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Characterization of anti-tumour myeloid cells in an orthotopic mouse model of mammary adenocarcinoma

Miguel Alexandre Ferreira Pinto

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Abbreviation list

APCs	Antigen presenting cells
ASC	Associated speck-like protein
ATMa	Anti-tumour macrophages
ATMc	Anti-tumour myeloid cells
ATNe	Anti-tumour neutrophils
ATP	Adenosine triphosphate molecule
BAFF	B cell–activating factor
BCG	Bacillus Calmette–Guérin
CD115	Colony-stimulating factor 1 receptor
cDMEM	Dulbecco's Modified Eagle's Medium with 10% FCS 1% penicillin/streptomycin
CDPs	Common DC precursors
cMoPs	Common monocyte progenitors
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte associated antigen 4
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DLBCL	Diffuse large B cell lymphoma
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
FACS	Fluorescence-activated cell sorting
FCS	Fetal Calf Serum
FcεR	IgE receptor

GM-CSF	Granulocyte macrophage colony-stimulatory factor
G-MDSC	Granulocytic MDSC
GMP	Granulocyte/macrophage progenitor
H60	Dominant minor antigen 60
HSCs	Haematopoietic stem cells
Hsps	Heat shock proteins
IFN	Interferon
IFN-γ	Interferon γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal injection
IT	Intra-tumour injection
IV	Intravenous injection
LLC	Lewis lung carcinoma
LPS	Lipopolysaccharides
M1	Classically-activated macrophage
M2	Alternatively-activated macrophage
mAb	Monoclonal antibody
MC	Myeloid cells
MCA	Methylcholanthrene
mCRPC	Metastatic castration-resistant prostate cancer
M-CSF	Colony-stimulating factor 1
M-CSFR	Colony-stimulating factor 1 receptor
MDPs	Macrophage and DC precursors
MDSC	Myeloid-derived suppressor cells

MFI	Mean fluorescent intensity
MHCI	Major histocompatibility complex class I
MHCII	Major histocompatibility complex class II
Mo-MDSC	Monocytic MDSC
NETs	Neutrophil extracellular traps
NK	Natural killer cells
NLR	NOD-like protein
NO	Nitric oxide
NOS	Nitric oxide synthase
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PD-1	Programed death 1
PDA	Pancreatic ductal adenocarcinoma
PMA	Phorbol 12-myristate 13-acetate
PSA	Prostate specific antigen
PTMa	Pro-tumour macrophages
PTMc	Pro-tumour myeloid cells
PTNe	Pro-tumour neutrophils
Rae-1	Retinoic acid early transcript 1
Rag2–/–	Recombination activating gene 2 deficient mice
rpm	Rotations per minute
SEM	Standard error of mean
TAA	Tumour associated antigen
TADCs	Tumour associated dendritic cells
TAM	Tumour associated macrophages

TAN	Tumour associated neutrophils
TCR	T cell receptor
TGF-β	Transforming growth factor β
TH1	T helper type 1 cells
TH2	T helper type 2 cells
TICAM1	Toll-like receptor adaptor molecule 1
TILs	Tumour infiltrating lymphocytes
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor α
Treg	Regulatory T cell
WT	Wild-type

Abstract

The tumour microenvironment is composed of haematopoietic and stromal cell types that interact dynamically with tumour cells, thereby impacting on cancer development and progression. The recent realization that infiltrating myeloid cells display pro-tumour features is reflected in the increase in myeloid-derived suppressor cells in the blood of cancer patients, which is associated with bad prognosis. Moreover, in mouse models, the depletion of pro-tumour myeloid cells (PTMc) inhibits tumour growth and reduces the level of immunosuppression in the tumour microenvironment, thus releasing the anti-tumour activities of CD4+ and CD8+ T lymphocytes.

To date, available strategies to manipulate PTMc include ablation (via antibody depletion or signalling blockade of myeloid cell survival factors) or inhibition of their pro-tumour features. However, myeloid cells can undergo anti-tumour programming in response to stimulating agents like tissue damage signals, TLR and costimulatory receptor agonists. Polarized anti-tumour myeloid cells (ATMc) display tumoricidal functions and efficiently produce inflammatory cytokines, such as TNF-α, IFN-γ, IL-12 and enzymes like iNOS, while expressing low levels of T cell suppressive cytokines such as IL-10. ATMc are responsible for the priming of CTLs thus inducing tumour regression. Despite these findings, information characterizing anti-tumour features, functions, phenotype and transcriptional program of ATMc are critically lacking. Consequently, this project aimed at developing novel approaches to “reprogram” myeloid cells into ATMc *in vivo* during tumour responses and characterize their anti-tumour features.

In this report we successfully demonstrate that myeloid cells can be induced to perform anti-tumour functions upon selective stimulation *in vivo*. In the orthotopic E0771 mammary tumour model, injection of TLR3 ligand dsRNA and agonist anti-CD40 mAb stimulated macrophages to produce anti-tumour cytokines and enzymes. We defined anti-tumour macrophages as CD11b+F4/80+Ly6C(+/-) cells that co-produced TNF-α and IL-1β. Our data suggest a crosstalk between macrophages and CD8+ T cells, promoting potent anti-tumour immune responses and leading to tumour complete eradication in 90% of the treated animals. This approach provides a solid platform to question whether different TLR agonists may trigger functional diversification of ATMc of various lineages such as neutrophils. Our findings open the way to dissecting the transcriptional program(s) that operate in ATMc, aiming at new strategies to manipulate MC in the context of cancer.

Keywords: Myeloid cells, TLR agonists, anti-tumour functions, orthotopic E0771 mammary tumour model

Resumo

O microambiente tumoral é composto por diversos tipos de células como as hematopoéticas ou do estroma que interagem com células tumorais, contribuindo para o desenvolvimento e crescimento do tumor. Recentemente, vários estudos têm demonstrado que as células mielóides podem apresentar funções pró-tumorais e que a sua presença no sangue de pacientes com cancro está associada a um mau prognóstico. Em modelos de ratinho, a eliminação de células mielóides com funções pró-tumorais (PTMc) inibe o crescimento tumoral e aumenta a atividade anti-tumoral dos linfócitos T CD4+ e CD8+.

Até à data, as estratégias disponíveis para a manipulação de células mielóides passam pela depleção, através de anticorpos ou inibição de vias de sinalização, ou pelo bloqueio das suas características pró-tumorais. Todavia, as células mielóides podem adquirir funções anti-tumorais em resposta a sinais de dano, ligandos de recetores de Toll (TLR) e moléculas co-estimuladoras. Deste modo, as células mielóides polarizadas para um fenótipo anti-tumoral (ATMc) apresentam funções tumoricidas produzindo citocinas inflamatórias como o TNF-α, IFN-γ, IL-12 e enzimas como o iNOS e expressando menor quantidade de citocinas supressoras de células T como a IL-10. Assim, as ATMc ativam células T citotóxicas (CTLs) que, por sua vez, eliminam células tumorais. Apesar destas descobertas, informações sobre as características, funções e fenótipo das ATMc são ainda muito escassas. Deste modo, o nosso projeto teve como objetivo desenvolver novas abordagens que permitam a manipulação das células mieloides *in vivo* durante respostas tumorais, caracterizando as suas funções anti-tumorais.

Neste estudo demonstrámos com sucesso que as células mielóides podem ser estimuladas a apresentar funções anti-tumorais *in vivo*. No nosso modelo ortotópico E0771 de células tumorais mamárias de ratinho, a injeção intra-tumoral do ligando dsRNA do TLR3 e do anticorpo agonista monoclonal anti-CD40, estimulou a produção de citocinas e enzimas anti-tumorais por macrófagos. Por conseguinte, definimos que os macrófagos anti-tumorais expressam os marcadores CD11b+F4/80+Ly6C(+/-) e que produzem TNF-α e IL-1β. Os nossos dados sugerem um *crosstalk* entre macrófagos e células T CD8+, gerando uma potente resposta imune anti-tumoral responsável pela completa erradicação do tumor em 90% dos animais tratados. Esta abordagem oferece um ponto de partida para o estudo de outros agonistas de TLR que estimulem funções anti-tumorais em diferentes linhagens de células mielóides como os neutrófilos. Os nossos resultados são promissores e permitem aspirar à descoberta dos programas de

transcrição responsáveis pela formação de células mielóides anti-tumorais (ATMc), fornecendo informações para a sua melhor manipulação no contexto tumoral.

Palavras-chaves: Células mielóides, agonistas de TLR, funções anti-tumorais, modelo ortotópico de células tumorais mamárias de ratinho

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Introduction

1. The immune system and its immune cell functions

The **immune system** is composed of several cell types with specialized roles in defending against infections and transformed cells (Nicholson 2016). In case of external aggression of host cells, immune cells can recognize foreign pathogens and generate a response able to fight them. Mucus and enzymes are the first barrier to prevent the foreign aggressors to establish an infection. If they penetrate, there is a recruitment of immune cells to the site of infection that phagocyte infectious agents, produce anti-microbial peptides and secrete cytokines able to initiate the process responsible for their elimination (Mackay et al. 2000).

Beutler states that all “true” immune system must be able to discriminate between self and non-self, tolerating self (sparing host tissues from damage) and attacking non-self (Beutler 2004). Accordingly to this point of view, Charles Janeway and Ruslan Medzhitov believe that the major decision to respond, or not, to a particular ligand or antigen is decided by the genome encoded innate immune system receptors present in the surface of immune cells. When the immune cells encounter pathogen-associated molecular patterns (PAMPs), such as flagellin, lipopolysaccharides (LPS) and/or nucleic acids like viral dsDNA/RNA and unmethylated CpG dinucleotides in bacterial DNA, they recognize them as non-self and create an immune response against the pathogens that have them. However, the immune system responds as well to damage-associated molecular patterns (DAMPs), such as nuclear or cytosolic proteins, heat shock proteins (Hsps), adenosine triphosphate molecule (ATP) outside cells and host cell’s DNA and RNA (Matzinger 2002). Therefore and in contrast to Janeway and Medzhitov, Polly Matzinger proposed the Danger model, which suggests that the immune system is more concerned with the damage done in host cells than with the foreignness of a certain pathogen. The immune system is then called into action by alarm signals released by injured tissues, rather than by the recognition of non-self pathogens or molecules (Matzinger 2002).

There are two distinct types of responses: **Innate and adaptive immunity** responses.

Innate immunity encompasses host defences at the time a danger signal is released. Its role is to limit spreading of an infection and constrain the aggression of infectious pathogens. It has no specificity for any type of infectious pathogen or transformed cells

and occurs as many times the alarm signal appears with similar amplitude and speed (Nicholson 2016) (Matzinger 2002).

Myeloid cells (MC); macrophages, dendritic cells (DCs), granulocytes (neutrophils, eosinophils and basophils) and mast cells; natural killer (NK) cells, γδ T cells and NKT cells are immune cells responsible for innate immune responses. Macrophages, DCs and neutrophils are able to phagocyte pathogens and produce inflammatory cytokines. Macrophages also promote wound healing and regulate adaptive immunity by activating T cells through the production of cytokines or functioning as antigen presenting cells (APCs). DCs are specialized APCs, effectively stimulating immune responses and promoting effector T cell differentiation (Janeway & Medzhitov 2002). Basophils, eosinophils and mast cells produce inflammatory mediators. NK cells are able to destroy infected and transformed cells, in particular those which do not display the major histocompatibility complex class I (MHCI) molecule in their surface. They induce apoptosis in transformed cells via production of perforin and granzyme (Bodduluru et al. 2015). Other pathway responsible for the induction of apoptosis is the Fas/Fas ligand pathway. The engagement of Fas by Fas ligand results in apoptosis, mediated by caspase activation (Trapani & Smyth 2002) (Waring & Müllbacher 1999). NKG2D is an activation receptor expressed on the cell surface of NK cells, NKT cells, γδ T cells, some cytotoxic CD8+ T cells and a small subset of CD4+ T cells associated with detection and elimination of transformed cells. NKG2D can recognize stress-induced molecules, such as retinoic acid early transcript 1 (Rae-1) and dominant minor antigen 60 (H60) displayed on the surface of transformed and infected cells, promoting their destruction (Smyth et al. 2005).

Adaptive immunity, as opposed to innate immunity, creates an immunological memory against specific pathogens or antigens. After an encounter with a given pathogen, it mounts faster and more robust responses, capable of protecting the host from secondary exposures and being the main immune system characteristic for vaccination (Beutler 2004) (Janeway & Medzhitov 2002). Adaptive immunity includes responses mediated by T cells and B cells against a specific target, following the exposure to a given antigen displayed by APCs. T cells are divided in αβ T cells and γδ T cells, according to the chains that constitute their T cell receptor (TCR). Naïve αβ T cells circulate in the blood and are activated in secondary lymphoid organs by APCs that process antigens and display them conjugated with MHCI and II molecules in their surface. T cells perform helper (CD4+ T cells) or cytotoxic (CD8+ T cells) functions. CD4+ T cells secrete cytokines and CD8+ T cells produce cytolytic enzymes. CD4+ T cells play

a central role in the immune responses, and are required for CD8+ T cell responses and B cell responses. Through the production of cytokines and chemokines, they can orchestrate immune responses by aiding B cells create antibodies, inducing macrophages to develop enhanced inflammatory and microbicidal activity, recruiting neutrophils to sites of infection and inflammation and inducing anti-tumour cytotoxic T lymphocytes (CTL) responses (Zhu & Paul 2008) (Melief 2013). CD4+ T cells can differentiate into T helper cells type 1 and type 2. T helper type 1 cells (TH1) produce interferon γ (IFN-γ), interleukin 2 (IL-2) and tumour necrosis factor (TNF), and are able to activate macrophages and induce cell-mediated cytotoxicity. T helper type 2 cells (TH2) CD4+ T cells produce large amount of IL-4, IL-10, and IL-13, and induce strong antibody responses, but inhibit several inflammatory functions of phagocytic cells (Romagnani 2000). A population of CD4+ regulatory T cells (Treg) are responsible for immunological self-tolerance by actively suppressing self-reactive lymphocytes. They express Foxp3, a key transcription factor for their development, and are identified as CD4+Foxp3+ T cells (Hori et al. 2003). The CD8+ T cells can become activated and classified as CTLs that induce cell-mediated cytotoxicity through perforin/granzyme or Fas/FasL apoptosis pathways. B cells secrete antigen-specific antibodies (immunoglobulins) that bind to viruses, microbial toxins and surface antigens, eliminating microorganisms and tumour cells.

Some lymphocytes of the adaptive immunity also display features of the innate immunity, developing a very rapid and pre-committed response to a given stimuli. These include γδ T cells and NK T cells, which represent a bridge between the two arms of the immune system. γδ T cells mainly populate epithelial tissues and respond to stress signals, recognizing stress-induced self-antigens (Hayday 2000). Similarly, NKT cells are a subset of T cell that have similar features with both, conventional T cells and NK cells (Godfrey et al. 2004). Thus, both γδ and NK T cells combine expression of a TCR, can recognize antigens without restriction to MHCI or II associated peptide ligands and express NK cell functional cytotoxic receptors on their cell surface. Thus, like NK cells, γδ T cells have cytolytic functions capable of eradicating infected and transformed cells (Groh et al. 1998) (Hayday 2009).

2. Myeloid cells lineages and functions in immunity

2.1. Myeloid cells lineages and functions

The immune cells present in the blood arise from pluripotent haematopoietic stem cells (HSCs) in the bone marrow. The HSCs can divide in two types of multipotent stem cells: a common lymphoid progenitor, which gives rise to NK cells and T and B lymphocytes, and a common myeloid progenitor, that gives rise to leukocytes, erythrocytes and platelets (Janeway et al. 2005). MC are composed of monocytes, macrophages, granulocytes (neutrophils, eosinophils and basophils), dendritic cells and mast cells. They are extremely short-lived (survive less than three days before undergoing apoptosis) when compared to lymphoid cells and perform important functions in the innate immune response.

Markers

CD11b is an integrin associated with various adhesive interactions of MC. It is expressed on monocytes, macrophages, granulocytes and in some DCs and B cells, thus being a marker for myeloid cells (McNally & Anderson 2002) (Cassado et al. 2015).

