lowered total antibody levels compared to sham surgery, the plasma concentrations of IgM and IgG remained significantly elevated in Clever-1$^{-/-}$ mice compared to wildtype mice even if the spleen was removed (**I: Fig. 3E, F**). Moreover, the antigen-specific humoral immune response against NP-Ficoll remained elevated even in splenectomized Clever-1$^{-/-}$ mice (**I: Fig. 3G, H**).

5.1.4 Clever-1 Deficiency Causes Delayed B Lymphopoiesis, B Lymphocytosis & Reduces Peritoneal B1 Cells

We extensively analyzed the frequencies of various B-cell subsets in the bone marrow, peritoneal cavity and blood by flow cytometry. We observed that B-cell development in the bone marrow was delayed in Clever-1$^{-/-}$ mice compared to wildtype mice, as demonstrated by the reduced frequencies of pre-pro, pre and early mature B cells, although we saw no differences in the frequencies of later-stage B cells in the bone marrow (**I: Table 1 & Supplementary Table 1**). Concomitantly, the frequency of peritoneal B1 cells was decreased and the frequency of total B cells in the blood was increased in Clever-1$^{-/-}$ mice compared to wildtype mice (**I: Table 1 & Supplementary Table 1**).

5.1.5 Clever-1$^{-/-}$ Macrophages Directly Enhance B-Cell Antibody Secretion

Even though B cells are specialized to produce antibodies and must be the final effectors causing elevated antibody concentrations in Clever-1$^{-/-}$ mice, they themselves do not express Clever-1. We reasoned that if we were observing a true, biologically relevant phenomenon caused by genetic Clever-1 deficiency, its primary source must obviously be the cells which in wildtype mice express Clever-1, namely endothelial cells or macrophages. We then turned to conditional Clever-1 knockout strains to analyze specifically which Clever-1^{+} cell type possibly regulates the altered B-cell biology of Clever-1$^{-/-}$ mice. By ELISA, we indeed observed elevated plasma IgG concentrations in myeloid-specific *Lyz2-Cre*/Clever-1$^{fl/fl}$ mice similar to full knockout Clever-1$^{-/-}$ mice when compared to wildtype mice (**I: Fig. 4A, B**) but not in *Tie2-Cre*/Clever-1$^{fl/fl}$ mice, which lack Clever-1 on the blood endothelium (data not shown). We confirmed by flow cytometry that Ly6C^{high} monocytes of wildtype mice expressed Clever-1, as is the case for their CD14^{high} human counterparts. Moreover, we observed that Clever-1$^{-/-}$ M2 BMDMs (bone-marrow-derived macrophages) responded to LPS stimulation more strongly than wildtype M2 BMDMs, observed as increased secretion of TNF by ELISA (**I: Fig. 4C–E**). We then performed coculture experiments with wildtype B cells and wildtype or Clever-1$^{-/-}$ monocytes. Coculturing B cells with Clever-1$^{-/-}$ monocytes induced significantly higher IgM secretion when compared to B cells cocultured with wildtype monocytes (**I: Fig. 4F**). This increase in IgM secretion was almost but not completely blocked by a neutralizing antibody against TNF (**I: Fig. 4F**). RNA-seq of wildtype and Clever-1$^{-/-}$ mice suggested that other factors that can increase antibody secretion in addition to TNF may also be upregulated as a result of genetic Clever-1 deficiency, resulting in an overall IFN-stimulated gene signature (**I: Supplementary Table 2**).

5.2 Clever-1 Blockade Re-educates TAMs & Activates Antitumour Immunity (II)

5.2.1 Macrophage Clever-1 Deficiency Improves Tumor Control

To dissect the contribution of macrophage-expressed Clever-1 on the growth of solid tumors, we measured the outgrowth of three subcutaneous or orthotopic syngeneic tumor models, LLC1, E0771 and EL4, in wildtype, Clever-$1^{-/-}$ and *Lyz2-Cre*/Clever-$1^{fl/fl}$ mice over fifteen days. LLC1 and EL4 are subcutaneously grown lung tumor and lymphoma models, respectively, whereas E0771 is an orthotopically grown breast tumor model. While Clever-$1^{-/-}$ mice showed significantly improved tumor control against LLC1 tumors, all three tumor models were almost completely eradicated in *Lyz2-Cre*/Clever-$1^{fl/fl}$ mice (**II: Fig. 1A–C, E, G, H**). The conditional Clever-1 knockout strain *Tie2-Cre*/Clever-$1^{fl/fl}$, where Clever-1 is deleted from the blood endothelium, did not show improved tumor control compared to wildtype mice (**II: Fig. 1H**). Increased tumor control was accompanied by a reduction in tumor-secreted G-CSF but an increase in the inhibitory checkpoint PD-L1 on surviving tumor cells (**II: Fig. 1D, F**).

5.2.2 Clever-$1^{-/-}$ Mice Overcome Cancer-Related Immunosuppression

To understand the mechanisms behind increased tumor control in *Lyz2-Cre*/Clever-$1^{fl/fl}$ mice, we analyzed the activation of the adaptive immune response both systemically and inside the tumor. Multiplex assays of plasma collected on day 15 showed significantly increased concentrations of the proinflammatory cytokines and chemokines IL-1β, IL-2, IL-12, TNF and CCL4 in Clever-$1^{-/-}$ mice (**II: Fig. 2A, B**). Tumors in *Lyz2-Cre*/Clever-$1^{fl/fl}$ mice showed massive infiltration by CTLs with an exhausted PD-1^{+} Lag3^{+} activation state and a concomitant increase in proliferating effector CTLs in the tumor-draining lymph nodes (**II: Fig. 2C–G**).

5.2.3 Tumor Control Requires Macrophages & CTLs

To confirm that the drastically increased tumor control we observed in *Lyz2-Cre*/Clever-$1^{fl/fl}$ mice with a complementary model *in vivo*, we created hematopoietic chimeras by reconstituting irradiated wildtype mice with bone marrow from DsRed^{+} wildtype or Clever-$1^{-/-}$ reporter mice (**II: Fig. 3A**). The outgrowth of LLC1 tumors was significantly restrained in Clever-$1^{-/-}$→wildtype chimeras compared to wildtype→wildtype chimeras and was accompanied by increased CTL infiltration

(**II: Fig. 3B–D**). TAMs in Clever-1$^{-/-}$→wildtype chimeras lost Clever-1 expression, reduced in numbers and acquired a more M1-like activation state, as observed by the increase in MHC class II and decrease in PD-L1 expression (**II: Fig. 3F–H**). To validate macrophages and CTLs as the effectors of tumor control in mice deficient of Clever-1, we used antibodies against CD115 or CD8, respectively, to systemically deplete these cells from wildtype and *Lyz2-Cre*/Clever-1$^{fl/fl}$ mice before tumor induction (**II: Fig. 3I**). Depletion of either macrophages or CTLs eliminated tumor control in *Lyz2-Cre*/Clever-1$^{fl/fl}$ mice (**II: Fig. 3J, K**).