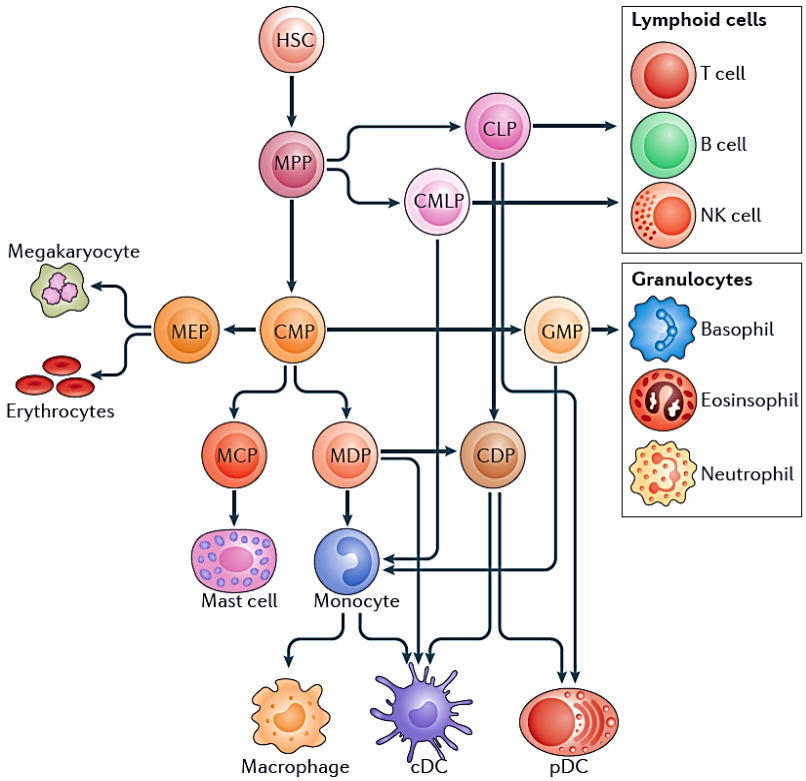


Figure 1: Myeloid cell lineages differentiation under normal physiological conditions. Adapted from Gabrilovich, Ostrand-Rosenberg and Bronte 2012

2.2. Monocyte differentiation and functions

Monocytes can be generated within the fetal liver and in the bone marrow in adult life. They are derived from macrophage and DC precursors (MDPs) and common monocyte progenitors (cMoPs), which arise from granulocyte/macrophage progenitor (GMP) in the bone marrow (Ginhoux & Jung 2014). Adult monocytes are present in the bone marrow, blood and spleen and their development and survival is dependent on colony-stimulating factor 1 (also referred as M-CSF). Mice deficient in M-CSF or its receptor, CSF1R (also known as CD115 or M-CSFR), exhibit severe monocytopenia (Dai et al. 2002). Monocytes patrol extravascular tissues and remain immature, being able to undergo a differentiation program. They acquire specific functions through the response to signals derived from damaged tissues, and lymphocytes. During infection and inflammation, monocytes migrate to affected tissues where they produce cytokines and differentiate into macrophages or DCs. Their migration and differentiation is determined by the inflammatory milieu where they carry out specific effector functions (Geissmann et al. 2010). During inflammation, they produce tumour necrosis factor α (TNF-α), IL-1β, inducible nitric oxide synthase (iNOS) and upregulate MHCII molecules (Biswas & Mantovani 2010) (Yang et al. 2014). Auffray and colleagues denote two types of monocytes: **Inflammatory monocytes**, recruited into inflamed tissues and lymph nodes, and **patrolling monocytes,** found in both resting and inflamed tissues (Auffray et al. 2007). **Inflammatory monocytes** express the cell surface protein Ly6C, L-selectin and the chemokine receptor CCR2 and can differentiate into inflammatory macrophages and DCs. Some studies suggest that they can also differentiate, mostly in the circulation, into Ly6Clow monocytes (Yona et al. 2013). By contrast **patrolling monocytes** express high levels of the chemokine receptor CX3CR1, LFA-1 integrin (and lack the expression of Ly6C, CCR2 and L-selectin). The different adhesion molecules and chemokine receptors expression, suggest different types of tissue trafficking. Intravital microscopy, revealed that monocytes within most blood vessels exhibited “constitutive crawling type motility” and, in contrast, “rolling” monocytes were observed only transiently in the mesenteric veins and not in arteries, giving rise to the two monocyte subsets (Auffray et al. 2007).

2.3. Macrophage ontogeny

Studies based on the inactivation of the transcription factor c-Myb, critical for HSCs development, showed that adult tissue-resident macrophages were derived during embryogenesis from yolk sac progenitors and not from blood monocytes (Schulz et al. 2012). Thus, macrophage populations can result from primitive haematopoietic

progenitors generated in the yolk sac and fetal liver and from bone-marrow monocytes (Lahmar et al. 2016) (Ginhoux & Jung 2014). **Yolk sac-derived macrophages** are the first to colonize the tissues but their population is later diluted with the arrival of **fetal liver-derived monocytes**. Brain microglia arise predominantly from yolk sac-derived macrophages while lung alveolar macrophages, liver Kupffer cells, epidermal Langerhans cells and heart macrophage populations are mainly resultant from fetal liver-derived monocytes. All tissues also contain adult **monocyte-derived macrophages** generated from the bone marrow (Ginhoux et al. 2010) (Ginhoux & Jung 2014). Despite differences in ontogeny, all tissue-resident macrophages are important for tissue integrity and manifest a suppressive function in steady-state conditions. For example, yolk sac–derived microglia and fetal liver–derived alveolar macrophages exert immunosuppressive functions by selectively inhibiting T cell activation and proliferation (Ginhoux et al. 2015). In contrast, during inflammation, blood monocytes are recruited and give rise to monocyte-derived macrophages and DCs that undergo programming of their functional properties in response to signals derived from microbes, damaged tissues, and activated lymphocytes (Biswas & Mantovani 2010). Altogether, these evidences suggest that it is the tissue that determine macrophage functions and not their origin (Guilliams & Scott 2017).

2.4. Macrophage functions during immune responses

Macrophages can be activated by a variety of stimuli, initiating and actively participating in the polarisation of immune responses (Duque & Descoteaux 2014). After exposed to inflammatory stimuli, macrophages secrete cytokines, such as TNF-α, IL-1, IL-6, IL-8, and IL-12, chemokines, leukotrienes, prostaglandins, and complement proteins. These molecules induce increased vascular permeability and recruitment of inflammatory cells, allowing for the extravasation of monocytes and neutrophils and infiltration of lymphocytes to the site of inflammation (Duque & Descoteaux 2014). **TNF-α** and **IL-1β** are two of the most prominent pro-inflammatory cytokines expressed by macrophages. **TNF-α** is a glycoprotein that was initially described for its ability to induce necrosis in tumours and is responsible for the destruction of transformed cells by macrophages (Carswell et al. 1975). **IL-1β** is a pro-inflammatory cytokine secreted by macrophages that help in the elimination of infectious agents. It is capable of inducing strong and durable CD4+ T cell responses and enhance their expansion and differentiation (Ben-Sasson et al. 2009). Upon toll-like receptors (TLR) activation, IL-1β is produced via the inflammasome, a multimolecular complex composed of NOD-like protein (NLR), apoptosis-associated speck-like protein comprehending a caspase recruitment domain (ASC) and caspase-1. Caspase-1 is activated through interaction with ASC and is responsible for the cleavage of

pro-IL-1β into its active form (Tschopp et al. 2003) (Guo et al. 2016). Macrophages also directly promote CD4+ and CD8+ T cell proliferation and maturation through their APC functions (Moser 2001) (Pozzi et al. 2005). Adoptive transfer of macrophages demonstrated that they could prime naive CD8+ T cells to proliferate and mature into both effector and memory cells (Pozzi et al. 2005).

Markers

Almost all mouse mature macrophages express a unique surface antigen - a epidermal growth factor glycoprotein - recognized by the F4/80 monoclonal antibody (mAb). F4/80 mAb bind to lung, spleen, thymus and peritoneal macrophages, blood monocytes and bone marrow derived macrophage precursors (Morris et al. 1991) (Austyn & Gordon 1981). F4/80 mAb also recognise eosinophils in several organs (Cassado et al. 2015) but they are distinguished from macrophages due to their expression of siglecF, a lectin receptor (Zimmermann et al. 2008).

2.5. Granulocyte origin and functions during immune response.

Granulocytes (neutrophils, eosinophils and basophils) differentiate from GMPs and are characterized by the presence of cytoplasmic granules and a particular nuclear morphology.

Neutrophils are the most abundant of the granulocytes and can be commonly referred as polymorphonuclear leukocytes. They are specialized in phagocytosis, having a complex machinery to engulf and destroy pathogens (Beutler 2004; Gabrilovich et al. 2012). They are short-lived cells, surviving in the circulation for only about eight to ten hours before undergoing apoptosis. Neutrophils can destroy pathogens through phagocytosis (uptake of opsonized and/or engulfed pathogens), production of reactive oxygen species and release of cationic peptides (degranulation), and formation of neutrophil extracellular traps (NETs) (Mayadas et al. 2014). They can also produce cytokines and chemokines, such as B cell–activating factor (BAFF), IL-23 and CCL3, able to regulate other immune cell migration and functions (Mayadas et al. 2014). Neutrophils were also reported as APCs, expressing low levels of MHCII molecules and costimulatory molecules after stimulation with IFN-γ, granulocyte macrophage colony-stimulatory factor (GM-CSF) and IL-3 (Abdallah et al. 2011).

Eosinophils mostly accumulate during parasitic infections and are weakly phagocytic. Upon activation, they kill parasites mainly by releasing cationic proteins and reactive oxygen metabolites into the extracellular fluid (Mackay et al. 2000). They also

promote inflammation by secreting leukotrienes, prostaglandins, and various cytokines. Eosinophils have been also reported to mediate cytotoxicity activity against some types of tumours and support tumour rejection when CD8+ T cells where present in the microenvironment (Carretero et al. 2015) (Gatault et al. 2015).

Basophils and mast cells have similar functions. Basophils are present mainly in the blood whereas mast cells are predominant in the skin, airways and intestine. Both types of cells possess high-affinity receptors for IgE (FcεR) and thereby become coated with IgE antibodies. In atopic allergies such as eczema, hay fever, and asthma, allergens bind to the IgE that cross-links the FcεR and triggers the cell to secrete inflammatory mediators such as histamine, prostaglandins, and leukotrienes producing a strong allergic and inflammatory response (Mackay et al. 2000).

Markers

Ly6G is a surface protein expressed on neutrophils, and transiently during developmental stages of monocytes. In 2004 it was confirmed the absence of cross-reactivity between murine Ly6C and Ly6G mAbs, allowing for a better discrimination of myeloid cells (Nagendra & Schlueter 2004). As described before, eosinophils can be differentiated from the other populations through their expression of siglecF (Zimmermann et al. 2008).

2.6. Dendritic cells ontogeny and functions during immune response.

DCs are terminally differentiated myeloid cells that specialize in antigen processing and presentation to T cells (Gabrilovich et al. 2012). DCs differentiate in the bone marrow from MDPs that derive in common DC precursors (CDPs). During infection or inflammation, they can also differentiate from blood monocytes (Geissmann et al. 2010).

DCs reside in tissues as immature cells that actively uptake tissue antigens and phagocyte pathogens, but are poor antigen presenters. They have low expression of MHC molecules and lack B7 co-stimulatory molecules. When functionally activated, DCs maturate and migrate to the lymph node where they synthesize MHC molecules and express B7 molecules, in order to effectively stimulate naïve antigen-specific T cells. They start to express a large number of adhesion molecules, which stabilizes the interaction between T cell TCR and peptides presented through MHC molecules. DCs also secrete cytokines like IL-12 and IL-18 to promote TH1 and cytotoxic CD8+ T cell responses (Greenwald et al. 2005) (Vanneman & Dranoff 2014) (Merad et al. 2013). CD40 signalling, was also reported to induce the upregulation of MHCII, co-stimulatory and adhesion

molecules in DCs, which make them more effective APCs (Ma & Clark 2010). CD40 ligand induces the production of massive amounts of IL-12 by DCs, resulting in enhanced T cell stimulatory capacity (Celia et al. 1996).

Markers

CD11c expression is a marker of DCs but it is not specific (being expressed also by macrophages, monocytes, NK cells, and activated T cells). However, by excluding macrophages with F4/80 mAb (DCs do not express F4/80) one should accurately visualize DCs using flow cytometry (Merad et al. 2013).

3. Macrophage polarization during inflammation

3.1. Macrophage polarization (M1 vs M2).

There are two distinct polarized macrophage states: the **classically-activated macrophage (M1)** and the **alternatively-activated macrophage (M2)** (Biswas & Mantovani 2010). **M1**, typically activated by IFN-γ and LPS, exhibit potent microbicide properties and promote strong IL-12-mediated **TH1** responses. In contrast, **M2**, induced by IL-4 and IL-13, support **TH2** associated effector functions and may play a role in the resolution of inflammation (Geissmann et al. 2010). Factors such as IL-10, glucocorticoids, and transforming growth factor β (TGF-β) induce phenotypes that partially overlap with the M2, contributing to the cytokines associated with M2 features (Ghassabeh et al. 2006).Thus, monocytes are polarized by the microenvironment or inflammatory milieu to mount specific functional programs accordingly to the cytokines that are available (Mantovani et al. 2002).

Classicaly-activated M1 macrophages are effector cells characterized by killing pathogens, eliminating tumour cells and as efficient APCs. M1 produce large amounts of pro-inflammatory cytokines, such as IL-12, IL-23 and TNF-α, iNOS and have higher expression of MHCII and costimulatory molecules (Mantovani et al. 2002). They are polarized during TH1 responses and their ability to produce IL-12, CXCL9 and CXCL10, drive the recruitment of TH1 cells, thereby amplifying the loop for type 1 responses (Biswas & Mantovani 2010). M1 macrophages metabolize arginine via nitric oxide synthase (NOS) to nitric oxide (NO) and citrulline. Thus, arginine metabolism is characterized by high levels of inducible iNOS in M1 macrophages (Rath et al. 2014).

Alternatively-activated M2 macrophages are characterized by high phagocytic activity, high expression of scavenging receptors, promoting angiogenesis, tissue

remodelling and repair and are associated with tumour progression (Mantovani et al. 2002) (Biswas & Mantovani 2010). They express large amounts of IL-10 and low quantities of IL-12. They express a selective set of chemokines, including CCL17, CCL22 and CCL24, which results in the recruitment of TH2 cells, Treg cells, eosinophils and basophils, amplifying polarized TH2 responses (Biswas & Mantovani 2010). M2 macrophages metabolize arginine, via arginase, to ornithine and urea. Thus, M2 are characterized by high production of arginase (Rath et al. 2014).

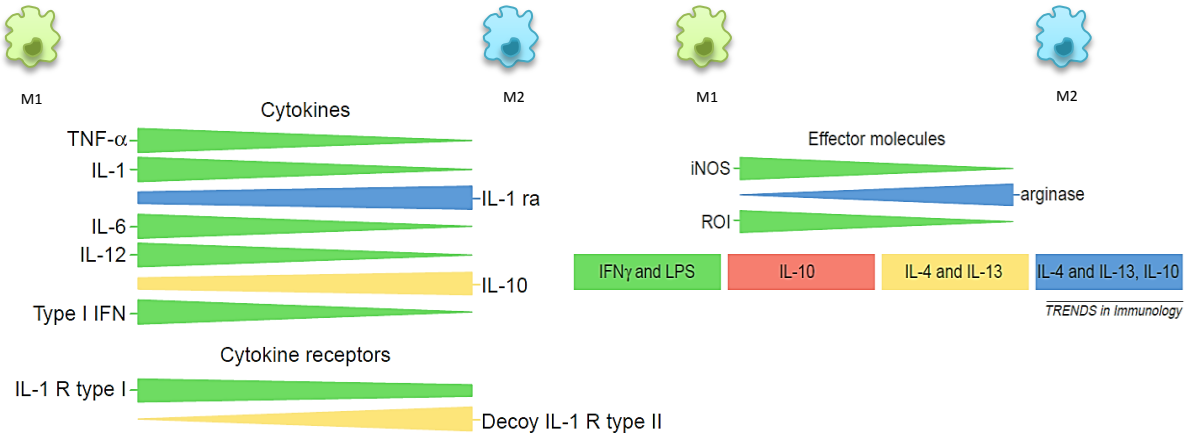


Figure 2: Characteristics of M1, M2 and M2 like polarized macrophages induced by IFN-γ and LPS (in green); IL-10 (in red); IL-4 and IL-13 (in yellow); and IL-4, IL-13, IL-10 (in blue). Adapted from Mantovani 2012

4. Immune cells control tumour development and growth

The concept that the immune system is very important in the destruction of cancer cells and that it can recognize and control tumour growth is not new. In 1893, William Coley used live bacteria as an immune stimulant to treat cancer. After stimulation, the immune system could recognize tumour cells and control tumour growth (Yang 2015). Lewis Thomas (1959) and Frank Burnet (1970) were the first to propose the concept of **tumour immune surveillance.** The tumour immune surveillance hypothesis defends that the immune system has the ability to identify and destroy nascent tumours, operating as a primary defence against cancer (Swann & Smyth 2007). Most recently was demonstrated that the immune system not only can protect the host against tumour development but also, by selecting for tumours of lower immunogenicity, has the capacity to promote tumour growth of resistant malignant cells (Dunn et al. 2004). This dual effect of the immune system on developing tumours prompted Gavin Dunn, Lloyd Old and Robert Schreiber to redefine the cancer immunosurveillance hypothesis into one they termed **cancer immunoediting**.

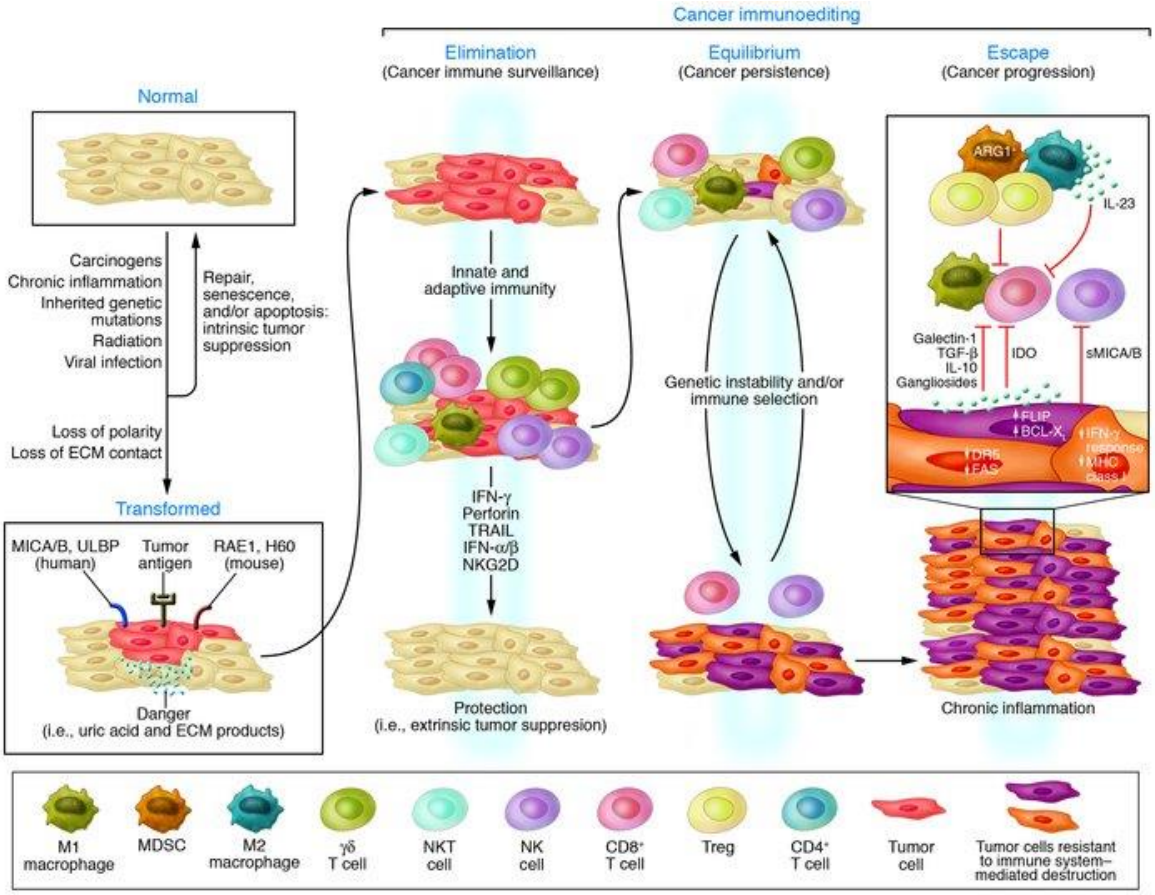


Figure 3: The three phases of cancer immunoediting: elimination, equilibrium and escape. Adapted from Swann and Smyth 2007

They envisaged that the cancer immunoediting process is comprised of three phases: **elimination, equilibrium, and escape** (Dunn et al. 2004). In the **elimination phase**, the immune system detects and eliminates tumour cells that have developed as a result of failed tumour suppressor gene activation or damage repair mechanisms. This phase is complete when all tumour cells are cleared, or incomplete, when only a portion of tumour cells is eliminated. If not all cancer cells are destroyed, it is predicted that they either remain dormant or continue to accumulate DNA mutations and/or changes in gene expression. The tumour cells can, for example, modify the expression of surface antigens evading immune recognition and, consequently, destruction. As this process continues, the immune system exerts a selective pressure by eliminating susceptible tumour clones, which is sufficient to **control tumour progression**. However, the process can result in the selection of tumour cell clones that are able to resist, avoid, or suppress the anti-tumour immune response, leading to the **escape phase**. During the escape phase the immune system is no longer able to contain the progressive tumour growth.