5.2.4 Clever-1$^{-/-}$ TAMs Acquire a Proinflammatory Activation State

To understand how macrophage-specific Clever-1 deficiency could lead to such dramatically increased tumor control, we analyzed the composition of the three main tumor-infiltrating myeloid cell populations—PMN-MDSCs, M-MDSCs and TAMs—by flow cytometry. While the numbers of PMN- and M-MDSCs were not significantly altered by Clever-1 deficiency, the number of TAMs was significantly decreased in tumors from Clever-1$^{-/-}$ and almost completely depleted in tumors from *Lyz2-Cre*/Clever-1$^{fl/fl}$ mice (**II: Fig. 4A, B**). We verified by flow cytometry that TAMs were the only myeloid cell population in wildtype tumors to express Clever-1. In the tumor models we used, approximately 20–40 % of TAMs on average were Clever-1^{+} (**II: Fig. 4C, Supplementary Fig. S3D**). The remaining TAMs in Clever-1$^{-/-}$ tumors had an increasingly M1-like activation state, although expression of the classical M2 marker CD206 was also increased (**Fig. 4D–F**). Clever-1$^{-/-}$ TAMs showed decreased induction of Nos2 after LPS stimulation but increased secretion of IL-12 both *in vivo* and *in vitro* (**II: Fig. 4G–I**). We used the Seahorse glucose stress test to analyze metabolic differences related to Clever-1 deficiency in macrophages. Both enriched Clever-1$^{-/-}$ TAMs and dexamethasone-polarized M2 macrophages cultured *in vitro* showed increased glycolysis and glycolytic capacity and a shift towards an M1-like activation state (**II: Fig. 5A–H**). Moreover, Clever-1$^{-/-}$ M2 macrophages showed increased sensitivity to the triggering of proinflammatory signaling pathways as measured by the phosphorylation kinetics of mTOR (mammalian target of rapamycin) and NF-κB after LPS stimulation (**II: Fig. 5J, K**).

5.2.5 Immunotherapeutic Clever-1 Blockade Improves Antitumor CTL Activation and Tumor Control

The functionally interfering antibody clone against Clever-1, mStab1-1.26, was previously reported to attenuate the growth of the subcutaneous mouse melanoma model

B16. In **II**, we further studied how the effects of immunotherapeutic Clever-1 blockade, targeting mainly the innate immune system, compared to PD-1 blockade, targeting mainly the adaptive immune system, and whether these treatments might synergize as increased tumor control (**II: Fig. 6A**). Clever-1 blockade showed at least equal efficacy to PD-1 blockade at improving tumor control and inhibiting metastatic spread in the immunologically cold tumor model LLC1 and the immunologically hot tumor models 4T1 and CT26.WT, with increased efficacy when the treatments were combined (**II: Fig. 6A–F & Supplementary Fig. 7A–H**). In the LLC1 model, Clever-1 blockade synergized with PD-1 blockade and increased tumor infiltration by proliferating CTLs (**II: Fig. 6G, H**). In the CT26.WT model, the combination of anti-Clever-1 with PD-1 blockade reduced the numbers of tumor-infiltrating $CD8^+$ T cells in spite of increased tumor control (**II: Supplementary Fig. 7I, J**). In both LLC1 and CT26.WT models, anti-Clever-1 treatment significantly reduced the numbers of TAMs, although combination with PD-1 blockade reverted the numbers of tumor-associated myeloid cell populations back to those of the irrelevant antibody control group (**II: Fig. 6I & Supplementary Fig. 7K**). Anti-Clever-1 treatment did not reduce the frequency of Clever-1^+ TAMs and Clever-1 was fully occupied by the immunotherapeutic antibody still at the experimental endpoint on day 15 (**II: Fig. 6J, Supplementary Fig. 7L, & Supplementary Fig. 8C**).

5.3 Clever-1 Blockade Stimulates Adaptive Immunity in Patients with Cancer (III)

5.3.1 Clever-1 Interacts with the Lysosomal Vacuolar ATPase Proton Pump

We wanted to understand the molecular mechanisms Clever-1 uses to regulate macrophage function in more detail. Initially, we incubated primary human M2 macrophages with two fluorochrome-conjugated antibody clones that recognize distinct epitopes on the Clever-1 protein, 9-11 and FP-1305, to analyze how they are taken up by cells and how they localize subcellularly. Both antibodies were rapidly taken into the cells and localized in vesicular structures. However, some of these vesicles were observed to contain only either one of the antibodies (**III: Fig. 1A, B**). We then performed coimmunoprecipitation/tandem mass spectrometry on primary human M2 macrophage lysates using unconjugated 9-11 and FP-1305 antibodies or isotype-matched irrelevant control antibodies (rat IgG_{2a} and human IgG_4, respectively). Pruning of the initial protein lists to remove nonspecifically binding and contaminating proteins resulted in two largely divergent protein interactomes, with 58 proteins specific for 9-11, 65 proteins specific for FP-1305 and 22 proteins shared by the antibodies (**III: Fig. 1C**). Clustering and functional enrichment analyses revealed one highly interlinked group of proteins involved in protein transport and localization and phagosome maturation that was specifically immunoprecipitated by 9-11 (**III: Fig. 1D, E**). The proteins in this cluster were subunits of the vacuolar ATPase (v-ATPase) (**III: Fig. 1D, F**). We validated that the v-ATPase subunits ATP6V1A, ATP6V0A1 and TCIRG1/ATP6V0A3 were indeed specifically immunoprecipitated with 9-11 by coimmunoprecipitation/western blotting, where all three proteins were immunoprecipitated by 9-11 and none by FP-1305 (**III: Fig. 1G**).

5.3.2 Clever-1 Regulates Lysosomal Acidification & Antigen Degradation

The v-ATPase is an ATP-dependent proton pump that uses ATP to transport H^+ ions from the cytosol into the lysosomal lumen. v-ATPase activity is regulated by the reversible assembly and disassembly of its V_0 and V_1 sectors on the lysosomal membrane, where they combine to form the functional v-ATPase multimer. For functional experiments, we used RNA interference to knock down Clever-1 from Clever-1^{high} KG-1 macrophages (**III: Fig. 2A**). Clever-1 knockdown significantly impaired steady-state acidification and DQ-ovalbumin antigen degradation in KG-1 macrophages (**III: Fig. 2B, C**). Furthermore, both the uptake and acidification of Clever-1 ligand acLDL were impaired by Clever-1 knockdown (**III: Fig. 2D, E**). We indepen-

dently validated these results with another siRNA (**III: Supplementary Fig. 1B**). In a similar fashion, incubating macrophages with FP-1305 decreased cellular acidification, while 9-11 increased it (**III: Fig. 2I**). Immunofluorescence confocal microscopy further validated the interaction between Clever-1 and the v-ATPase subunit ATP6V0A1 (**III: Fig. 2F, G**). Clever-1 and ATP6V0A1 colocalized especially with the addition of Clever-1 ligand acLDL, which recruited ATP6V0A1 to LAMP (lysosomal-associated membrane protein)-1^{+} lysosomes (**III: Fig. 2F–H**, left panel). When Clever-1 was knocked down, the recruitment of ATP6V0A1 to lysosomes was inhibited (**III: Fig. 2H**, right panel).