Various animal experiments were performed in order to test the effect of the immune system in controlling tumour formation (Corthay 2014). One of the first approaches was to eliminate specific and essential components of the mouse immune system and observe them for the development of tumours (Swann & Smyth 2007). In 2001, Shankaran and colleagues describe that the immune response functions as an effective tumour-suppressor system, showing that primary **immunodeficiency** in mice is associated with increased cancer risk. In their experiment, recombination activating gene 2 deficient mice (Rag2–/–), which lack both B and T cells, developed chemically methylcholanthrene (MCA) induced tumours earlier than wild-type (WT) mice and with greater frequency (60% vs 20%) (Shankaran et al. 2001). Rag2–/– mice had higher incidence of spontaneous adenocarcinoma of the intestine and lung and intestinal adenomas when compared to WT mice. When Rag2–/– mice were also deficient for STAT1, an important transcription factor mediator of type I and type II interferon (IFN) signalling, tumour incidence was increased and the spectrum of tumours was augmented with breast adenocarcinomas and colon adenocarcinomas (Shankaran et al. 2001). Mice deficient for NKG2D also had a greater incidence of MCA-induced fibrosarcomas than control mice (Smyth et al. 2005). This is due to the NKG2D ligands upregulation on tumour cells, such as Rae1, not being recognized by primed cytotoxic T cells and NK cells, which fail to eliminate cancer cells (Diefenbach et al. 2001). These results show that distinct cell surface ligands, expressed often by tumour cells, but not by normal cells, can be associated with malignant transformation and allowing immune cells to recognize and kill tumour cells. It was also described that mice lacking γδ T cells were highly susceptible in developing cutaneous carcinomas (Girardi et al. 2001). Upon exposure to carcinogens, skin transformed cells express ligands for NKG2D like Rae-1 and H60 allowing for NKG2D+ γδ T to recognize these ligands in the surface of skin carcinoma cells, killing them (Girardi et al. 2001).

A number of **clinical observations** also provided evidence supporting the importance of innate and adaptive immune system in allowing or controlling tumour development. Clinical trials showed that patients with primary immunodeficiency had higher risk of developing lymphoproliferative disorders like non-Hodgkin's lymphomas (60%), Hodgkin's disease (23%) and leukaemia (6%) (Salavoura et al. 2008). Organ transplant patients subjected to immunosuppressive treatments had three times the number of neoplasms when compared with age-matched controls (Penn 1988). **Tumours infiltrated with T cells** have been associated with improved prognoses (Haanen et al. 2006) (Zhang et al. 2003) (Galon et al. 2006). An association between patient prognosis and tumour infiltrating lymphocytes (TILs) was first observed in patients with melanoma. The presence of tumour associated antigen (TAA) specific TILs improved survival in advanced-stage

resected melanoma patients (Haanen et al. 2006). Moreover, in ovarian cancer the presence of TILs correlates with improved progression-free survival and overall survival and is associated with increased expression of IFN-γ and IL-2 (Zhang et al. 2003). In colon-rectal cancer the presence of TH1, cytotoxic and memory T cells was related with low incidence of tumour recurrence (Galon et al. 2006). Treg cells demonstrate an inverse correlation between presence of tumour infiltrating Treg cells and patient survival by suppressing endogenous TAA specific TILs cell immunity (Curiel et al. 2004).

5. Cancer Immunotherapy

The development of novel immunotherapeutic approaches, which aim at stimulating cytotoxic T cells, have revolutionized treatments against cancers. These treatments include infusion of re-activated tumour-infiltrating T lymphocytes, immune checkpoint inhibitors and vaccination strategies (Vanneman & Dranoff 2014).

5.1. Adoptive T cell transfer therapy

Adoptive transfer of TILs was shown to induce long-lasting responses and complete regression of metastatic melanoma tumours (Rosenberg et al. 2011). In combination with IL-2, the response rate was increased in approximately 15%, with 5% durable complete responses. A clinical trial from Rosenberg and colleagues resulted in 56% clinical responses and 22% complete responses of treated patients. However and despite TILs can be harvested from a variety of cancers, with the exception of melanoma TILs adoptive transfer rarely demonstrate tumour elimination (Hinrichs & Rosenberg 2014). To overcome this problem various types of genetically engineered T cells have been created. Adoptive transfer of autologous T cells that are gene transduced to express antigen-specific receptors represents a promising therapy to provide tumour-specific immunity to cancer patients (Hinrichs & Rosenberg 2014). The most promising results from engineered T cells come from the chimeric antigen receptor CAR T cells. CARs are constructed of antibody single-chain variable fragments joined with TCR and costimulatory molecule domains, which confers T cells the ability to bind more efficiently to cell surface antigens. As an example, CAR T cells have been created with good results to target B-cell malignancies using anti-CD19 single Ab chain (Kochenderfer et al. 2010). Novel strategies to target other cancer types are being developed.

5.2. Immune checkpoint inhibitors

A promising novel strategy to treat cancer consists in potentiating the naturally occurring immune response of the patient through **blockade of immune checkpoint molecules**. Once activated, T cells upregulate on their cell surface two co-inhibitory molecules: cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and programed death 1 (PD-1). CTLA-4 binding counters early T-cell activation, leading to a decrease of IL-2 production and inhibition of cell cycle progression. Similarly, PD-1 has been described as a negative regulator of immunity that limits the production of IFN-γ and T cell proliferation, and increasing T-cell apoptosis. It is hypothesised that CTLA-4 acts early in tolerance induction while PD-1 acts late in long-term tolerance maintenance (Fife & Bluestone 2008). CTLA-4 inhibits T-cell activation in lymphoid organs, while PD-1 contributes to T-cell exhaustion in peripheral tissues indicating that CTLA-4 and PD-1 play complementary roles in regulating adaptive immunity (Fife & Bluestone 2008). For the purpose of cancer immunotherapy, monoclonal antibodies have been generated to potentiate the ongoing anti-tumour immune response of the patient, through blockade of CTLA-4, PD-1, or PD-1 ligand (PD-L1) (Corthay 2014) (Dunn et al. 2004) (Swann & Smyth 2007).

Ipilimumab is an antibody against CTLA-4 for treatment of patients with metastatic melanoma. Treated patients showed a prolonged overall survival compared to non-treated patients (Hodi et al. 2010). Nivolumab is an antibody against PD-1 that has been shown to produce durable tumour regression responses in clinical trials (Topalian 2012). On the basis of their distinct immunologic mechanisms of action and supportive preclinical data, some clinical trials were made to combine nivolumab with ipilimumab. In patients with advanced melanoma, combined therapy showed rapid and deeper clinical tumour responses in comparison with the previous experiences with either agent alone (Wolchok et al. 2013).

5.3. Vaccination therapies

Therapeutic vaccines are used to treat cancer patients, promoting the recognition and elimination of tumour cells by the host immune system. The anticancer vaccines have several subtypes depending on their constitution and action mechanisms: dendritic cells, carbohydrates, genetic (DNA or RNA), whole cells, and peptides/proteins all belong to cancer vaccines (Yang 2015). Many vaccines have promising results in clinical trials and some are already in use in the clinic. From the most promising vaccines we can discriminate: Sipuleucel-T™, already approved by the FDA in 2010, an autologous dendritic cell vaccine destined for mCRPC patients (Cheever & Higano 2011) (Kantoff et

al. 2010), Oncovax™, an autologous vaccine derived directly from the patients tumour in combination with Bacillus Calmette-Guérin (BCG) as adjuvant (Huang et al. 2015); NeuVax™, a peptide vaccine used for the treatment of HER2+ breast and ovarian cancer (Chablani 2013); ValloVax™, an anti-angiogenic vaccine derived from placental endothelial cells (Wagner et al. 2015); and PROSTVAC™, a genetic vaccine for the treatment of metastatic castration-resistant prostate cancer (mCRPC), that combines prostate specific antigen (PSA) and molecules able to stimulate T cell recognition and killing of PSA expressing cells (Mandl et al. 2014).

Despite the clear efficacy of immunotherapy, regrettably only a small proportion of patients benefit from complete response and long-term tumour remission. This is partially explained because tumour cells have the ability to avoid recognition and elimination by the immune system, which represents a major drawback for the actual immunotherapy strategies (Yang 2015).

6. Tumour cells manipulate immune cells

In 2000, Hanahan and Weinberg proposed six hallmarks of cancer, tumour cells unique and complementary capabilities, required for its growth and metastatic dissemination: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. In the 2011 revised version of the hallmarks of cancer, Hanahan and Weinberg added two more emerging hallmarks: reprogramming of energy metabolism and evading immune destruction. The addition of the last hallmark was critical due to the arguments (discussed above) showing major contributions of immune cells to tumour destruction or development (Hanahan & Weinberg 2011).

6.1. Tumour microenvironment

Tumours are not isolated masses of proliferating cancer cells but composed of distinct haematopoietic and stromal cell types that participate in heterogeneous interactions with one another, contributing to tumour promotion (Galdiero et al. 2013) (Elpek et al. 2014). Tumours depend on the availability of blood vessels and inflammatory infiltrative cells. Angiogenesis is essential for tumour growth, allowing the tumour access to nutrients and growth factors present in the blood. Cancer cells produce cytokines and chemokines that are mitogenic and/or chemoattractants for granulocytes, monocytes/macrophages, fibroblasts and endothelial cells. The infiltration of these cells

and the factors they produce potentiate tumour growth, stimulate angiogenesis, induce fibroblast migration and maturation, and enable metastatic spread via venous or lymphatic vessels (Coussens & Zena 2002).

6.2. Tumour manipulation of immune cells

Tumour infiltrating immune cells can be anti-tumour, able to eliminate cancer cells mostly in the early stage of tumour formation, or pro-tumour, allowing and favouring tumour growth and progression (Mantovani 2014). The tumour microenvironment is able to damper these early pro-inflammatory features and more importantly convert them into potent immunosuppressive cells (Galdiero et al. 2013) (Fridlender et al. 2012) (Gabrilovich et al. 2012). There is growing evidence that the tumour-mediated upregulation of immunosuppressive cytokines like TGF-β and IL-10, promote aberrant differentiation of myeloid lineage cells (Fridlender et al. 2009). In this case, myeloid cell progenitors in the bone marrow give rise to immature myeloid cells with immunosuppressive potential, often referred to as **myeloid-derived suppressor cells (MDSC)** (Elpek et al. 2014).

The recent realization that infiltrative myeloid cells display pro-tumour features is demonstrated by an increase in MDSC in the blood of cancer patients associated with bad prognosis in various types of tumours, such as pancreatic (Xu et al. 2016) bladder (Eruslanov et al. 2012), oesophageal and gastric cancers (Gabitass et al. 2011) and colorectal carcinoma (Zhang et al. 2013). Moreover, in a spontaneous mouse model, the depletion of pro-tumour myeloid cells (PTMc) inhibits tumour growth and reduces the level of immunosuppression in the tumour microenvironment, allowing for increased activity of CTLs (Pekarek et al. 1995). These immature immunosuppressive myeloid cells are divided into monocytic MDSC (Mo-MDSC - CD11b+ GR1mid Ly6Chi Ly6G–) and granulocytic MDSC (G-MDSC - CD11b+ GR1hi Ly6Clow Ly6G+) (Youn et al. 2008) (Movahedi et al. 2008).

6.3. MDSC infiltrate tumour and differentiate into tumour-associated macrophages and neutrophils

Kusmartsev and colleagues demonstrated that GR1+ immature myeloid cells start to accumulate in tumour tissues 16 hours after being adoptively transferred, being potential precursors of tumour-associated myeloid cells. The findings give strength to the hypothesis that MDSC infiltrate tumours and differentiate into mature **tumour-associated macrophages (TAM) or tumour-associated neutrophils (TAN)** (Kusmartsev et al. 2005). However, Fridlender and co-workers describe TAN not as ‘‘tissue-based G-

MDSC'', but as a distinct subset of neutrophils, differing from naive steady-state neutrophils and G-MDSC by the dissimilarities of their transcriptomic profile (Fridlender et al. 2012). It is still not clear whether TAM and TAN derive from MDSC that infiltrate tumours or whether they are blood-derived monocytes and neutrophils converted into TAM and TAN, respectively by high local concentrations of immunosuppressive cytokines, such as TGF-β and IL-10 (Fridlender et al. 2009).

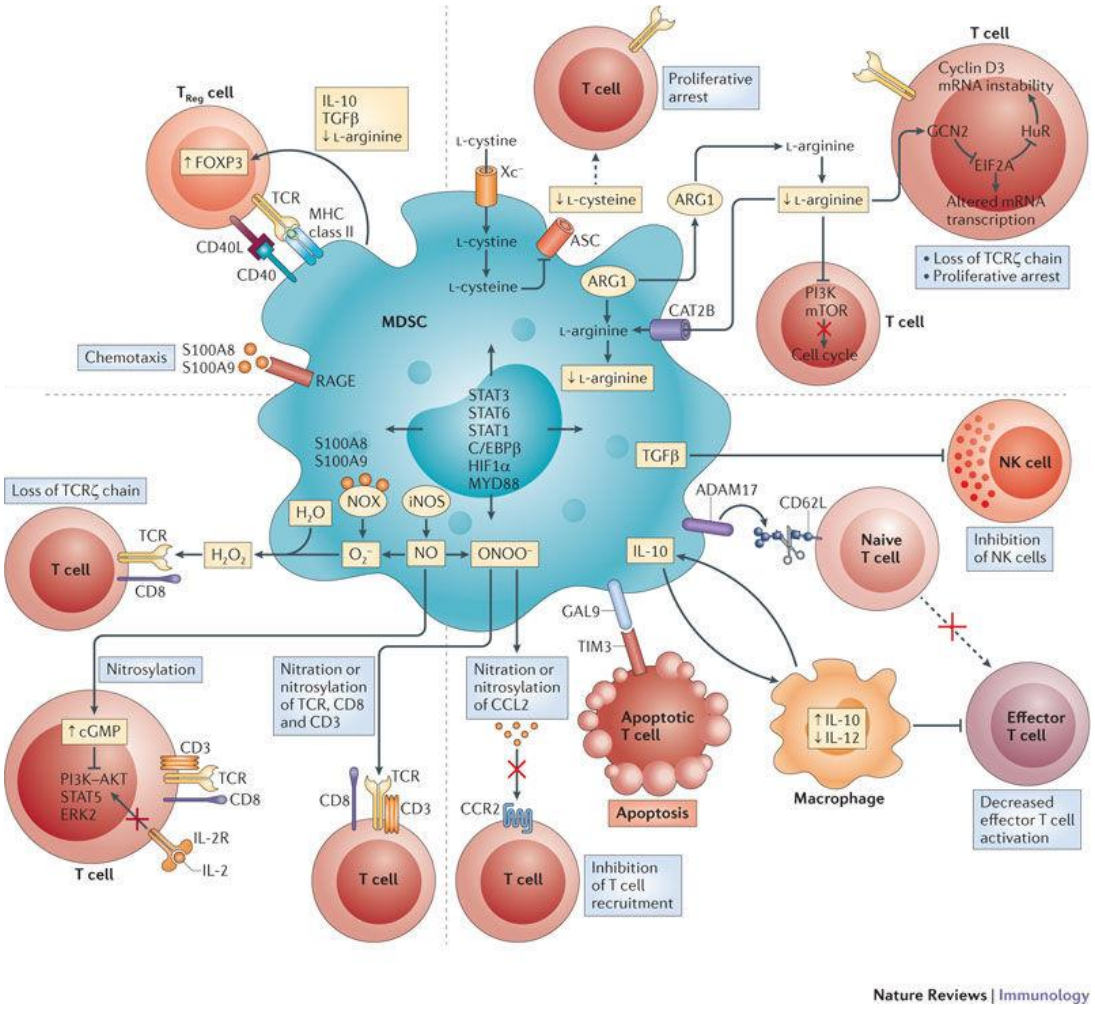


Figure 4: MDSC mechanisms responsible for the inhibition of T cell activation and proliferation. Adapted from Gabrilovich, Ostrand-Rosenberg and Bronte 2012

6.4. Tumour-associated macrophages

Similar to the macrophage polarization into a M1 or M2 phenotype during infection/inflammation, **TAM** can also have different functions, inhibiting or promoting tumour growth (Biswas & Mantovani 2010) (Mantovani 2014). Macrophages isolated from mouse and human tumours share M2 macrophages properties (Biswas et al. 2006). *In vivo* analysis of TAM through immunohistochemistry and confocal microscopy show their

high expression of IL-10, scavenger receptors, angiogenic factors and phagocytosis-related molecules and low expression of IL-12, exhibiting similar functions between M2 macrophages and TAM. Unexpectedly, TAM upregulated IFN inducible chemokines, such as CXCL9, CXCL10 and CXCL16, associated with TH1 responses (Biswas et al. 2006).