5.3.3 Clever-1 Blockade Renders Suppressive Macrophages Immunostimulatory

Degradative enzymes within the lysosomal lumen require an acidic pH to function efficiently. Indeed, limiting phagolysosomal fusion preserves peptide epitopes compatible with class I MHC molecules for cross-presentation. To analyze the ability of wildtype and Clever-$1^{-/-}$ bone-marrow-derived macrophages (BMDMs) to cross-present peptide-derived antigens, we fed them class-I-compatible ovalbumin-derived peptide SIINFEKL, full-length recombinant ovalbumin protein or ultraviolet-irradiated EG.7 cells, which are ovalbumin-expressing EL4 cells, and stained them with the 25-D1.16 antibody, which recognizes the complex of class I MHC and SIINFEKL. Clever-$1^{-/-}$ macrophages cross-presented more antigen after all treatments (**III: Supplementary Fig. 2A**). We then tested whether antibody treatment increased the stimulatory capacity of primary human macrophages in a mixed leukocyte reaction. Preincubating M2 macrophages with FP-1305 increased their ability to stimulate CTL proliferation to a level comparable with M1 macrophages as measured by flow cytometry (**III: Fig. 2J**). Preincubation with FP-1305 also increased T_H-cell proliferation, but the effect was less robust (**III: Supplementary Fig. 2B**). We did not observe any increases in T-cell proliferation when M2 macrophages were preincubated with 9-11.

5.3.4 Clever-1 Blockade Promotes the Proinflammatory Re-Education of Monocytes in Patients with Cancer

The phase I/II clinical trial MATINS (Macrophage Antibody to Inhibit Immune Suppression) started recruiting patients in 2018. MATINS investigates the safety and efficacy of the recombinant human IgG_4 antibody against Clever-1, referred to initially as FP-1305 but since named bexmarilimab, on patients with solid cancers refractory to available therapies. Eligible indications were selected on the basis of Clever-1's association with survival in the Cancer Genome Atlas database, analyzing

biobank materials for tumors with the highest numbers of Clever-1^+ TAMs and previously published research. According to the trial design, patients receive intravenous doses of bexmarilimab once in every three weeks, each three-week period constituting one treatment cycle. Additionally, the patients donate blood samples before receiving the first dose of bexmarilimab (D0), the day after receiving the first dose (D1) and once weekly after that (D7, D14). These blood samples were delivered to us for analyzing how the patients' antitumor immune responses, inasmuch as can be measured from the blood, react to bexmarilimab over time. The patients' $CD14^{high}$ monocytes had highest Clever-1 expression, which was comparable to healthy donors, while other immune cells in the blood did not express Clever-1 (**III: Supplementary Fig. 4A; Supplementary Table 1**). To analyze whether bexmarilimab altered myeloid cells in the blood, we performed CyTOF with a custom antibody panel on patients' D0 and D7 samples (**III: Fig. 3A, B; Supplementary Fig. 4B; Supplementary Table 2**). A single dose of bexmarilimab significantly downregulated CD206 and CD163 on $CD14^{high}$ monocytes as well as transiently decreased CD14 itself (**III: Fig. 3C, D**). Clever-1^+ monocytes were not depleted by bexmarilimab, even though Clever-1 was well-occupied initially after bexmarilimab administration, although Clever-1 occupancy decreased over time (**III: Supplementary Table 1**). In some patients, cell-surface Clever-1 even transiently increased (**III: Fig. 3D**). We also performed RNA-seq on $CD14^+$ monocytes isolated from D0, D1 and D7 samples. Perhaps unexpectedly, we saw little differences between D0 and D1, but D7 samples from different patients converged in principal component analysis and had overlapping transcriptional changes compared to D0 (**III: Fig. 3E, F; Supplementary Fig. 4C**). IPA on these differentially expressed genes revealed that bexmarilimab downregulated the LXR/RXR (liver X receptor/retinoid X receptor) and PPAR (peroxisome proliferator-activated receptor) signaling pathways and upregulated proinflammatory signaling in $CD14^+$ monocytes (**III: Fig. 3G, H**). The magnitude of this effect positively correlated with Clever-1 occupancy.

5.3.5 Clever-1 Blockade Promotes T_H1 Immunity in Patients with Cancer

We performed CyTOF with another custom antibody panel on D0 and D7 samples to analyze whether bexmarilimab induced changes in circulating T cells (**III: Fig. 4A, B; Supplementary Table 3**). The most pronounced effect we observed was the upregulation of CD25 and CXCR3 on naïve T_H cells and CTLs, effector CTLs and effector memory CTLs (**III: Fig. 4C, D; Supplementary Fig. 5**). In general, we noted that activation markers were upregulated and checkpoint molecules downregulated throughout most T-cell clusters we identified. We also observed increased expression of Ki67 and perforin (**III: Fig. 4E, F**) and increased production of IL-2

and IFNγ (**III: Fig. 4G**) in the CTLs of some patients. Overall, a single dose of bexmarilimab increased the absolute numbers of NK cells, B cells and CTLs and decreased the numbers of T_{REG} cells in most patients (**III: Fig. 5A, B**). Moreover, plasma levels of IFNγ and CXCL10 increased after bexmarilimab administration especially in patients whose pretreatment levels of these cytokines were low (**III: Fig. 5C**). We also treated PBMCs isolated from D0 or D1 samples with LPS *in vivo* and noticed that the secretion IFNα and IFNβ increased while the secretion of soluble CD163 increased in response to bexmarilimab (**III: Fig. 5D**).

5.3.6 Clever-1 Blockade May Promote CTL Activation in a Subset of Patients with Cold Tumors

As a "case study," we analyzed blood samples from one patient with colorectal cancer who had a partial response to bexmarilimab (**III: Fig. 6A**) with scRNA-seq coupled with TCR sequencing. Pretreatment, the patient's tumor contained very high numbers of Clever-1^+ and $CD163^+$ TAMs but few infiltrating CTLs (**III: Fig. 6B**). On the fourth treatment cycle, we observed clonal expansion of *GZMA*^{high} CTLs that also upregulated, for example, CD16 and perforin (**III: Fig. 6C; Supplementary Fig. 6A–C**), as well as the emergence of *TCF7*^{high} CTLs in the patient's blood (**III: Supplementary Fig. 6A, B**). Unfortunately, a posttreatment biopsy of the tumor was not available and we were unable to validate whether the partial response resulted from tumor infiltration by the expanded CTL clones. We did, however, analyze tumor-infiltrating CTLs in the paired pre- and posttreatment biopsies that were available. These were from five patients (Pt)—Pt5, Pt7, Pt11, Pt13 and Pt21—whose tumors had not responded to bexmarilimab. We saw increased peritumoral CTLs in Pt11's and increased tumor-infiltrating granzyme B^+ CTLs in Pt13's biopsy (**III: Fig. 6D, E**). The pretreatment biopsy of Pt11 contained almost no CTLs at all, whereas that of Pt13 contained only peritumoral CTLs. Additionally, we noticed that the amount of Clever-1^+ TAMs in Pt11 and Pt13's biopsies actually decreased posttreatment, whereas two of the patients who did not respond to bexmarilimab and whose tumor-infiltrating CTLs did not increase, either—Pt7 and Pt21—actually had very few Clever-1^+ TAMs in pretreatment biopsies. Their numbers remained unchanged also in the posttreatment biopsies (**III: Fig. 6D, F**).