Despite some authors describing TAM as having an M2-like phenotype, because of their mixed anti or pro-tumour functions they will be referred as **anti-tumour macrophages (ATMa) or as pro-tumour macrophages (PTMa)**. PTMa perform many functions, including extracellular matrix remodelling, promotion of tumour cell invasion and metastasis, angiogenesis and immune suppression. They express high amounts of TGF-β, arginase-1, IDO, and IL-10, known for being immunosuppressive molecules (Mantovani 2014). Additionally, PTMa have been shown to express PD-L1 (B7-H1) in some types of cancer, associated with immunosuppressive functions (Kuang et al. 2009). In a report from Kuang and colleagues, PD-L1+ macrophages from stroma of patients with hepatocellular carcinoma, effectively induced dysfunctional T cells with low cytotoxicity and with impaired capacities for proliferation and production of IL-2 and IFN-γ (Kuang et al. 2009).

6.5. Tumour-associated neutrophils

TAN have been proposed as promotors of malignant transformation, tumour progression and angiogenesis through the production of granule proteins, cytokines and angiogenic factors (Mantovani 2014). However, they have been also associated with anti-tumour functions upon stimulation with type I IFN (Jablonska et al. 2010) or after TGF-β blockade (Fridlender et al. 2009) (Zhang et al. 2016). Therefore, TANs can have pro-tumour or anti-tumour functions, thus being referred henceforth as **anti-tumour neutrophils (ATNe) or as pro-tumour neutrophils (PTNe)**. PTNe from human lung cancers secreted CCL17, a Treg cell chemoattractant chemokine that promotes the inhibition of cytotoxic T cell activity and enables tumour immune evasion (Mishalian et al. 2014). Depletion of PTNe was shown to inhibit tumour growth and reduce the level of immunosuppression in the tumour microenvironment, allowing for increased activity of CTLs (Pekarek et al. 1995) (Fridlender et al. 2009).

6.6. Different tumour types have different infiltrative myeloid cells

In a study made by Elpek and colleagues, TAM, TAN, and tumour-associated DCs (TADCs) were represented in all tumours but at different ratios and MDSC accumulation was also tumour specific. In B16 melanoma tumours, myeloid cells comprised only 40% of

tumour-infiltrating leukocytes compared with over 75% in 4T1 triple negative breast cancer and Her2+ breast cancer (Elpek et al. 2014). They also found that the percentage of TAM within CD45+ cells was significantly increased in Her2+ tumours (~75%) when compared to 4T1 (~23%) and B16 (~20%) tumours. TAN within CD45+ cells, however, were significantly increased in 4T1 tumours (~28%) when compared to Her2+ (~2%) and B16 (~2%) tumours. Therefore, this and other studies propose a distinct myeloid composition in different tumours. Differences in tumour infiltrating myeloid cells are due to a combination of tumour-specified growth factors, cytokines and chemokines, suggesting that each tumour may create a distinctive microenvironment (Elpek et al. 2014) (Youn et al. 2008).

6.7. Myeloid cells are anti-tumour early in tumour development and become pro-tumour at a later stage of tumour progression

Myeloid cells present at early stages of tumour development have anti-tumour functions, delaying tumour growth. If the tumour continues growing and becomes established in the host, the myeloid cells are manipulated by the tumour cells and become pro-tumour, losing their anti-tumour capabilities (Mishalian et al. 2013) (Sagiv et al. 2015) (Shen et al. 2016). In diffuse large B cell lymphoma (DLBCL), TAM were reported in having a shift from anti-tumour to pro-tumour functions. They produced more legumain, an endopeptidase associated with tumour progression, in late stages of tumour development when compared to early stages. Thus, TAM in the late stage of tumour growth were associated with worst prognosis in DLBCL patients (Shen et al. 2016).

In a model of transplantable Lewis lung carcinoma (LLC) and malignant mesothelioma (AB12), TAN at early stages of tumour growth are more cytotoxic, whereas later they acquire an immunosuppressive phenotype (Mishalian et al. 2013). The production of chemokines and cytokines, such as CXCL2, IL-1β, IL-10 and, interestingly, IL-12, were up-regulated in TAN isolated from established tumour, compared with early tumours. In 4T1, E0771 (triple negative breast adenocarcinoma) and AT-3 (epithelial prostatic carcinoma) transplantable tumours, neutrophils acquired a previously non-existent suppressive capacity, limiting the proliferation of CD8+ T cells. This shift was due, in part, to the presence of TGF-β in the tumour microenvironment (Sagiv et al. 2015).

7. Myeloid cells can be reprogrammed to have anti-tumour functions

In order to achieve tumour remission, most strategies targeting MC predominantly block their PTMc functions, rather than potentiating their anti-tumour functions. For instance, inhibition of PI3Kg (Kaneda et al. 2016) and blocking TGF-β, IL-10 or CSF-1R signalling result in decreased tumour growth (Ruffell & Coussens 2015) (Zhang et al. 2016). However, PTMa can be reprogrammed by immunological stimuli, such as IFN-γ, toll like receptor ligands and cytokines into ATMa, capable of inhibiting tumour growth and induce T cell anti-tumour responses (Duluc et al. 2009) (Shime et al. 2012) (Biswas & Mantovani 2010).

Polarized anti-tumour macrophages efficiently produce inflammatory cytokines (TNF-α, IL-1β, IL-6) and T cell activation cytokines (IL-12) but low expression of T cell suppressive cytokines (IL-10) (Galdiero et al. 2013) and are able to control tumour growth. Many studies suggest that IL-1β promoted tumour growth and metastasis in animal and human breast cancer models (Guo et al. 2016) and angiogenesis (Carmi et al. 2013). However, in other articles, IL-1β is associated with anti-tumour effector activity produced by ATMa. IL-1β was reported to extend the cytotoxic state of macrophages and monocytes and may play a role in the defence against malignant cells, acting as an auto stimulating factor (Onozaki et al. 1985). Alongside IL-1β, iNOS role in tumour immunity is also not clear. In one hand, it was associated with malignant transformation, angiogenesis, and metastasis formation (Lechner et al. 2005). But on the other hand, iNOS production by macrophages have been reported in having cytostatic and cytotoxic effects on tumour cells (Stuehr & Nathan, 1989). Like PTMa, PTNe can be reprogrammed into ATNe through TGF-β blockade and injection of low levels of INF-β (Fridlender et al. 2009) (Mantovani 2014). CD8+ T cell activation was increased after TAN depletion but after TGF-β blockage, depletion of TANs decreased CD8+ T cell activation, showing their anti-tumour functions (Fridlender et al. 2009).

The **reprogramming of MC** *in situ* requires **two signals**, **one danger signal** provided via **TLR ligands** and a **priming signal** provided via a **costimulatory agonist** (anti-CD40).

In a Lewis lung carcinoma transplantable model, intraperitoneal or subcutaneous injection of **TLR3 ligand** (PolyI:C) resulted in tumour regression by converting TAM into ATMa. TAM infiltrating the tumour respond very rapidly to TLR3 ligand and produce inflammatory cytokines. **TNF-α** was increased in the tumour and serum within 1 hour after injection of PolyI:C, followed by tumour necrosis and growth suppression (Shime et al. 2012). It was also reported that mouse bone marrow- derived macrophages cultivated in

vitro with TLR3 ligand for 24 hours upregulated the expression of the pro-inflammatory cytokines **TNF-a** and **IL-1b** and the enzyme **iNOS** (Liu et al. 2016). This showed that TLR3 ligand can directly induce an anti-tumour phenotype in macrophages.

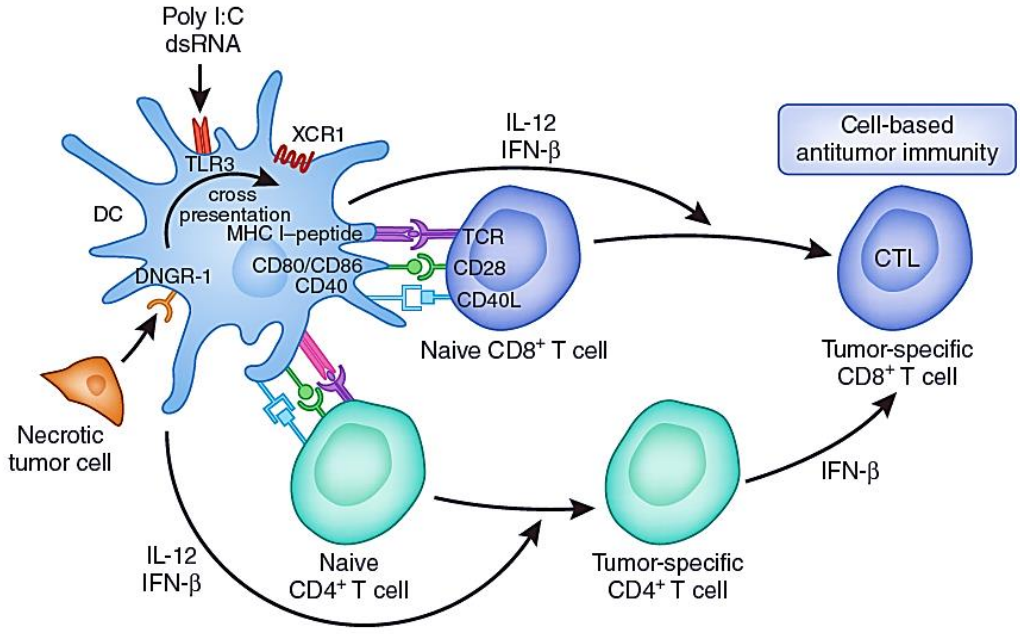


Figure 5: Anti-tumour vaccine strategy based on TLR3 stimulation of DCs. Adapted from Gallois and Bhardwaj 2010.

Other experiments showed the agonistic CD40 antibodies potential to generate anticancer immunity. **CD40** have the ability to activate APCs and promote antitumor T cell responses, through activation of ATMc (Vonderheide & Glennie 2013). CD40-activated macrophages were reported to infiltrate tumour and have tumoricidal functions, facilitating control of tumour growth in a clinical trial of pancreatic ductal adenocarcinoma (PDA) and in a KPC mice model (spontaneously develop PDA tumours with features of human PDA) (Beatty et al. 2011).

In the same line of thought, Guiducci and colleagues found that adenoviral delivery of CCL16, combined with the TLR9 ligand CpG and anti–IL-10 receptor antibody intra-tumour treatment, rejected TSA mammary adenocarcinomas, MCA38 colon carcinomas, and 4T1 tumours. CCL16 promoted an accumulation PTMa and immature DCs in the tumour that, upon treatment, were converted in ATMa and mature DCs. Upon intra-tumour treatment, macrophages produced IL-12, TNF and iNOS and DCs secreted IFN-γ. The high production of TNF by macrophages induced a massive haemorrhagic tumour necrosis that, associated with the concomitant DCs migration to draining lymph nodes, primed CTLs and induced tumour eradication (Guiducci et al. 2005).

Despite these findings, information characterizing anti-tumour features, functions, phenotype and transcriptional program are critically lacking (Elpek et al. 2014). Consequently, this project aims at developing novel approaches to manipulate myeloid cells *in vivo* during tumour responses. The hypothesis lays on the versatility of the myeloid cells and that even when converted into pro-tumour subsets, MC can be “reprogramed” to perform anti-tumour functions.

Objectives

To date, protocols allowing manipulation of myeloid cells *in vivo* are scarce, and the available strategies include ablation or inhibition of their pro-tumour features. However, studies from our laboratory demonstrate that myeloid cells can have anti-tumour capabilities. One of the last published articles from our laboratory, in *Cancer Immunology Research*, identified a patrolling monocyte/NK cell crosstalk induced by primary tumours that strongly inhibits experimental metastasis formation in the lung (Kubo et al. 2017). Patrolling monocytes are found to be the critical source of IL-15, an essential cytokine for NK cell activation and metastasis inhibition.

Another ongoing study indicates that neutrophils could inhibit pro-tumour γδ T cell functions in the tumour environment, adding to the main proposal in our laboratory that myeloid cell populations can crosstalk with lymphocyte subsets to promote anti-cancer immunity.

Our goal in this study was to manipulate myeloid cells *in vivo* in the tumour microenvironment. We planned to induce ATMc using stimulating agents, such as agonists of costimulatory molecules and TLR *in vivo*. They were be intra-tumour (IT) injected to act on local myeloid cells as a “reprogramming” agent, allowing the polarization of *in situ* MC into ATMc. We also assessed if the mechanism of tumour elimination is dependent on myeloid subsets and triggers potent anti-tumour adaptive T cell response.

In this thesis, we aimed to:

- Demonstrate that myeloid cells can have anti-tumour functions upon stimulation *in vivo*.
- Characterize anti-tumour myeloid cell phenotype and functions.
- Identify which myeloid cell population is capable of promoting potent anti-tumour immune responses, leading to tumour complete eradication *in vivo*.
- Decipher which lymphocyte subsets crosstalk with anti-tumour myeloid cells and are capable of killing tumour cells *in vivo*.

Methods

1. Mice

C57BL/6J and BALB/c mice were purchased from Charles River Laboratories. JHT-/- were purchased from Instituto Gulbenkian da Ciência. Mice were kept in specific pathogen-free facilities at Instituto de Medicina Molecular (iMM; Lisboa, Portugal). All experimental procedures were performed in compliance with guidelines approved by the local ethics committees.

2. Cell lines and tumour models.

The E0771 and 4T1 triple negative mammary adenocarcinoma tumour cell lines were a kind gift from Dr. Sergio Dias (iMM), the CT26 colon cancer tumour cell line was a kind gift from Dr. Gonçalo Bernardes (iMM) and the B16F0 melanoma tumour cell line was purchased from ATCC. Tumour cells were defrosted and maintained/expanded in Dulbecco's Modified Eagle's Medium (Gibco; Life Technologies), supplemented with 10% (v/v) of Fetal Calf Serum (FCS) (Gibco; Life Technologies) and 1% (v/v) penicillin/streptomycin (Sigma) (cDMEM) at 37ºC and 5%CO2. For long term storage, cells were ressuspended in FCS with 10% dimethyl sulfoxide (DMSO) and maintained in -80ºC.

3. *In vivo* tumour transplantation

E0771 tumour cells were harvested in the exponential growth phase. After one wash with phosphate buffer saline (PBS) (Gibco; Life Technologies) and incubation with Triple Express for 3/5min at 37ºC (Gibco; Life Technologies), tumour cells were ressuspended in PBS at a concentration of 20x10^6 / ml. One million tumour cells were injected in the mammary fat pad of female C57BL/6J mice, in a volume of 50ul. Tumour volume was measured using a calliper and calculated as (length × width × width)/2 (mm3). To induce tumour regression and complete eradication, 50µg of TLR3 ligand (PolyI:C LMW) (1mg/ml; Invivogen; tlrl-picw) and 15µg of agonist anti-CD40 mAb (0.3mg/ml; BioXcell; FGK4.5) were injected IT for *in vivo* activation of myeloid cells. Tumour implantation and treatment for 4T1 tumour cell experiments were performed similarly. CT26 and B16F0 were injected subcutaneous and only 5x10^4 cells were injected to implant B16F0 melanoma tumours.

4. *In vivo* depletion of cell lineages

- For depletion of neutrophils, 200µg of anti-Gr1 (1.3mg/ml; BioXcell; clone RB6-8C5) mAb was injected IP and 50µg injected IT (0.3mg/ml) every 1, 2 or 3 days and always 1 day before TLR3 ligand and anti-CD40 injection.
- For macrophage ablation, 150µg of anti-CD115 (Bio X Cell, clone AFS98) and 200µg of clodronate-containing liposomes (Liposoma; Lip-01) were injected IV and 50µg of anti-CD115 and 40µg of clodronate-containing liposomes were injected IT every 1, 2 or 3 days and always 1 day before TLR3 ligand and anti-CD40 injection.
- For CD4+ and CD8+ T cell depletion, 1000µg of anti-CD4 (clone YTS169) (6.6mg/m) or 1000µg of anti-CD8 (clone YTA 3.1.2) (6.6mg/m) were injected IP once a week. Both Abs were in-house produced and kindly provided by Dr Luis Graca (iMM).

5. Flow Cytometry analysis.

To assess myeloid cell or CD4+ and CD8+ T cell anti-tumour effector functions, mice were sacrificed and tumours were resected and weighed. Tumours were collected on day 18 (for kinetic day 2 and 3 myeloid cell experiment), on day 22 (for CD4+ and CD8+ T cell experiment) and on day 25 to visualize differences in tumour size between treated and non-treated groups after tumour implantation. Tumours were cut and digested for 30 minutes at 37ºC with shaker at 1000 rotation per minute (rpm) in 1.5ml DMEM plus collagenase I (0.4mg/ml; Wortington), collagenase IV (1 mg/ml; Wortington) and DNase (10µg/ml; Sigma) per tissue. If tumours were enriched with erythrocytes, 500µl of RBC Lysis Buffer (Biolegend 420301 10x) was added after tumour digestion to osmotically lyse them.

To assess myeloid cell depletion, blood samples were collected from mice facial vein (~ 5 drops) into Eppendorf tubes with 50µl of heparin at day 12, 18 and 24 after tumour transplantation. 500µl of RBC lysis (BioLegend 420301 10x) was added to each blood sample and Eppendorf's were shaken for 10 minutes. This process was repeated 2 more times to make sure the sample was haemolysed.

For fluorescence-activated cell sorting (FACS) analysis of tumour and blood samples, surface staining was done in 96 well plates. Tumour or blood cells were incubated with 50µl of RPMI-1640 medium (Gibco; Life Technologies) supplemented with 10% (vol/vol) FCS, 1% (vol/vol) penicillin/streptomycin, 1% (vol/vol) and nonessential amino acids, 10 mM HEPES, 50 uM 2-mercaptoethanol, 1% (vol/vol) pyruvate and gentamycin (10µg/mL),

in the presence of 2% normal mouse serum, with the antibodies presented in table 1 (expect TNF-α, iNOS, IL-1β, IFN-γ and Foxp3), for 1 hour at room temperature and in the dark.

After surface staining, cells were treated with Zombie Violet (BioLegend) or LIVE/DEAD Fixable Near-IR stain to exclude dead cells, for 15min at 4ºC.