6 Discussion

6.1 Myeloid Clever-1 Is an Endogenous Immunosuppressive Molecule

The scavenger receptor Clever-1 is expressed on specialized endothelial cells in various organs as well as myeloid innate immune cells of the monocyte–macrophage lineage. In previous studies, Clever-1 expression has been strongly associated with an immunosuppressive M2 activation state on, for example, placental macrophages. However, at first, Clever-1 was thought to mostly facilitate the migration of specific immune cells, such as neutrophils and T_{REG} cells, and modify the extracellular matrix through scavenging (Karikoski *et al.*, 2009; Palani *et al.*, 2011; Riabov *et al.*, 2016; Shetty *et al.*, 2011). Later, our group discovered that Clever-1 expressed on monocytes and macrophages inhibits the overactivation of T cells through the downregulation of proinflammatory cytokines and possibly also direct cell-to-cell contact (Palani *et al.*, 2016; Tadayon *et al.*, 2021). However, whether the regulatory activity of Clever-1 extended to humoral immunity was completely unknown. Humoral immunity includes antibodies generated against TD and TI-1 antigens encountered through infections or vaccinations as well as constitutively produced natural antibodies against TI-2 antigens. Generation of the first typically requires the concomitant activation of T_H and B cells in secondary lymphoid organs, whereas the latter two generally require no T-cell help. Thus, our purpose was to investigate the putative suppressive effects of Clever-1 on both elicited and natural humoral responses. To this end, we used wildtype and Clever-$1^{-/-}$ mice. We found that the baseline levels of IgM and IgG in blood plasma were already elevated in Clever-$1^{-/-}$ mice compared to wildtype mice. Additionally, the humoral responses of Clever-$1^{-/-}$ mice against both TD and TI antigens were also increased. This was most clearly the case for the TI-2 carbohydrate antigen NP-Ficoll, whose hapten-conjugated polysucrose structure mimics the highly repetitive pneumococcal polysaccharide and activates B cells through excessive BCR clustering. Both the initial IgM response as well as later class switching to IgG_3, which, in the mouse, is the default class produced against TI-2 antigens after IgM, were increased in Clever-$1^{-/-}$ mice in a statistically significant manner.

Our attention, at first, focused on the spleen, because the spleens of Clever-1$^{-/-}$ mice were significantly enlarged and it is where the MZB cells that chiefly mediate TI-2 responses reside, hinting it could be the source of this phenomenon. However, the only statistically significant difference we observed in our analyses of different splenic B cells at baseline was the unexpected reduction in the frequency of MZB cells in Clever-1$^{-/-}$ mice, which persisted even after immunization with our model antigens. Still, the frequencies of GCB and plasma cells increased robustly in response to NP-Ficoll, even though these subsets are canonically more related to TD than TI-2 responses. We thought that the scavenging and subsequent degradation of antigen would be decreased in the absence of Clever-1, which could have increased the amount of antigen available for MZB cells, thus explaining the elevated TI-2 response. However, Clever-1 was neither expressed on any splenic macrophage population nor did its deficiency increase or decrease the accumulation of intravenously delivered TI-2 antigen to the MZ. This somewhat contradictory data led us to question whether the spleen was, in fact, at all required for the phenomenon we observed. Indeed, our experiments showed that it was not, since the relative increase in baseline levels of both IgM and IgG as well as the increased TI-2 response against NP-Ficoll in Clever-1$^{-/-}$ mice persisted even if the spleen was surgically removed. Yet, our exhaustive flow cytometric analyses of various B-cell subsets in the blood, bone marrow and peritoneal cavity did not offer easy explanations, either, as the only significant differences we observed between wildtype and Clever-1$^{-/-}$ mice were mainly reductions in the frequencies of, for example, peritoneal B1 cells, which also mediate TI-2 responses—apart from the overall frequency of B cells in the blood, which was elevated in Clever-1$^{-/-}$ mice.

We analyzed the baseline antibody levels in the blood plasma of our *Lyz2-Cre*/Clever-1$^{fl/fl}$ and *Tie2-Cre*/Clever-1$^{fl/fl}$ mice. Because we observed an increase in IgG levels similar to that in Clever-1$^{-/-}$ mice only in the macrophage-specific knockout strain, we thought that perhaps the increased proinflammatory activity of monocytes and macrophages in mice deficient of Clever-1 could be behind the augmented humoral responses we observed, since macrophages are known to regulate B-cell activation through, for example, the secretion of proinflammatory cytokines. Supporting this hypothesis, our research group had recently reported that transient Clever-1 knockdown in human monocytes upregulated the secretion of several proinflammatory cytokines, including TNF, one major B-cell activator (Boussiotis *et al.*, 1994; Palani *et al.*, 2011). We verified that genetic Clever-1 deletion also led to increased TNF secretion from Clever-1$^{-/-}$ BMDMs of mouse origin. Finally, our coculture experiments with wildtype B cells and wildtype or Clever-1$^{-/-}$ monocytes demonstrated that Clever-1$^{-/-}$ monocytes could actually directly induce greater antibody secretion from B cells, which was almost but not completely reversed by neutralizing TNF. Thus, the intrinsic proinflammatory activity of Clever-1$^{-/-}$ monocytes and macro-

phages, coupled with the increased overall absolute numbers of B cells per mouse in Clever-$1^{-/-}$ mice, could well explain the increased basal levels of IgM and IgG. Moreover, since neutralizing TNF did not completely abrogate the effect, it is probable that Clever-$1^{-/-}$ monocytes and macrophages release more of other proinflammatory mediators that increase antibody production, as is suggested by the IFN-stimulated gene signature of Clever-$1^{-/-}$ mice.

Clever-$1^{-/-}$ mice do not present with defects under physiological conditions nor are they more susceptible to infections. In fact, pathology has only been reported when both *Stab1* and its homolog *Stab2* have been knocked out simultaneously, which results in glomerulofibrotic nephropathy (Schledzewski *et al.*, 2011). Moreover, in the disease models that have been applied to Clever-$1^{-/-}$ mice, they have presented aggravated fibrosis and delayed resolution in response to liver injury and improved tumor control against models of breast cancer, lymphoma and melanoma (Karikoski *et al.*, 2014; Rantakari *et al.*, 2016; Riabov *et al.*, 2016). In all three cases, the effects were linked to Clever-1's function as an adhesion and scavenger receptor, because the migration of immune cells and clearance of extracellular molecules were altered in its absence. However, we concluded here that Clever-1 actually does not regulate humoral responses through its function as a scavenger receptor, by which it could pilfer antigens from B cells, nor does Clever-1 directly regulate humoral responses in the spleen, either. Instead, our results implicated that myeloid Clever-1, specifically, functions additionally as an endogenous immunosuppressive molecule that inhibits the proinflammatory overactivation of monocytes and macrophages. One consequence of Clever-1 disruption is the release of the proinflammatory cytokine TNF that consequently increases antibody production. Moreover, the enlarged spleens of Clever-$1^{-/-}$ mice appeared to be of immunological origin and not caused by, for example, congestion or tumorous growth, so the systemic increase of proinflammatory cytokines could also be their underlying cause. What remains to be explained, however, is the curious increase in GCB and plasma cells in response to TI-2 but not TD antigen. Possibly, this phenomenon could still be explained by altered antigen availability in the absence of Clever-1 outside of the spleen, perhaps to DCs, and the activity of endothelial Clever-1, the loss of which on the lymphatic endothelium increases the capacity of mature DCs to activate T_H cells in lymph nodes. Also, it is very likely that Clever-1 deficiency increases the secretion of proinflammatory cytokines other than TNF, both directly and indirectly. Such candidates could be IL-1 or IFNγ, which, in the mouse, induce class switching from IgM to IgG_3 against TI-2 antigens (Fukao *et al.*, 2021; Snapper *et al.*, 1992).

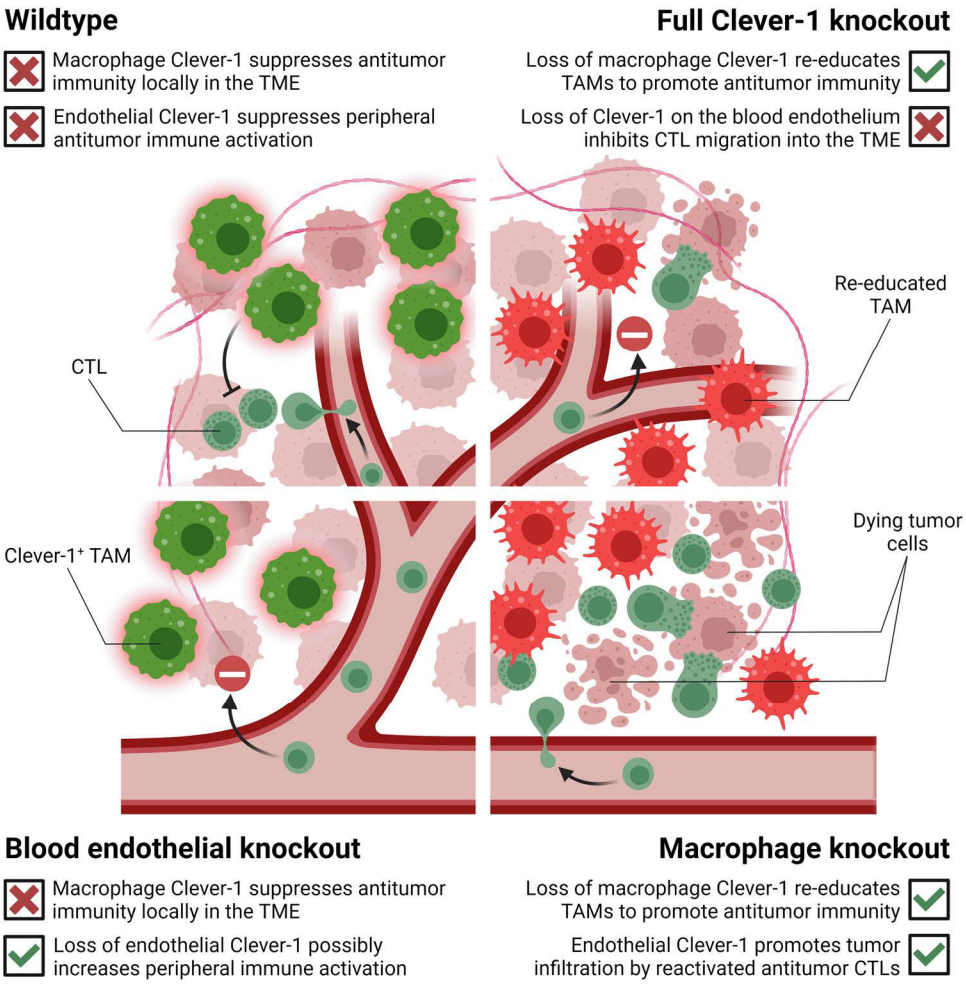
6.2 Clever-1 Interference Re-educates TAMs to Activate Antitumor Immunity

Reports from our group as well as others have indicated that, in addition to regulating the proinflammatory activity of monocytes and macrophages, Clever-1 also participates in maintaining the cancer-related immunosuppression that enables tumor progression (Karikoski *et al.*, 2014; Riabov *et al.*, 2016). In these works, Clever-1 was described to contribute to tumor progression through its functions as an adhesion receptor on the endothelium and a scavenger receptor on macrophages. However, other reports from our group as well as then-unpublished observations led us to question the paradigm of Clever-1, especially when expressed on myeloid cells, as a simple scavenger facilitating waste management (Palani *et al.*, 2011; Tadayon *et al.*, 2021). Indeed, we had discovered that the disruption of Clever-1 on monocytes or macrophages increases their capacity to directly activate T_{EM} cells of the T_H1 but not the T_H2 subset (Palani *et al.*, 2016)—the results I discuss above extend this ability also to humoral immunity. Because of the now well-recognized shortcomings of checkpoint blockade, novel approaches to treat patients whose cancers are refractory to these and other currently available therapies are urgently needed. Thus, we wanted to investigate in more detail how Clever-1 participates in the regulation of immune responses especially in the context of cancer. Considering its expression on M2-like TAMs as well as its general immunosuppressive effect on both innate and adaptive immunities, we hypothesized that Clever-1 could present a novel immunotherapeutic drug target whose functional interference would, additionally, re-educate M2-like TAMs and, consequently, reactivate antitumor immunity. Using various tumor models, conditional knockout mice, bone marrow chimeras and cell depletion *in vivo*, we found that TAM-expressed Clever-1, in particular, controls macrophage-mediated antitumor immune responses both locally in the tumor and systemically in the draining lymph nodes.

Overall, the results I present here corroborate and, importantly, explain many previous findings (Karikoski *et al.*, 2014; Riabov *et al.*, 2016) and demonstrate that Clever-1 blockade can indeed bolster antitumor immunity to break through cancer-related immunosuppression. Notably, we were able to demonstrate that macrophages re-educated by Clever-1 disruption are still indispensable for the initiation of antitumor immunity, which, in the end, is apparently executed by reactivated antitumor CTLs (**Figure 3**). Quite recently, specific DC subsets and their ability to activate antitumor immunity have received much attention. While it is not my intention to discount the significance of DCs, considering that they are, for example, reportedly much more efficient at priming naïve T cells than TAMs are on a per-cell basis (Broz *et al.*, 2014; Roberts *et al.*, 2016), tumors seem often to acquire the means of excluding these cross-presenting DCs out of the TME altogether (Spranger *et al.*, 2015).

Figure 3. Proposed model for the effects of Clever-1 deficiency on antitumor immune responses. *Top left.* Tumors in wildtype mice are populated by Clever-1$^+$ TAMs (tumor-associated macrophages) that suppress the activity of antitumor CTLs (cytotoxic T lymphocytes). Although the Clever-1$^+$ blood endothelium of the tumor vasculature promotes immune cell infiltration into the TME (tumor microenvironment), Clever-1 on the lymphatic endothelium suppresses immune activation in the periphery, i.e., the tumor-draining lymph vessels and lymph nodes (see also Tadayon *et al.*, 2021). *Top right.* In the full Clever-1 knockout, TAMs deficient of Clever-1 are re-educated to promote antitumor immunity in the TME. Peripheral immune activation is likewise increased. However, immune cell infiltration into the TME is inhibited by the loss of Clever-1 on the blood endothelium of the tumor vasculature, altogether resulting in improved but limited tumor control. The phenotype of full knockout mice resembles the effects of antibody-mediated Clever-1 interference the most, because antibody treatment can partially block also endothelial Clever-1 and thus limit the treatment's efficacy. Major differences in TAM activation states between full knockout and antibody-treated mice are also apparent (see text for details). *Bottom left.* Tumors in blood endothelial Clever-1 knockout mice grow comparably to tumors in wildtype mice. Clever-1$^+$ TAMs remain to suppress antitumor immunity, while loss of Clever-1 on the tumor vasculature inhibits immune cell infiltration. *Bottom right.* Macrophage Clever-1 knockout mice show remarkably improved tumor control over wildtype and even full Clever-1 knockout mice. Macrophages deficient of Clever-1 are re-educated to promote antitumor immunity through CTL reactivation, while Clever-1 on the blood endothelium remains to mediate immune cell infiltration into the TME, resulting in near-complete tumor clearance. Created with BioRender.com.