For myeloid cell intracellular cytokine staining, tumour cells were stimulated with TLR4 ligand (LPS from E. coli 055:B5) (1µg/ml; Invivogen; tlrl-b5lps), IFN-γ (50ng/ml; Peprotech; 315-05), brefeldin-A (10µg/mL; Sigma) and 5 µM monensin (eBioscience) for 4 hours at 37ºC. For T cell intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 200 ng/mL; Sigma), ionomycin (1µg/mL; Sigma), brefeldin-A (10 µg/mL; Sigma), and 5 µM monensin (eBioscience) for 4 h at 37 °C. For effective cytokine intracellular staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer set (eBioscience), following the manufacturer's instructions, and incubated overnight at 4ºC in the dark with TNF-α, iNOS, IL-1β, IFN-γ and Foxp3 antibodies from table 1. Data was acquired on a FACS Fortessa (BD Bioscience) and analyzed using FACS Diva and FlowJo software (Tree Star).

Table 1: Antibodies (clone and manufacturer) used in flow cytometry analysis

Antibodies	Clone	Manufacturer
CD45	30-F11	Biolegend
CD11b	M1/70	Biolegend
Ly6G	1A8	Biolegend
SiglecF	E50-2440	BD Bioscience
F4/80	BM8	eBioscience
Ly6C	HK1.4	Biolegend
CD11c	N418	Biolegend
IL-1β	NJTEN3	eBioscience
TNF-α	MP6-XT22	eBioscience
iNOS	CXNKT	eBioscience

Antibodies	Clone	Manufacturer
MHCII	M5/114.15.2	Biolegend
CD3	17A2	Biolegend
CD4	GK1.5	Biolegend
CD8	53-6.7	Biolegend
IFN-γ	XMG1.2	Biolegend
PD-1	J43	eBioscience
Lag3	C9B7W	Biolegend
Foxp3	FJK-165	eBioscience
PD-L1	10F.9G2	Biolegend

6. In vitro assay

In order to confirm that TLR3 ligand and Anti-CD40 does not affect the proliferation of E0771 tumour cell line we organized an *in vitro* assay. Five million tumour cells were incubated with cell track violet (CTV; 2µg/ml; Invitrogen; Clone C7001) in 1ml of PBS for 20min at room temperature and in the dark. After incubated, cells were centrifuged at 1000rpm for 10 minutes and washed twice with 10ml of PBS to remove all CTV outside cells. Tumour cells were ressuspended in cDMEM and 5x10^4 cells were distributed per well in a 6 well plate and incubated with TLR3 ligand (50ng/ml) plus anti-CD40 (1µg/ml) or IFN-γ (5ng/ml) for 72 hours. Seventy two hours later, cells were washed with 500µl of PBS and incubated with 500µl of Triple Express for 3/5min at 37ºC. cDMEM was added to stop trypsin action and cells were transferred to Eppendorfs. After one centrifugation at 2000rpm for 8 minutes, supernatant was discarded and cells were ressuspended with 200µl of cDMEM. FACS analysis and data was acquired on a FACS Fortessa (BD Bioscience) and analyzed using FACS Diva and FlowJo software (Tree Star).

7. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc.). In tumour growth measurement graphs, means and standard error of mean (SEM) are plotted. In other graphs presented in this study, each individual value is plotted, alongside the means of each group, and analysed by a two-tailed unpaired t test with 95% confidence intervals or one-way ANOVA followed by Bonferroni's multiple comparison test; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ were considered to be statistically significant.

Results

1. *Intra-tumour injection of TLR3 ligand and anti-CD40 induced tumour remission in vivo, resulting in tumour-free long-term survival.*

Mice were transplanted with 10^6 E0771 breast adenocarcinoma tumour cells in the mammary fat pad. Tumour growth was evaluated *in situ* by direct measurement with a calliper.

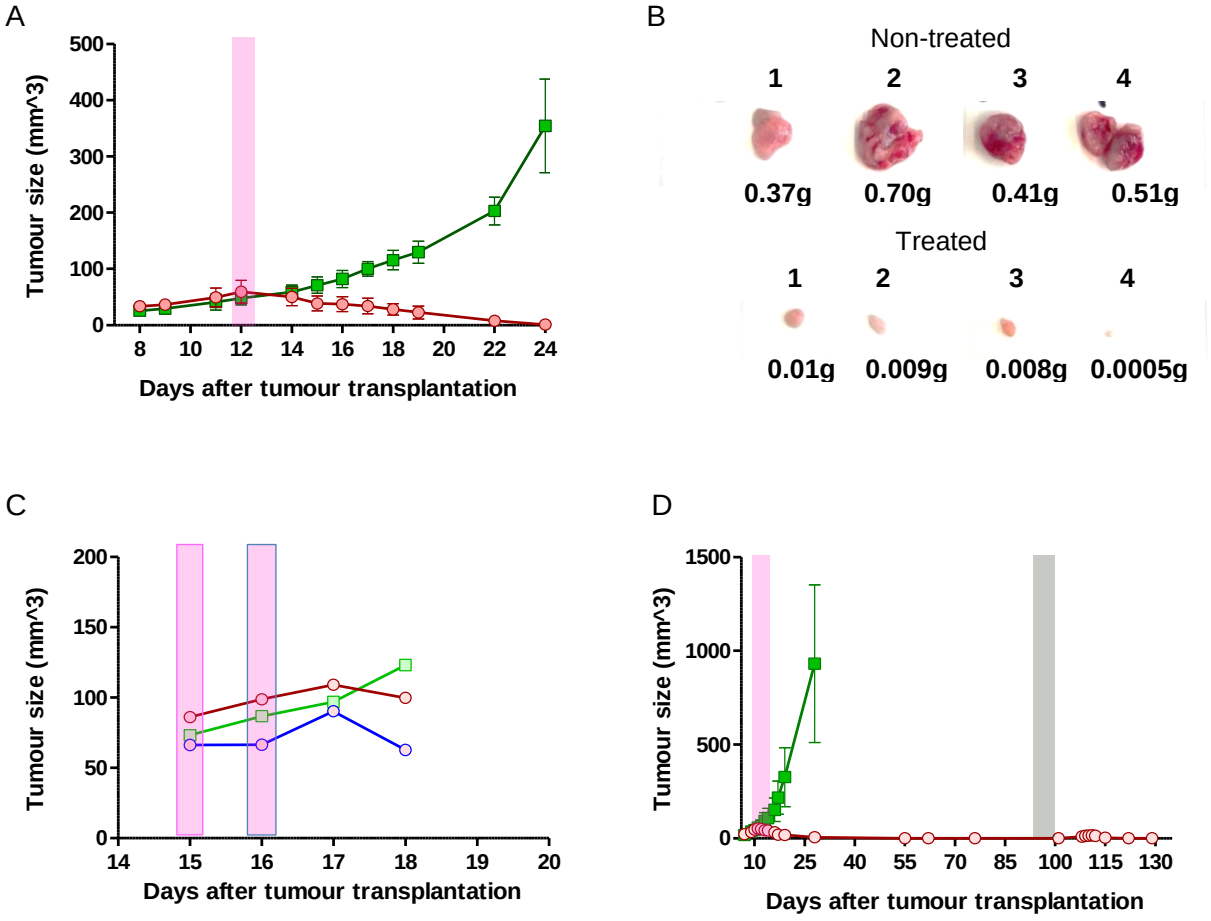


Figure 6: TLR3 ligand and anti-CD40 intra-tumour injection induced tumour remission, resulting in tumour-free long-term survival. (A) E0771 tumour growth in non-treated mice (green square; n=6) or mice treated (red circular; n=6). On day 12 (highlighted in the graphic), mice were injected with TLR3 ligand plus anti-CD40 intra-tumour. **(B)** Tumour size and weight of mice treated with PBS and with ATMc-treatment (referred to as ADJ in the graph), 24 days after tumour cell implantation **(C)** E0771 tumour growth in non-treated mice (green square; n=4), mice treated on day 15 (red circular with red outline; n=4) and mice treated on day 16 (red circular with blue outline; n=4) **(D)** E0771 tumour growth in non-treated mice (green square; n=3) or mice treated (red circular; n=7) on day 12. Mice were re-injected on day 92 with 10^6 E0771 tumour cells.

When the tumour size reached 100mm3, mice were treated with an IT injection (50µl) of TLR3 ligand (Poly I:C) (50µg) and anti-CD40 (15µg). The treatment is supposed to target MC, thus will be henceforth called anti-tumour myeloid cell treatment (ATMc-treatment). IT injection of ATMc-treatment consistently led to a dramatic reduction in the tumour burden and, in 90% of the cases, to the complete eradication of the tumour (Fig. 6A). Twenty four days after tumour cell implantation, treated and non-treated mice were euthanized and their tumours harvested. Tumours were weighted and were significantly bigger in non-treated mice in comparison to the treated 12 days before tumour resection (Fig. 6B). As soon as 2 to 3 days after one unique injection of ATMc-treatment, there is an indication of tumour regression, suggesting that the MC activation occurs in the first 3 days upon ATMc-treatment (Fig. 6C). After tumour eradication, mice received a second injection of 10^6 E0771 tumour cells on the opposite mammary gland on day 92 after the first tumour transplantation. The tumour cells were not able to establish a tumour mass, showing that the mice had an immunologic memory against E0771 tumour cells (Fig. 6D).

2. *TLR3 ligand and anti-CD40 did not affect the proliferation of E0771 tumour cell line in vitro*

In order to confirm that the effect of the treatment was not due to a direct effect on tumour cells, E0771 cell line was incubated with CTV to measure their proliferation using FACS.

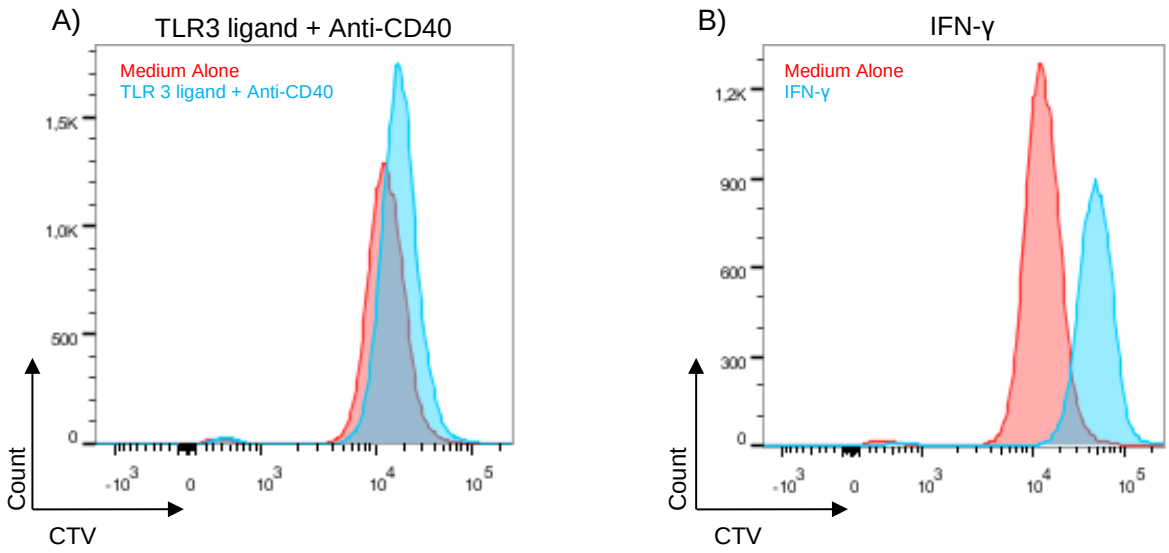
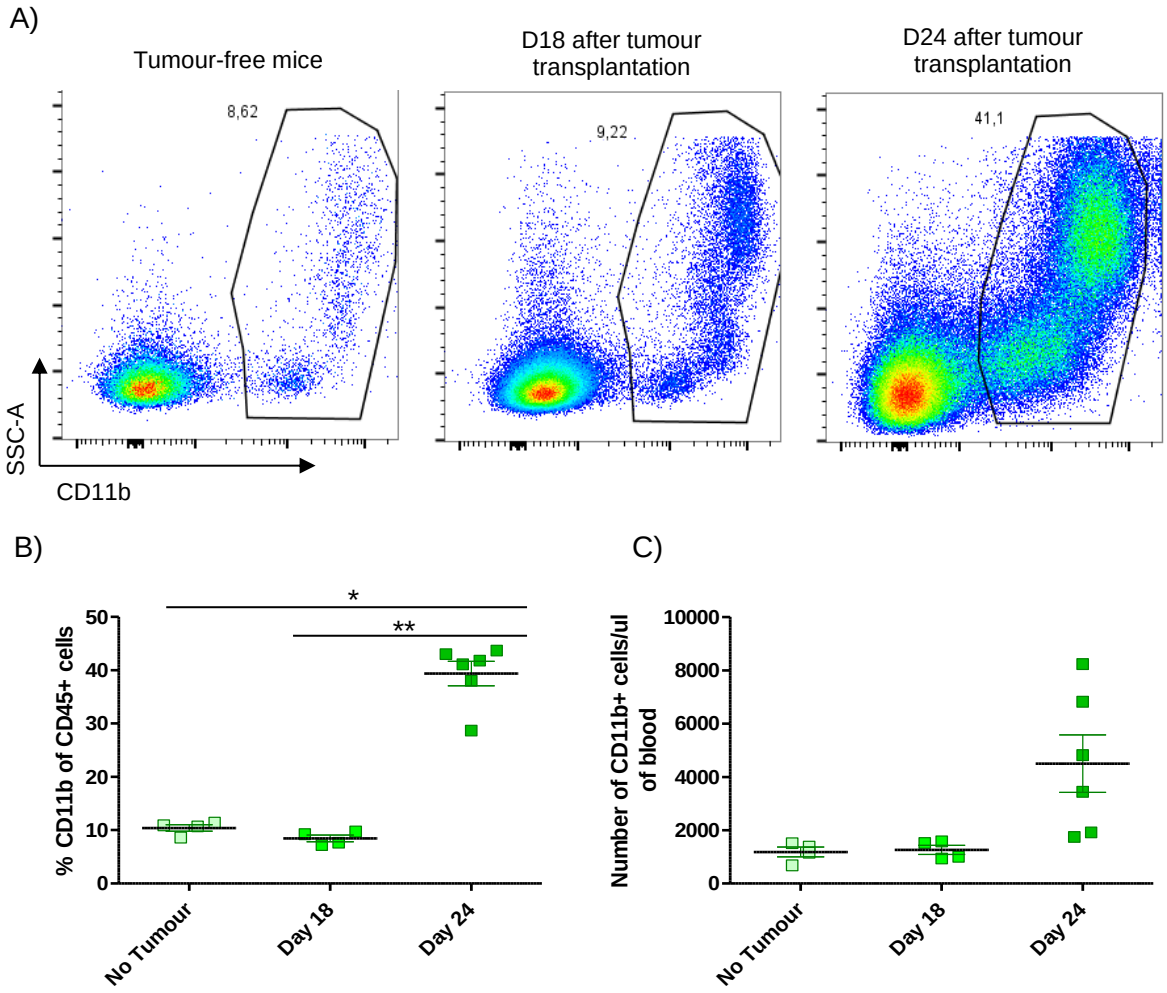


Figure 7: TLR3 ligand and anti-CD40 does not affect the proliferation of E0771 tumour cell line *in vitro*. Representative FACS plots of E0771 tumour cells incubated with TLR3 ligand and anti-CD40 **(A)** and IFN-γ **(B)** for 3 days.

Fifty thousand tumour cells were incubated with TLR3 ligand plus anti-CD40 for 3 days in a 6 well plate or with IFN-γ (as a positive control to know how MC-stimulators might affect tumour cell line proliferation *in vitro* (Möbus et al. 1993) (Zhao et al. 2013)). FACS analysis indicate that ATMc-treatment do not affect E0771 tumour cell proliferation, suggesting no direct impact of TLR3 ligand and anti-CD40 in E0771 tumour cell remission (Fig. 7A). As expected, IFN-γ affected E0771 proliferation *in vitro* (Fig. 7B).

3. Myeloid cells accumulate in the blood during tumour progression.

Myeloid cells can display pro-tumour features and are increased in the blood of cancer patients (Xu et al. 2016) (Eruslanov et al. 2012) (Gabitass et al. 2011) (Zhang et al. 2013). In the E0771 tumour model, the frequency of myeloid cells increased in the blood of tumour-bearing mice accordingly to tumour growth (Fig. 8A-B).