The modes of treatment proposed for their reintroduction require, for example, injections of growth factors directly into the tumor parenchyma or laborious manipulation of patient-derived immune cells *ex vivo*. TAMs, however, typically vastly exceed DCs in tumors (Broz *et al.*, 2014), and even though some TAM subsets have the potential to activate cytotoxic T_{EFF} cells and produce CTL memory (Modak *et al.*, 2022; Pozzi *et al.*, 2005), the majority are extremely potent immunosuppressors both individually and through sheer numbers (Hamilton *et al.*, 2014). For example, TAMs can physically restrict CTLs from reaching their target cells or directly induce CTL apoptosis (Peranzoni *et al.*, 2018; Saio *et al.*, 2001). Somewhat paradoxically, then, wholesale TAM depletion has yielded clinical benefits only in rare, specific cases. One explanation for this phenomenon could be that some subsets of macrophages that are susceptible to CD115 inhibition are, in fact, indispensable for



eliciting tumor control (House *et al.*, 2020; Stromnes *et al.*, 2019; Zhang *et al.*, 2020)—not necessarily through priming naïve T cells but, for example, through restimulating antitumor effector CTLs in the TME or through regulating DC activity. This is in line with the results I present here, according to which the efficient antitumor immune response achieved through macrophage-specific Clever-1 disruption was completely reversed by macrophage depletion. Thus, our results emphasize the importance of specific macrophage subsets for the generation of antitumor T-cell responses.

In the preclinical tumor models we used, we detected Clever-1 expression on approximately 20–40 % of TAMs. Based on the results I present here, Clever-1 expression appears to define a subset of TAMs capable of freezing the cancer–immune cycle in place—recently, a corresponding immunosuppressive *STAB1*$^{+}$ TAM sub-

cluster was identified by scRNA-seq in human breast cancer (Timperi *et al.*, 2022). Moreover, we observed that genetically deleting Clever-1 increased the proinflammatory activation state of the remaining TAMs, although all the effects of Clever-1 disruption did not neatly fit into the classical M1–M2 dichotomy. For example, although Clever-$1^{-/-}$ TAMs expressed more class I and II MHC molecules, less PD-L1 and secreted more IL-12 and TNF, which, taken together, would increase T_H1-cell and CTL infiltration and activation, these changes were accompanied by the upregulation of CD206, a canonical M2 marker, and downregulation of NOS2, a canonical M1 marker. Given the many nonconforming TAM subsets that have been detected *in vivo*, it is probably better to consider the coexpression of multiple molecules and how they might work together rather than the expression of single molecules in order to see the forest for the trees. Even though tumor-expressed CD206 is associated with negative outcomes, it has also been reported to transfer soluble antigens into endosomes specialized in cross-presentation (Burgdorf *et al.*, 2007)—thus mediating CTL activation, as was recently reported to occur also *in vivo* (Modak *et al.*, 2022). Upregulation of the M1-associated NOS2 would not necessarily be all good news, either, as NO produced by NOS2 has been reported to suppress M1 polarization, thus limiting its own production through a negative feedback loop, disturb antigen presentation and induce CTL apoptosis (Saio *et al.*, 2001; Sicher *et al.*, 1994). Additionally, our results show that tumor control is significantly more improved in *Lyz2-Cre*/Clever-$1^{fl/fl}$ over Clever-$1^{-/-}$ mice. We attribute this phenomenon to Clever-1 expressed on the endothelium, which is retained in the former strain. A recent publication from our group details the contradictory functions of endothelial Clever-1 (Tadayon *et al.*, 2021). Shortly, the loss of Clever-1 on the lymphatic endothelium increases DCs' capacity to activate T cells, which cancels out the inhibitory effect the loss of Clever-1 on blood and lymphatic endothelium has on immune cell migration. However, in *Lyz2-Cre*/Clever-$1^{fl/fl}$ mice, immunostimulatory capacity is increased because of Clever-$1^{-/-}$ macrophages, while endothelial Clever-1 remains to mediate immune cell migration to draining lymph nodes and into the tumor. Altogether, Clever-1 deficiency appears to polarize an immunostimulatory TAM subset that does not fully conform with either an M1 or an M2-like activation state. Detailed transcriptional studies, for example, scRNA-seq, could be of interest to determine the global alterations to tumor-infiltrating immune cells and TAM subclusters' gene signatures that are induced by Clever-1 disruption.

In addition to genetic deletion, we achieved significant improvement in tumor control by blocking Clever-1 with the antibody clone mStab1-1.26. The efficacy of Clever-1 blockade rivaled that of PD-1 blockade especially in the cold lung tumor model LLC1 (Lechner *et al.*, 2013), where both monotherapies reduced the numbers of TAMs and PMN-MDSCs and increased the frequency of activated CTLs in tumors. While the former effect can of itself partially explain the latter, it did not

appear to result from Clever-1$^+$ TAM depletion, since their frequency among the remaining TAMs was unchanged. The epitope mStab1-1.26 recognizes on mouse Clever-1 is absent from human Clever-1 and *vice versa* for the human-specific clone 3-372. However, both antibodies inhibit acLDL uptake and upregulate the secretion of, for example, CCL3 and TNF in their respective target species. Thus, we believe that mStab1-1.26 interferes with murine Clever-1 function in a similar way as 3-372 with human, supporting the translational relevance of the mouse tumor models. Interestingly, Clever-1 blockade did not fully recapitulate the Clever-1$^{-/-}$ activation state—possibly, the steric hindrance or conformational change caused by the antibody does not completely inhibit Clever-1's activity. For example, we previously published that the antibody apparently does not increase baseline antibody levels (Karikoski *et al.*, 2009), unlike genetic Clever-1 deletion. As one notable difference between Clever-1 and PD-1 blockade, Clever-1 interference actually decreased the number of tumor-infiltrating CTLs, possibly because of blocking endothelial Clever-1. However, the frequency of activated CTLs was higher, similarly to PD-1 blockade. Another notable difference was the massive reduction in PD-L1$^+$ nonimmune tumor cells in response to Clever-1 but not PD-1 blockade, suggesting that the PD-L1 checkpoint is not upregulated as a resistance mechanism against Clever-1 interference, despite tumor control apparently being mediated by CTLs. Curiously, in LLC1 tumors, the combination of Clever-1 and PD-1 blockade appeared to reverse the positive reductions in tumor cells and tumor-associated myeloid cells achieved with Clever-1 interference by itself, producing worse tumor control than either treatment alone. However, the combination was most efficacious against the highly metastatic 4T1 breast cancer cell line and yielded best tumor control—by an admittedly small margin—in the hot colon tumor model CT26.WT (Lechner *et al.*, 2013). Possibly, the abundance of PD-L1$^+$ tumor cells, brought about by PD-1 blockade in LLC1 tumors, rendered simultaneous Clever-1 blockade ineffective. The magnitude of response did not, at least, depend on the amount of Clever-1$^+$ TAMs, of which CT26.WT tumors contained approximately double compared to LLC1 tumors. Further research, overall, is required to elucidate what factors regulate the response to Clever-1 blockade—results from the MATINS trial hint that hot tumors, in particular, would be less likely to respond to monotherapeutic Clever-1 interference. Moreover, one important parameter could be the tumor's organ of origin, because tumor-draining lymph nodes in different anatomical locations do not have equal ability to activate tumor-reactive CTLs (Horton *et al.*, 2021). Thus, it must be noted that the responses against LLC1 and CT26.WT tumors could have also been different had they been grown orthotopically instead of subcutaneously in the flank.