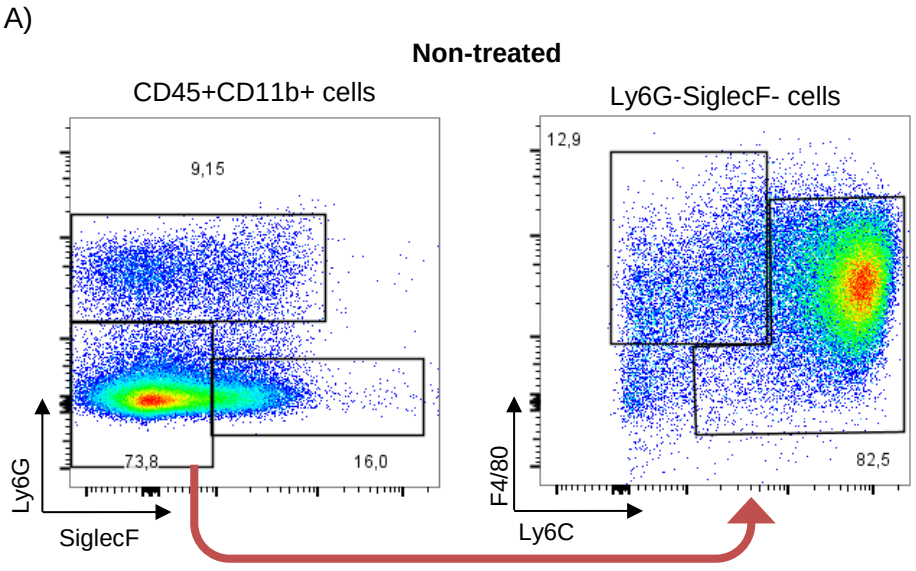
<u>Figure 8</u>: Myeloid cells accumulate in the blood of tumour-bearing mice in later stages of tumour development. (A) Representative FACS plots of blood samples collected from tumour-free mice and

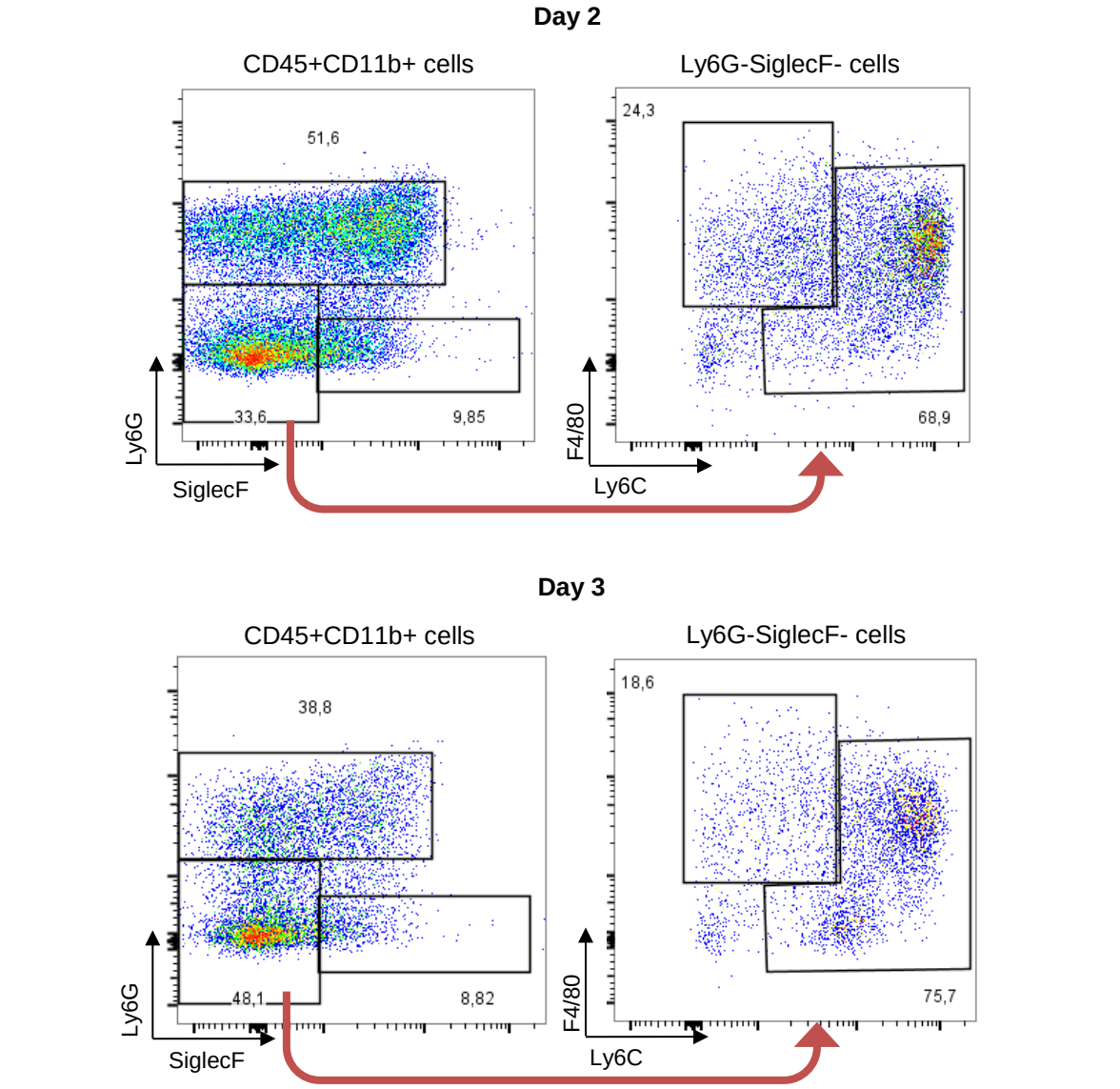
from tumour-bearing mice at day 18 and day 24 after implantation. Pre-gated on CD45+ cells, the plots represent the percentage of CD11b+ myeloid cells. **(B)** Frequencies and **(C)** numbers of myeloid cells per µl of blood volume from tumour-free mice and from tumour-bearing mice at day 18 and day 24 after tumour implantation.

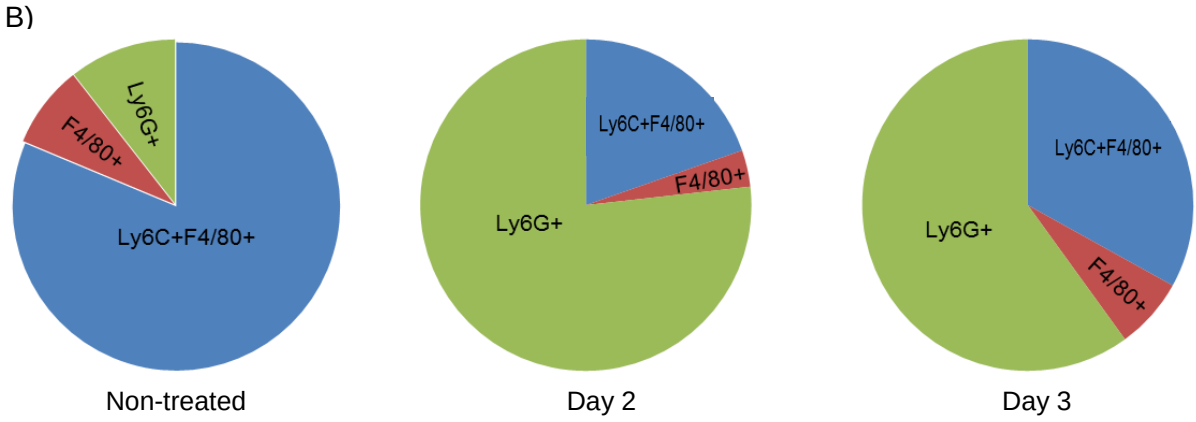
After day 24 of E0771 tumour cell transplantation, there was an accumulation of CD11b+ cells in the blood of tumour-bearing mice in comparison to the previous analysed day 18 after tumour implantation and to tumour-free mice (Fig. 8C). These results indicate a possible pro-tumour role of myeloid cells in later stages of tumour establishment.

4. TLR3 ligand and anti-CD40 changed the composition of tumour-infiltrating myeloid cells

To understand if TLR3 ligand and anti-CD40 IT injection was able to activate MC, tumours from non-treated and treated mice, day 2 and day 3 after ATMc-treatment, were harvested. There was an increase of the frequency of tumour infiltrating neutrophils (Ly6G+ cells) and a decrease in the percentage of F4/80+Ly6C+ macrophages in treated mice in comparison to the non-treated (Fig. 9A). The percentage of neutrophils increased from 11% (non-treated mice) to 60% in the tumour infiltrate of treated mice (Fig. 9A-B). Conversely, macrophages decreased from 81% (non-treated mice) to 33% in the treated mice (Fig. 9AB). The frequency of F4/80+Ly6C- macrophages did not change upon treatment (Fig. 9A-B). Thus, as expected, MC are affected by intra-tumour treatment with TLR3 ligand plus anti-CD40 mAb.



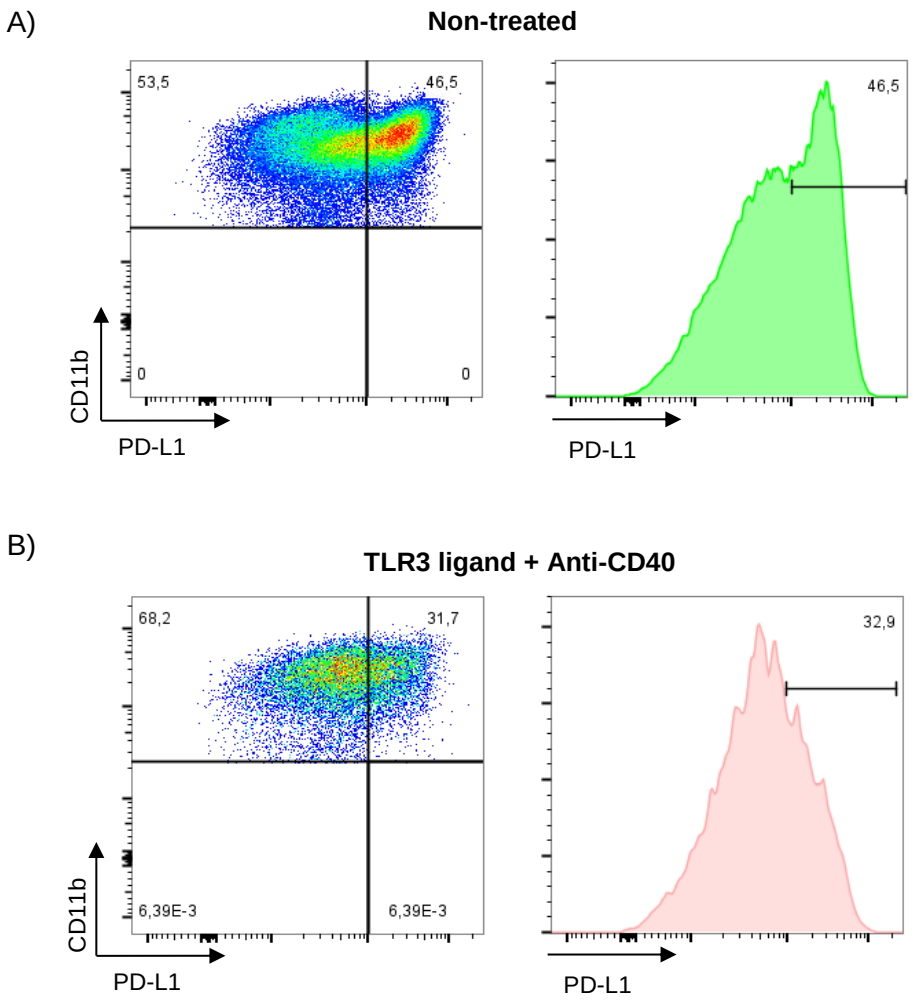


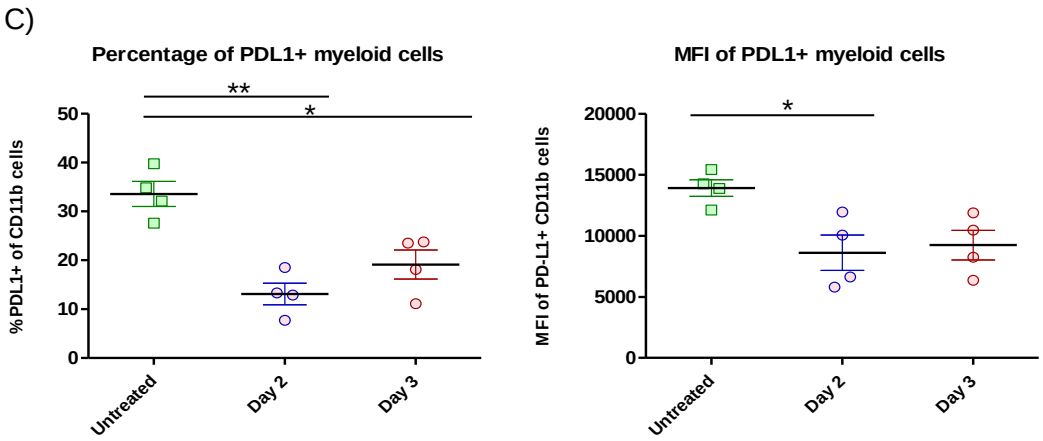


<u>Figure 9</u>: ATMc-treatment induced variations in the myeloid cell infiltrate. (A) Representative FACS plots of myeloid cell infiltrate in tumour harvested from non-treated mice and mice treated 2 days and 3 days before tumour collection. **(B)** Representative pie charts of myeloid cell infiltrate differences between non-treated and day 2 and day 3 treated mice**.**

5. *TLR3 ligand and anti-CD40 decreased the frequency of PD-L1high myeloid cells in the tumour.*

Given that the treatment changed the composition of tumour-infiltrating MC, the phenotype of these cells was assessed and, in particular, the expression of a determinant immunosuppression marker, PD-L1. There was a reduction in the percentage of PD-L1high expressing MC in the tumour, 2 and 3 days after ATMc-treatment injection, in comparison to the non-treated (Fig. 10A-C). In the tumour of non-treated mice, the percentage of PD-L1high MC was about 47%, whereas in the treated tumours this population decreased to 25% (2 days after) and 28% (3 days after) (Fig. 10A-C). The mean fluorescent intensity (MFI) was also lower in the MC from treated tumours compared to the non-treated mice (Fig. 10A-C). Thus, the treatment induced the downregulation of the immunosuppression marker PD-L1, showing a probable shift in the MC functions towards a more anti-tumour phenotype.

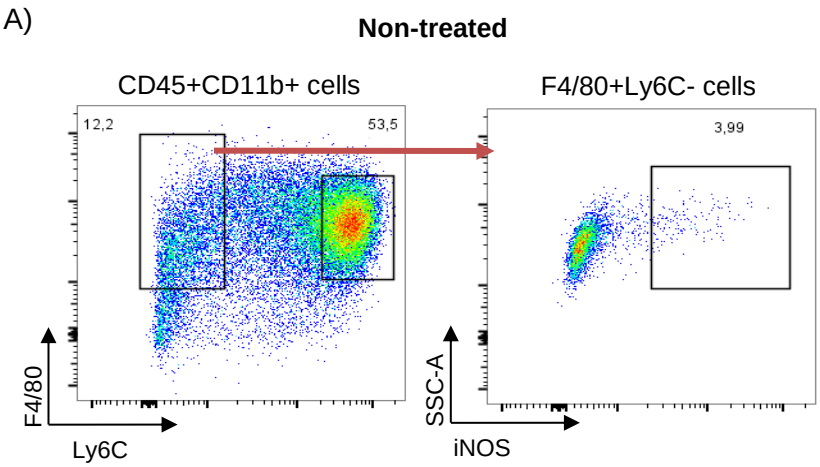




<u>Figure 10</u>: TLR3 ligand and anti-CD40 decreased the percentage of PD-L1high tumour infiltrating myeloid cells (A) Representative FACS plots from tumour of non-treated and **(B)** treated animals on day 2 and 3 of PD-L1 expression by CD11b+ cells (pre-gated on CD45+ cells) and their corresponding MFI, harvested on day 19 after tumour implantation **(C)** Graphs of the frequency of PD-L1high MC (on the left) and their MFI (on the right) of non-treated (green square) and treated animals on day 2 (blue outlined circles) and 3 (red outlined circles) after ATMc-treatment.

6. *Macrophages produce TNF-α, iNOS and IL-1β in response to TLR3 ligand and anti-CD40 treatment*

To further determine if other anti-tumour functions were acquired by MC simultaneously to the diminution of expression of PD-L1, changes in production of cytokines and enzymes, such as TNF-α, iNOS and IL-1β were assessed using FACS analysis. Three days after ATMc-treatment there was already a remission in the tumour of treated animals, suggesting that the activation of MC had already happen. Thus, anti-tumour functions were evaluated on day 3 after treatment IT injection.



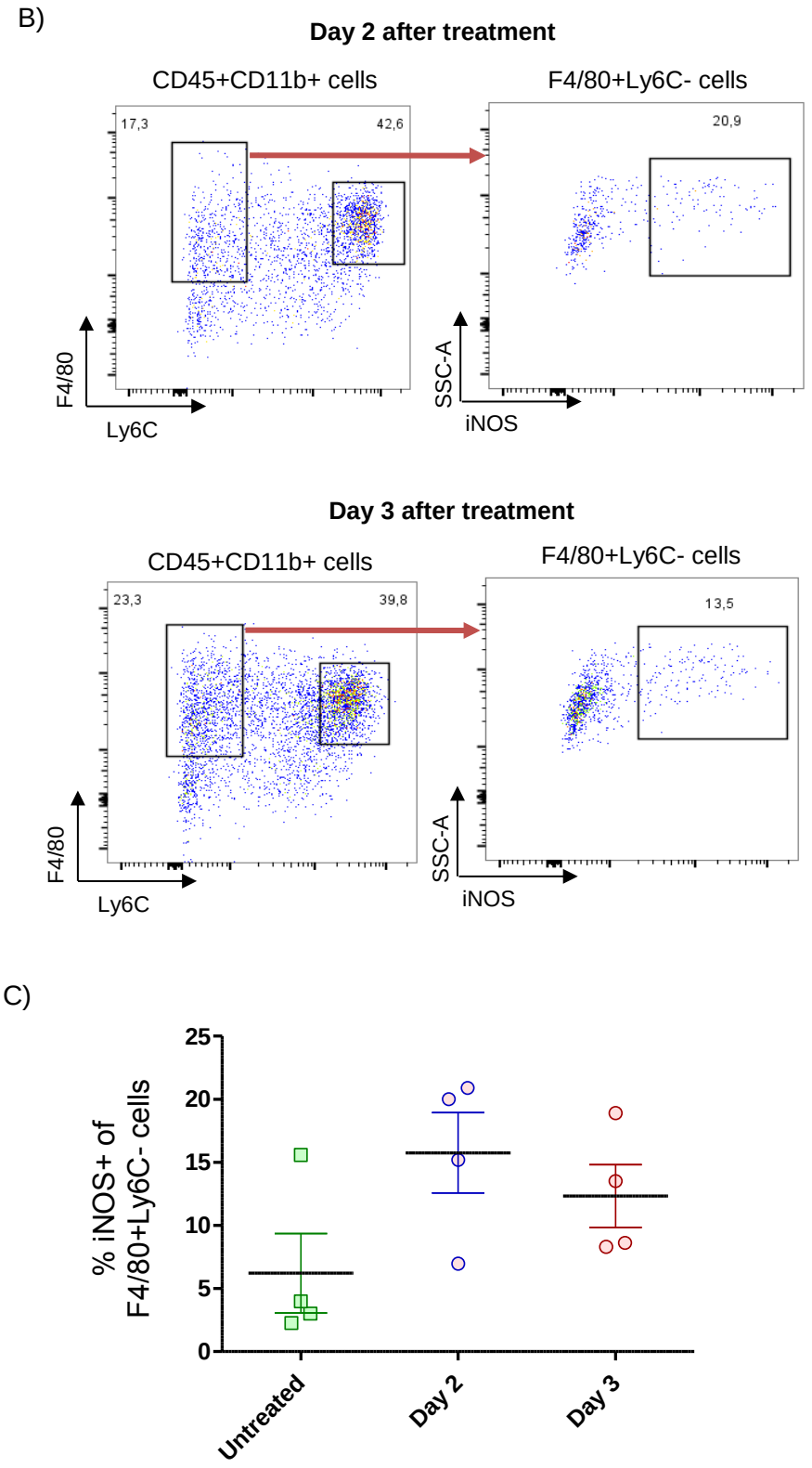


Figure 11: Macrophages produce iNOS upon TLR3 ligand and anti-CD40 injection. Representative FACS plots of non-treated **(A)** and treated 3 days before tumour harvest **(B)** on day 19 after tumour implantation**,** showing the frequency of F4/80+Ly6C- population expression of iNOS on non-treated and treated samples **(C)** Graphs displaying the frequency of iNOS by F4/80+Ly6C- cells in non-treated (green squares) and treated animals (blue and red outlined circles).