Regarding the mechanism of TAM conversion after Clever-1 disruption, we observed that Clever-1$^{-/-}$ TAMs isolated from tumors had increased glycolytic metabolism over oxidative phosphorylation. Increased reliance on glycolysis—that is, the

catabolism of glucose into pyruvate and adenosine triphosphate (ATP)—typically occurs in response to M1 polarization (Hard, 1970). Consistently with this, we noted that Clever-$1^{-/-}$ macrophages differentiated and M2 polarized *in vitro* were sensitized to the M1 stimulus LPS, which manifested as increased and prolonged phosphorylation of NF-κB and mTOR, both of which would support M1 polarization. Indeed, Clever-$1^{-/-}$ macrophages cultured *in vitro* had less PD-L1 expression and IL-10 secretion. NF-κB is a well-known proinflammatory signaling pathway downstream of, for example, TLRs, whereas mTOR can be activated by both LPS and IL-4 as well as the excess of amino acids or glucose, although constitutive mTOR signaling seems to strongly favor M1 polarization (Byles *et al.*, 2013; Kimura *et al.*, 2016). Because Clever-1 cycles from the cell membrane to various endolysosomal subcompartments, it could well cross paths with mTOR complexes, which, in their active conformations, are localized on the membranes of intracellular vesicles, particularly on lysosomes (Kzhyshkowska *et al.*, 2004a; Sancak *et al.*, 2010). In fact, the results I present here demonstrate that Clever-1 clearly associates with the multimeric v-ATPase complex, whose activity is regulated by the reversible assembly of its subunits on the lysosomal membrane, where it uses energy from the breakdown of ATP to transport protons into the lysosome (Forgac, 2007). Accumulation of protons or hydrogen ions, H^+, is what turns the lysosome acidic, which, in turn, activates lytic enzymes that degrade the lysosome's contents. Both M1 and M2 macrophages require v-ATPase for their function, and active v-ATPase itself activates mTOR (Bidani *et al.*, 2000; Rao *et al.*, 2019; Zoncu *et al.*, 2011). Availability of the v-ATPase's subunits is regulated by their subcellular localization, and specific subunits, ATP6V0A1 in particular, regulate phagolysosomal fusion and acidification (Bagh *et al.*, 2017; Peri & Nüsslein-Volhard, 2008; Saw *et al.*, 2011). To ensure that antigen presentation even takes place, phagosomal fusion with v-$ATPase^+$ lysosomes must actually be transiently inhibited, as protein antigens would otherwise be eliminated before they have time to be loaded onto MHC molecules (Alloatti *et al.*, 2015). Consistently, Clever-$1^{-/-}$ macrophages cross-presented more soluble and cell-associated antigen. Thus, because Clever-1 deficiency also reduced the association of both its canonical ligand acLDL and the v-ATPase subunit ATP6V0A1 with lysosomes, impaired lysosomal acidification and improved cross-presentation, we propose that Clever-1 can effectively sweep antigens "under the rug" by bringing together its antigenic ligands and active v-ATPase in the phagolysosomal subcompartment. Why mTOR activation increased in the absence of Clever-1, when the expectation would be for it to decrease when the v-ATPase is less active, remains an open question. One simple explanation could be that other pathways that drive M1 polarization and converge at mTOR become more active when Clever-1 is disrupted. Interestingly, the macrophage-specific v-ATPase subunit ATP6V0D2 promotes microbial clearance, M1 polarization and antitumor immunity by increasing autophago-

lysosomal fusion and mTOR activation through the v-ATPase (Li *et al.*, 2019; Liu *et al.*, 2019; Xia *et al.*, 2019). Although ATP6V0D2 was not in our Clever-1 interactome—instead, it contained its substitute, ATP6V0D1—perhaps tacking together specific combinations of subunits to assemble the v-ATPase is another intricate mechanism by which scavenger receptors regulate the fate of and responses against phagocytosed substances. Altogether, the results I present here demonstrate that Clever-1 disruption re-educates immunosuppressive macrophages, impairs lysosomal acidification and promotes cross-presentation, which results in the increased capacity of macrophages to activate tumor-reactive CTLs.

6.3 Clever-1 Blockade May Promote Antitumor Immunity in a Subset of Patients with Cold Tumors

The mouse antibody clone 3-372, specific for human Clever-1, inhibits the uptake of acLDL and induces the secretion of TNF from monocytes and blocks the adhesion of immune cells to Clever-1^+ endothelial cells. Thus, we consider it a functionally interfering antibody against human Clever-1, similar to mStab1-1.26 for the mouse. For the purposes of treating human patients, however, a mouse antibody would not necessarily be suitable. Therefore, the fully human IgG_4 antibody bexmarilimab, also referred to as FP-1305, was developed from the 3-372 clone's complementarity-determining region. Based on preclinical characterization, bexmarilimab retains its parental antibody's specific binding to Clever-1, inhibits acLDL uptake and induces the secretion of TNF especially when coincubated with LPS—however, it does not inhibit the phagocytosis of apoptotic cells or bacteria (Hollmén *et al.*, 2022). Furthermore, to limit Fcγ-related immune responses, bexmarilimab contains the L237E mutation, which, in practice, makes it unable to bind either activating FcγRs or C1q (Hollmén *et al.*, 2022). Thus, any effects bexmarilimab has should result from Clever-1 binding, although it does retain low affinity for the inhibitory FcγRII, which could be relevant considering that TAM re-education by antibody-mediated interference of MARCO relies on its FcγRII-mediated internalization (Eisinger *et al.*, 2020). In preclinical studies, bexmarilimab did not show signs of provoking cytokine storms or other toxicities. Since the end of 2018, the tolerability, safety and efficacy of bexmarilimab has been studied on patients with solid cancers refractory to checkpoint blockade in the phase I/II clinical trial MATINS. By January 2022, altogether 193 patients had been enrolled to MATINS and 138 patients belonging to 11 cancer cohorts treated with bexmarilimab, which has been well-tolerated even at the highest dose levels, the most common adverse events related to treatment being fatigue, abdominal pain and anemia in approximately 20–30 % of patients. Importantly,

bexmarilimab has shown promising increases in disease control rate—the sum of partial responses and stabilized diseases—in, on average, 34 % of patients with breast, gastric or hepatocellular cancer, cholangiocarcinoma or cutaneous melanoma who had not responded to previous lines of therapy (Bono *et al.*, 2022).

To extensively profile these patients' ongoing systemic immune responses, inasmuch as can be determined from the blood, we used CyTOF with two custom antibody panels before and after the administration of bexmarilimab. Notably, a single dose of bexmarilimab induced the M2-to-M1 re-education of monocytes, which was rather reminiscent of mStab1-1.26's effect on the mouse. We observed this as the downregulation of CD206 and CD163, fundamental M2 markers, and the upregulation of proinflammatory genes in bulk RNA-seq of $CD14^+$ monocytes. Importantly, patients' $CD14^+$ monocytes expressed Clever-1 at a comparable level to healthy controls. After administration of bexmarilimab, the Clever-1 on patients' monocytes was also well-occupied, although Clever-1 occupancy did clear to an extent over the following two weeks. Interestingly, in a subset of patients, Clever-1 was immediately significantly less occupied by bexmarilimab than in others. Indeed, the magnitude of M2-to-M1 re-education positively correlated with Clever-1 occupancy, suggesting that this effect was specifically mediated by the binding of bexmarilimab to monocyte Clever-1. Therefore, for predicting positive responses, it would be of interest to find out what sink could swallow up bexmarilimab so rapidly in some patients. Moreover, we saw that bexmarilimab downregulated the LXR/RXR and PPAR nuclear receptor signaling pathways in the RNA-seq data. We hypothesize that this effect is secondary to the inhibited scavenging of lipoproteins caused by Clever-1 interference, although the downregulation of these pathways can also underlie the increased proinflammatory activity of monocytes in patients treated with bexmarilimab. For example, increased LXR/RXR activity was recently linked to the polarization of "fat" TAMs with upregulated scavenger receptors and cholesterol metabolism that were associated with negative outcomes (Donadon *et al.*, 2020). Inhibiting this pathway secondhand to Clever-1 blockade could circumvent the possible off-target effects of inhibiting LXR/RXR systemically with, for example, small-molecule inhibitors, as these receptors are also central regulators of the function of the liver and spleen.