The production of iNOS was higher in the F4/80+Ly6C- macrophages infiltrating the tumour of treated mice when compared to non-treated (Fig. 11A-C).

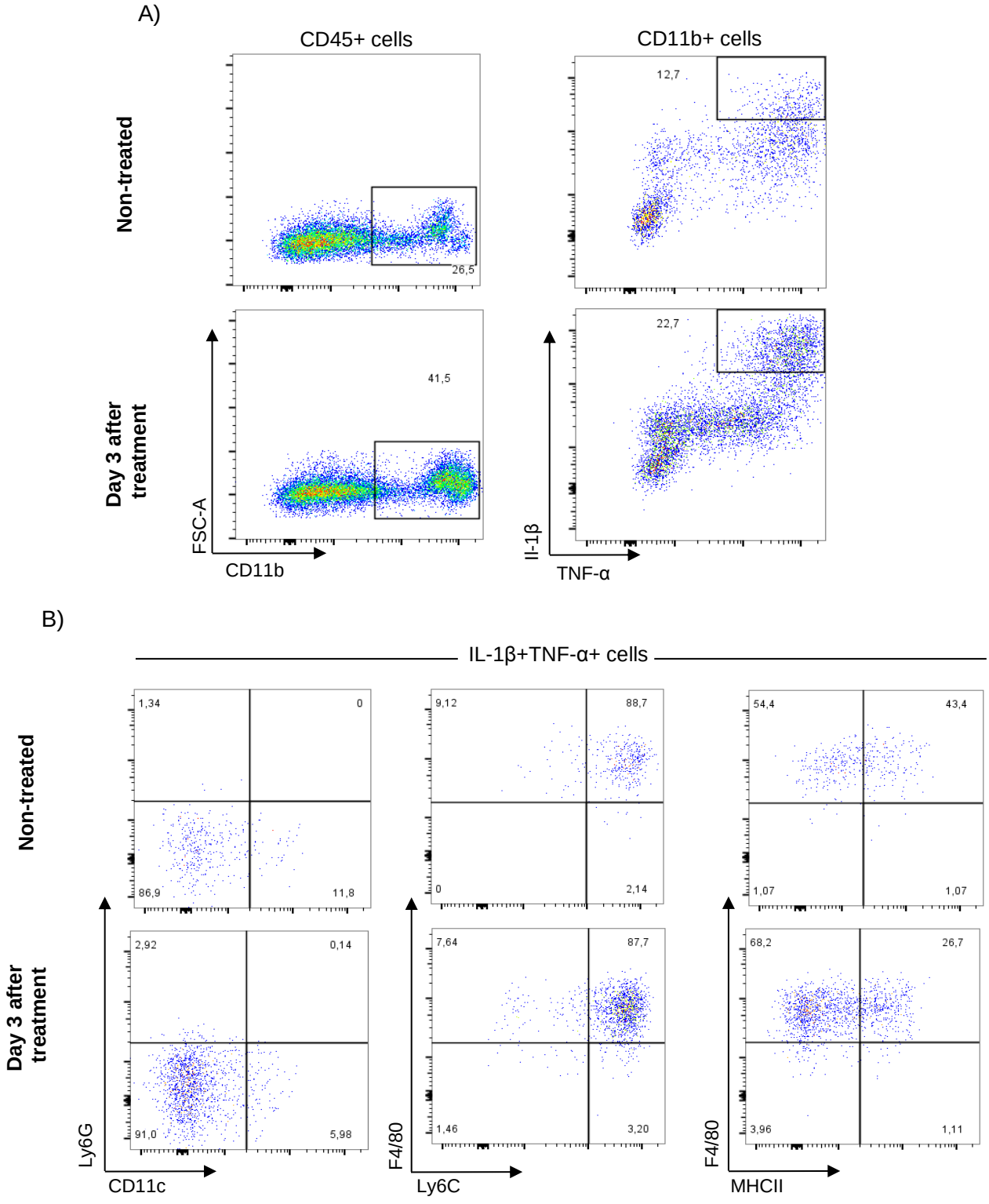
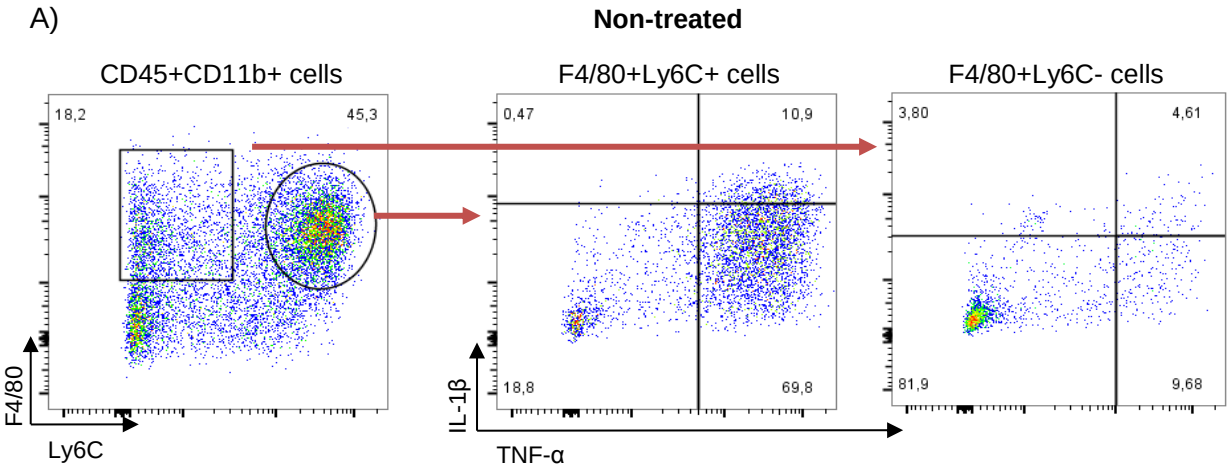
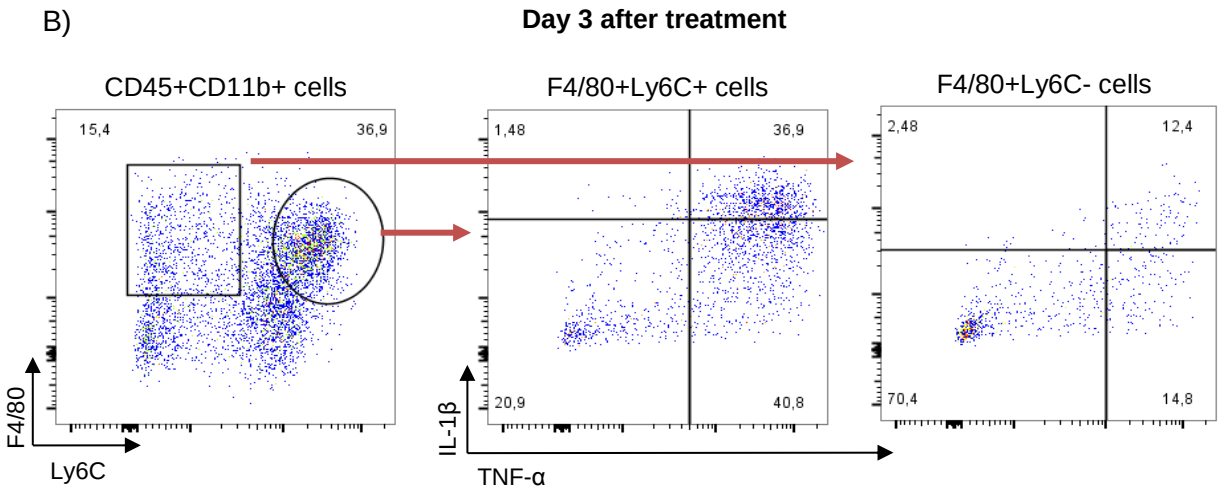


Figure 12: TNF-α and IL-1β double producing cells were F4/80+Ly6C+/- macrophages. Representative FACS plots of non-treated and treated mice 3 days before tumour harvest, on day 19 after tumour implantation, showing the frequency of **(A)** TNF-α+IL-1β+ cells and **(B)** Ly6G+, CD11c+, F4/80+, Ly6C+ and MHCII+ cells from IL-1β+TNF-α+ producing cells**.**

TNF-α and IL-1β double producing cells were not neutrophils (Ly6G-) or dendritic cells (CD11c-) (Fig. 12A-B). Ninety percent of the double producing TNF-α and IL-1β cells were F4/80+Ly6C+ and 10% were F4/80+Ly6C-. Unexpectedly, the expression of MHCII was slightly higher in the non-treated samples in comparison to the treated.

Therefore and accordingly to the previous results, there was an increase in the percentage of F4/80+Ly6C+ and F4/80Ly6C- macrophages that co-produced high levels of TNF-α and IL-1β in tumour of treated mice in comparison to the untreated mice (Fig. 13A-C).





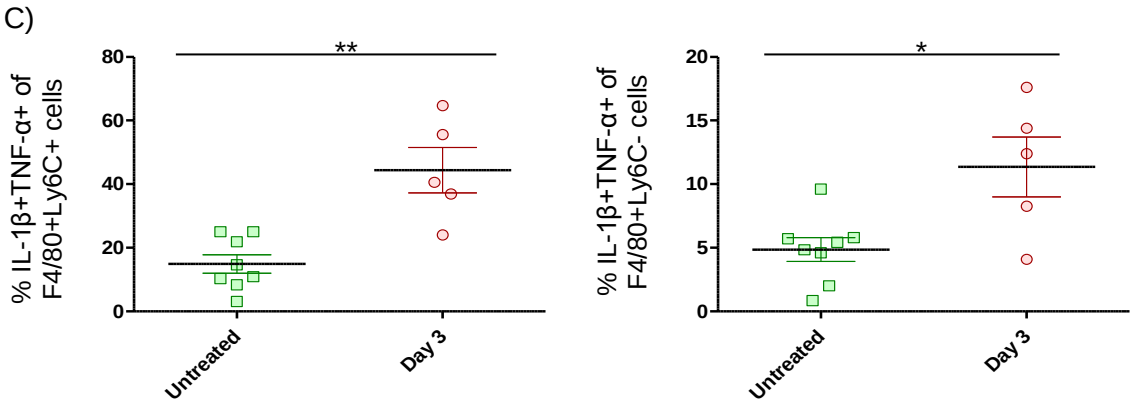


Figure 13: F4/80+Ly6C+/- macrophages co-produce TNF-α and IL-1β upon TLR3 ligand and anti-CD40 injection Representative FACS plots of **(A)** non-treated and **(B)** treated 3 days before tumour harvest, on day 19 after tumour implantation. The frequency of F4/80+Ly6C+ and F4/80+Ly6C- macrophages as well as their expression of TNF-α and IL-1β on non-treated and treated samples is displayed **(C)** Graphs representing the frequency of TNF-α and IL-1β produced by F4/80+Ly6C+ (on the left) and F4/80Ly6C- (on the right) cells in non-treated (green squares) and treated animals (red outlined circles).

Thus, although there was a higher increase in tumour-infiltrating neutrophil, phenotypic and functional changes preferentially occurred in macrophage subsets. This led to the assessment of the role of different myeloid lineages in tumour regression by using depletion strategies.

7. Macrophages are required for tumour eradication, being target of TLR3 ligand and anti-CD40 treatment

To understand which MC population is activated and play critical role for tumour rejection upon ATMc-treatment, macrophage and neutrophil populations were depleted. B cell role was also assessed because of their capacity to constitutively express TLR receptors and up-regulate CD40 ligand upon activation. For that, JHT-/- homozygote mice that have no mature B cells were used. Treatment induced tumour complete eradication in all mice lacking B cells, showing that they were not required for the treatment-induced tumour elimination (Fig. 14A).

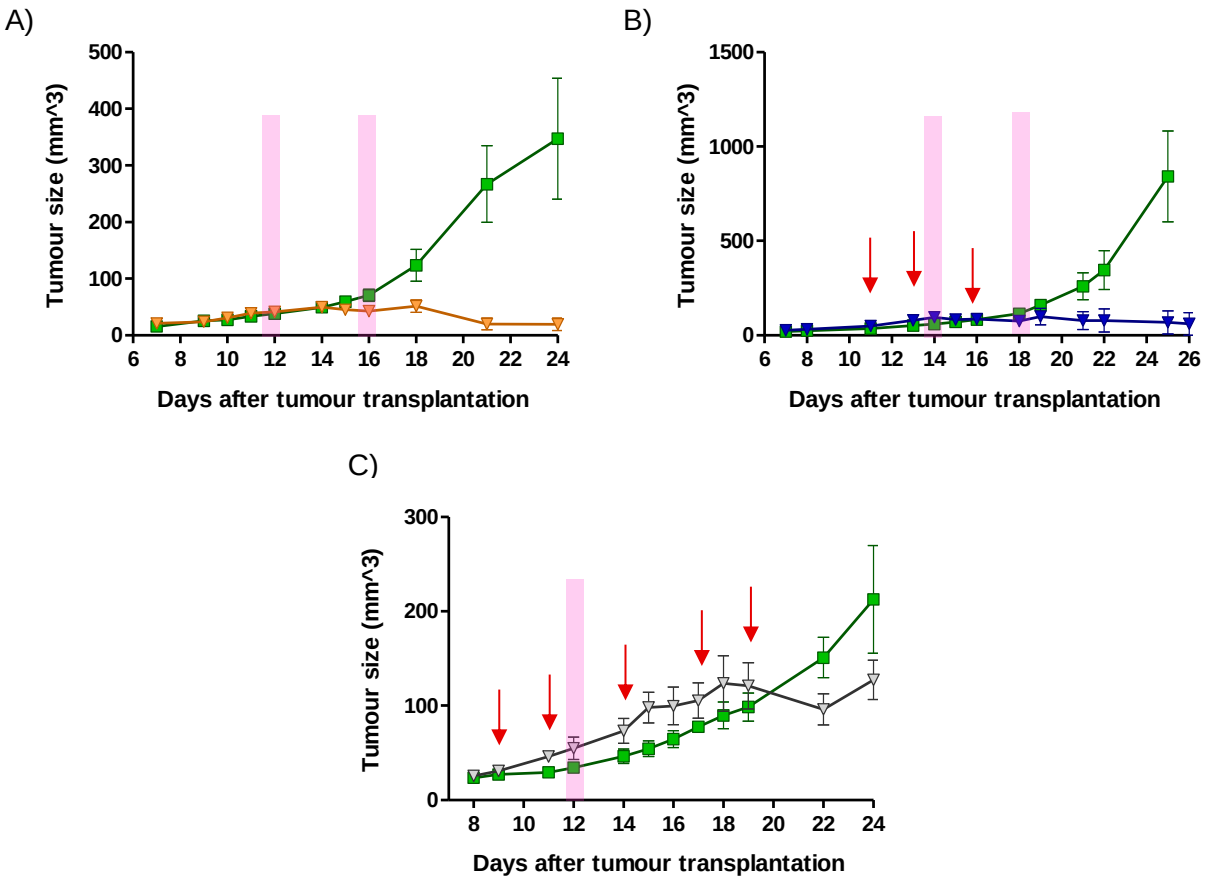


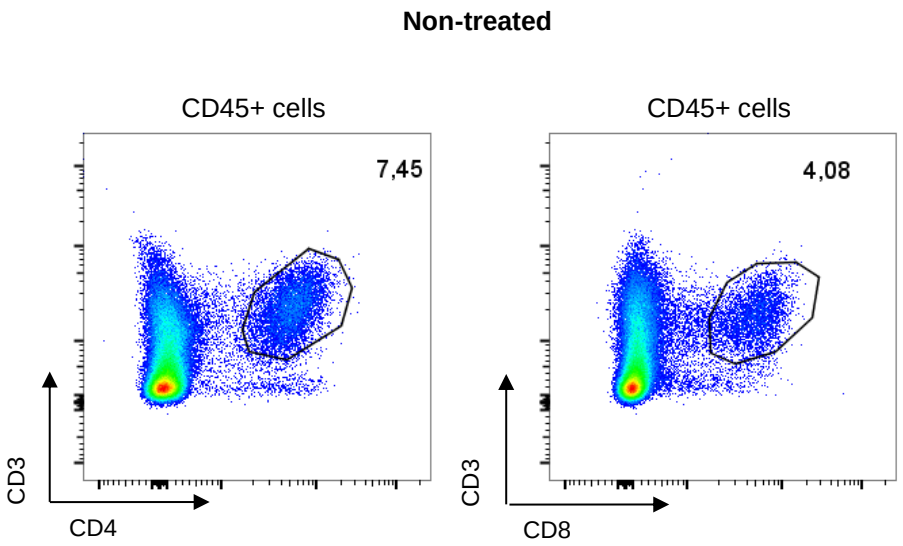
Figure 14: Macrophages are the target of ATMc-treatment (A) E0771 tumour growth in JHT-/- mice (green square; n=2) or JHT-/- treated mice (orange triangle; n=5) on day 12 and 16 after tumour implantation. **(B)** E0771 tumour growth in non-treated mice (green square; n=4) or mice treated on day 14 and 18 and depleted with anti-Gr1 on days 11, 13, 16 (red arrows) after tumour implantation (blue triangle; n=4). **(C)** E0771 tumour growth in non-treated mice (green square; n=6) or mice treated on day 12 and depleted with anti-CD115 plus clodronate-loaded liposomes on day 9, 11, 14, 17, 19 (red arrows) after tumour implantation (grey triangle; n=5).