As a very positive signal, we observed that bexmarilimab led to robust peripheral immune activation in most treated patients. Indeed, peripheral T-cell activation can predict positive responses also to checkpoint blockade (Wu *et al.*, 2020). Various activation markers such as CD25, CD69 and CXCR3 were upregulated by bexmarilimab in most T-cell clusters we identified with CyTOF, while several immune checkpoint molecules, including CTLA4, LAG3 and PD-L1, were downregulated on T_H-cell clusters. Additionally, the numbers of NK and B cells also increased while the numbers of T_{REG} cells decreased in most patients, but we observed the most

prominent effects on clusters of naïve T_H cells and CTLs as well as effector CTLs, which significantly upregulated the proliferation marker KI67 and activation markers CD25 and CXCR3 in response to bexmarilimab. CD25, the α chain of the IL-2 receptor, is required for T cells to respond to IL-2—after TCR stimulation, it moves onto the cell membrane to enable the clonal expansion of activated T cells. As an exception, CD25 is constitutively located on the surface on T_{REG} cells, which deplete IL-2 from the extracellular space. However, bexmarilimab did not affect CD25 expression on T_{REG} cells. Expression of the chemokine receptor CXCR3, on the other hand, is upregulated on T_H1 cells and CTLs. Its ligands, CXCL9, CXCL10 and CXCL11—all induced by IFNγ and associated with the M1 activation state—promote $CXCR3^+$ T-cell migration into inflamed peripheral tissues, including tumors if they express these chemokines (De Simone *et al.*, 2019). Bexmarilimab also increased the systemic levels of both IFNγ and CXCL10. Interestingly, this effect was more pronounced in patients whose pretreatment levels of these cytokines were low, suggesting that Clever-1 blockade could be more efficient in immunologically cold patients (Bono *et al.*, 2021; Koivunen *et al.*, 2022). This was also the case with mouse models, where the cold tumor LLC1, which is high in Clever-1^+ TAMs but low in CTLs, had a significantly greater response to mStab1-1.26 than the hot tumor CT26.WT, which is high in CTLs already without treatment. Moreover, in a subset of patients, CTLs upregulated perforin and the secretion of IL-2 and IFNγ in response to bexmarilimab, supporting increased cytotoxic potential. Thus, we showed that a single dose of bexmarilimab is sufficient to break peripheral immunosuppression and activate T cells in a subset of patients with highly pretreated cancers that are refractory to currently available standard-of-care.

The indications eligible for the MATINS trial were preselected to include cancers that are most likely to contain Clever-1^+ TAMs and in which Clever-1 expression significantly associates with negative outcomes. It was therefore unfortunate that, in the trial itself, pretreatment tumor biopsies from several patients actually had quite low Clever-1 expression. Thus, adding some threshold frequency of Clever-1^+ TAMs to the inclusion criteria could possibly result in more consistent responses, since a drug is, unsurprisingly, much more likely to work when its target is also present. For example, in the pretreatment sample of one patient with colorectal cancer who had a partial response to bexmarilimab treatment, we noted that the primary tumor contained a very high amount of Clever-1^+ TAMs and very few CTLs. After receiving bexmarilimab, this patient presented with the expansion of a CTL clone that was high in granzyme A, suggesting cytotoxic activity. Unfortunately, the patient in question developed several inflammatory disorders and had to discontinue the study before donating a posttreatment biopsy from which we could have validated whether the expanded granzyme A^+ CTL clone actually infiltrated the tumor. We could, instead, analyze Clever-1 expression and CTL infiltration in paired pre-

and posttreatment tumor sections from five other patients—none of whom, however, had clinical responses to bexmarilimab. Nevertheless, we observed that bexmarilimab was more likely to decrease the number of Clever-1^+ TAMs the higher their number pretreatment was. Moreover, bexmarilimab appeared to convert one seemingly immune-excluded tumor hot by inducing tumor infiltration of peritumoral CTLs and turn one immune desert into an immune-excluded tumor by increasing the accumulation of peritumoral CTLs. If the conclusions we drew from mouse models translate to humans, the execution of bexmarilimab's antitumor activity would ultimately depend on CTLs. Because many standard lines of therapy the patients may have gone through before enrolling in MATINS include drugs that also deplete T cells, a low CTL count could simply be insufficient to produce a clinical response even if bexmarilimab activates these CTLs. Additionally, it seems that some patients' T cells could simply be unresponsive to bexmarilimab-induced IFN stimulation (Boukhaled *et al.*, 2022). Moreover, Clever-1 interference in the absence of Clever-1^+ TAMs could potentiate its plausible inhibitory effect through blocking Clever-1 on the tumor or lymphatic endothelium, which could suppress both peripheral T-cell activation as well as tumor infiltration by peripherally activated CTLs while, because of the lack Clever-1^+ TAMs, having no cold-to-hot converting effect on the TME. Nevertheless, an increased disease control rate in a third of patients is, actually, rather positive in comparison to results from other recent clinical trials investigating experimental cancer immunotherapies. Thus, the finetuning of eligibility criteria to preselect the patients most likely to benefit from bexmarilimab treatment as well as the discovery of biomarkers indicative or predictive of positive responses are the next challenges in the continued clinical development of Clever-1 blockade.

7 Summary

The work I present in this PhD thesis clarifies many previously unknown aspects of the scavenger receptor Clever-1 in the suppression of immune responses and supports further clinical investigation of Clever-1 blockade as an immunotherapeutic cancer treatment. We investigated how myeloid Clever-1 participates in the regulation of adaptive immunity and found that myeloid Clever-1 is an endogenous immunosuppressive molecule that limits monocyte and macrophage overactivation and, consequently, inhibits both B- and T-cell responses. We evaluated the suitability of Clever-1 as an immunotherapeutic drug target preclinically and produced proof-of-concept of M2-to-M1 macrophage re-education and activation of antitumor immunity following antibody-mediated Clever-1 interference. Importantly, we demonstrated that both macrophages and CTLs, the latter of which we determined to ultimately mediate the antitumor effect, were indispensable for improved tumor control. We elucidated the molecular mechanisms by which Clever-1 regulates the suppressive activation state and function of macrophages and discovered that Clever-1 putatively suppresses cross-presentation by increasing v-ATPase activity and lysosomal degradation of protein antigens. Finally, we studied the effects of immunotherapeutic Clever-1 blockade on the immune responses of patients with cancers refractory to available standard-of-care lines of treatment and obtained first clinical evidence that Clever-1 blockade may promote antitumor immunity especially in a subset of patients with immunologically cold tumors. Overall, the results of this PhD thesis support continuing the clinical assessment of Clever-1 blockade as a novel immunotherapy of cancer.

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