Upon depletion of neutrophils, using anti-Gr1 (200µg) mAb injected intraperitoneal (IP) and IT (50µg), ATMc-treatment was still able to induce tumour complete eradication in all cases (Fig. 14B). By contrast, the protective effect of the treatment disappeared with the depletion of macrophages, using depleting anti-M-CSFR (anti-CD115 - 1mg/ml) and clodronate-containing liposomes, both injected intravenously (IV) (150µg and 200µg, respectively) and IT (50µg and 40µg, respectively) (Fig. 14C). The depletion schedule is described in the supplementary data (Sup Fig. 1A) and neutrophil and macrophage depletion was confirmed by blood analysis using FACS on the day of the first ATMc-treatment (data not shown). These findings confirmed the crucial role of treatment-induced macrophages in controlling and promoting tumour eradication.

8. *TLR3 ligand and anti-CD40 induced an increase in the frequency of CD4 and CD8 T cell infiltrate*

Given that TLR3 ligand plus anti-CD40 treatment induced long-lasting tumour-free survivors, a lymphocyte-dependent anti-tumour response was probably induced. Thus, phenotype and functions of tumour-infiltrating T cells were assessed in non-treated and treated mice. Tumours were harvested 10 days after ATMc-treatment and the percentage of CD4+ and CD8+ T cells was assessed (Fig. 15A-D).

A)



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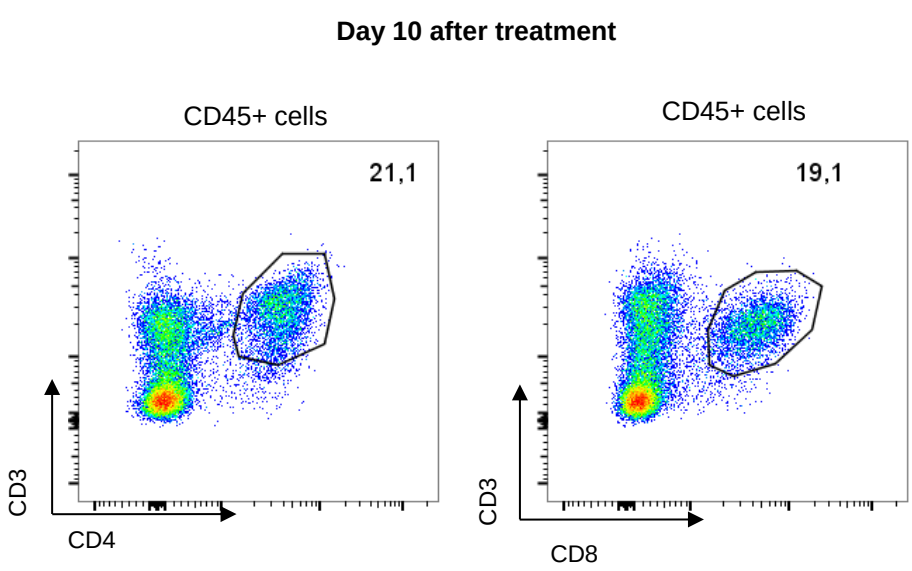


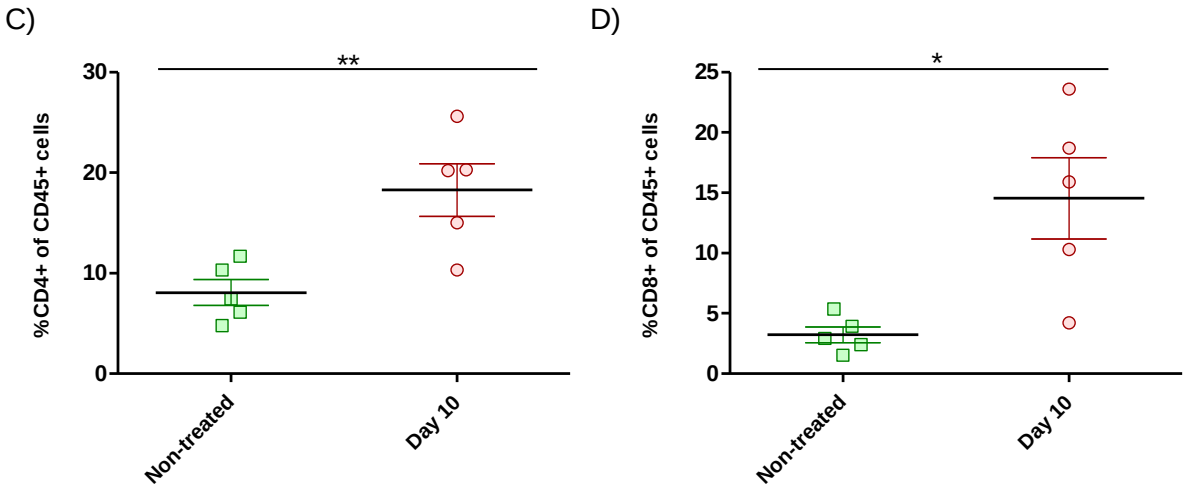
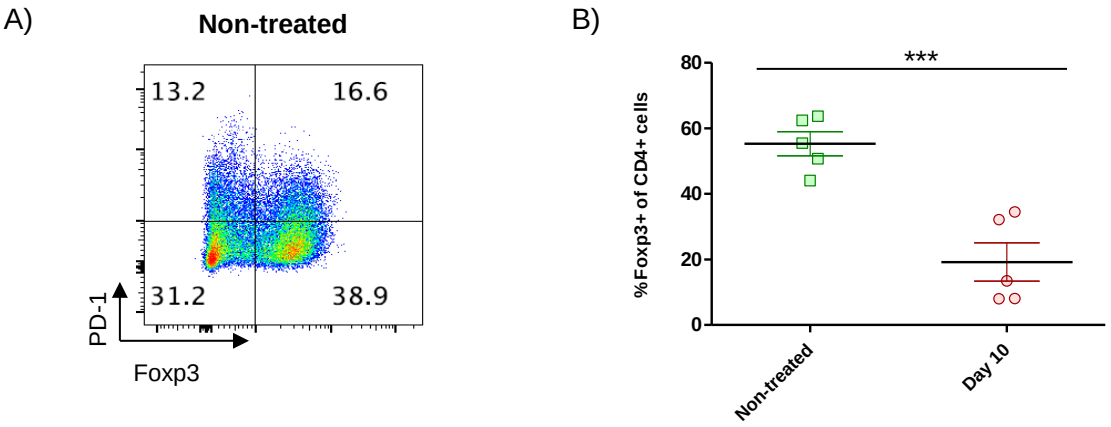


Figure 15: TLR3 ligand and anti-CD40 induced an increase in T cell infiltrate. Representative FACS plots of the frequency of CD4+ T cells CD8+ T cells in **(A)** non-treated and **(B)** treated samples on day 10 before tumour harvest. Graphs displaying the frequency of CD4+ T cells **(C)** and CD8+ T cells **(D)** infiltrating the tumour in non-treated (green squares) and treated mice 10 days before tumour harvest (red outlined circles).

TLR3 ligand and anti-CD40 treatment induced an increase in CD4+ and CD8+ T cell infiltrate in the tumour. Tumour harvested from treated mice had more than the double in the frequency of tumour infiltrating CD4+ T cells and three times more in the frequency of CD8+ T cells (Fig. 15C-D).

Then, regulatory Foxp3+ CD4+ T cells that carry immunosuppression in tumour context were specifically detected. The results clearly show a reduction in the frequency of Treg cells infiltrating the tumours of treated mice in comparison to the non-treated (Fig. 16A-B).



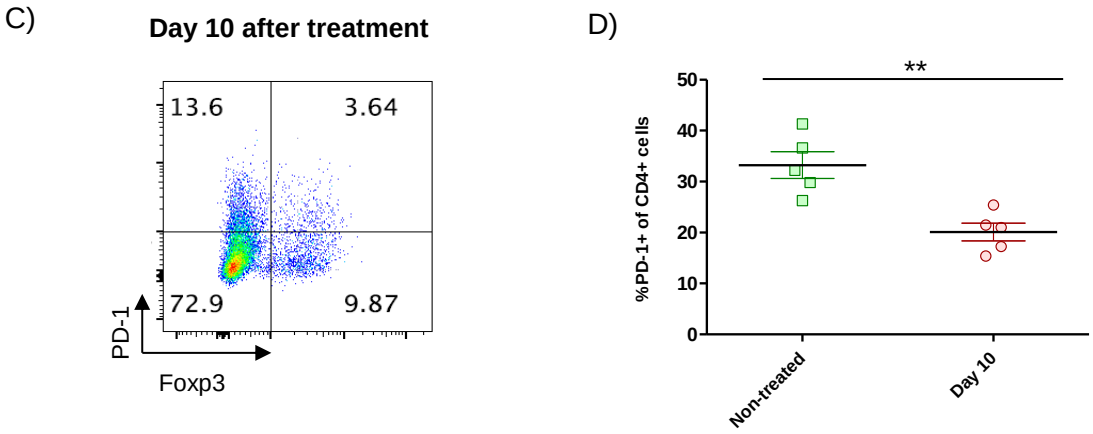


Figure 16: TLR3 ligand and anti-CD40 induced a reduction in the percentage of Treg cells and dysfunctional CD4+ T cells infiltrating the tumour. (A) Representative FACS plots and **(B)** graphs of the frequency of Foxp3+ CD4+ Treg cells in non-treated and treated samples on day 10 before tumour harvest. **(C)** Representative FACS plots and **(D)** graphs of the frequency of PD-1+ CD4+ T cells in non-treated and treated samples on day 10 before tumour harvest. In all graphs, non-treated (green squares) and treated (red outlined circles) animals are represented.

It was further assessed weather the treatment affected the proportion of dysfunctional T cells. As visualised by the expression of PD-1, there was a reduction in the frequency of dysfunctional PD-1+ CD4+ T cells in treated mice compared to the non-treated (Fig. 16C-D). Therefore, ATMc-treatment induced clear changes in the CD4+ T cell responses, promoting an increase in the frequency of CD4+ and CD8+ T cells and a decrease in regulatory and dysfunctional CD4+ T cells.

9. TLR3 ligand and anti-CD40 induced an increase in the frequency of CD8+ T cell effectors and a reduction of tumour-infiltrating exhausted CD8+ T cells

Exhaustion markers and anti-tumour functions of CD8+ T cells were similarly evaluated on tumours harvested on day 10 after ATMc-treatment. CD8+ T cells were stained against the exhausted/dysfunctional markers PD-1 and Lag-3. Tumours from treated mice had a decrease in frequency of infiltrating CD8+ T cells expressing both PD-1 and Lag3 markers, when compared to CD8+ T cells from untreated mice tumours (Fig. 17A-C).

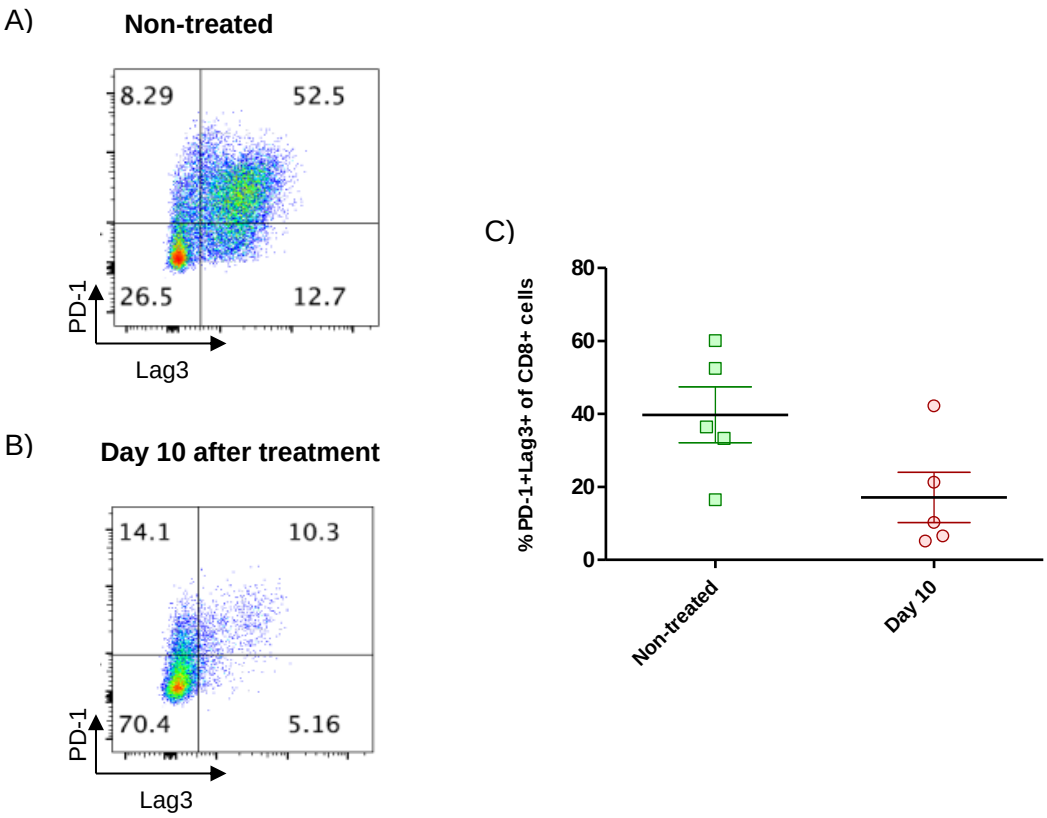


Figure 17: TLR3 ligand and Anti-CD40 induced a reduction in the numbers of dysfunctional CD8 T cells infiltrating the tumour. Representative FACS plots of the frequency of PD-1+Lag3+CD8+ T cells in **(A)** non-treated and **(B)** treated samples on day 10 before tumour harvest. **(C)** Graphs of the frequency of exhausted CD8+ T cells in the tumour in non-treated (green squares) and treated (red outlined circles) animals.

To assess CD8+ T cell anti-tumour functions, cells were assessed for IFN-γ and TNF-α production by intracellular FACS staining. Tumours from treated mice had an increase in frequency of CD8+ T cells capable of producing high amounts of IFN-γ and TNF-α in comparison to the untreated mice (Fig. 18A-C). These results from exhaustion markers and anti-tumour capabilities suggested that CD8+ T cells were the main effector cell subset leading to tumour elimination. To confirm the major role of CD8+ T cells, CD4+ and CD8+ T cells were independently depleted.

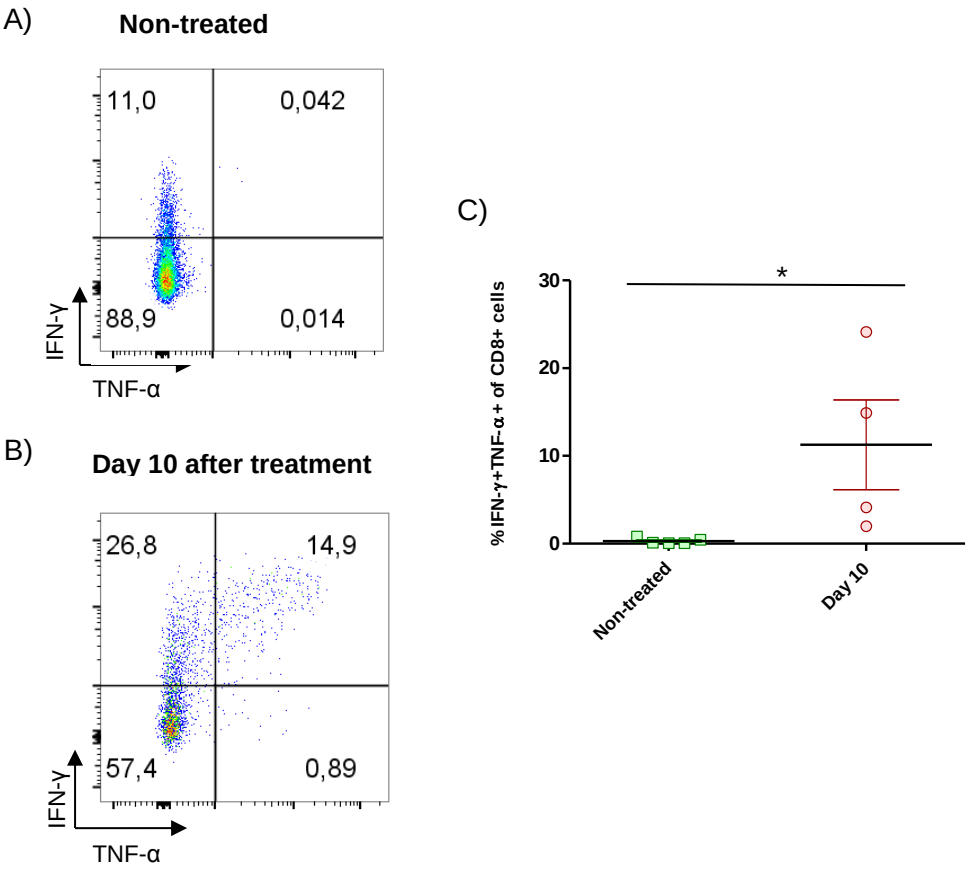


Figure 18: TLR3 ligand and anti-CD40 induced an increase in frequency of double producing IFN-γ and TNFα CD8+ T cells. Representative FACS plots of the frequency of IFN-γ+TNF-α+ CD8+ T cells in **(A)** non-treated and **(B)** treated samples on day 10 before tumour harvest. **(C)** Graphs of the frequency of double producing IFN-γ and TNFα CD8+ T cells in the tumour of non-treated (green squares) and treated (red outlined circles) animals.

10. CD8+ T cells, not CD4+ T cells, are required for tumour eradication upon TLR3 ligand and anti-CD40 treatment

To further identify the CD4+ T cell and/or CD8+ T cell imperative role in tumour killing, these lymphocytes were depleted with anti-CD4 and anti-CD8 mAbs. 1mg of anti-CD4 and anti-CD8 (6.67mg/ml) mAbs were injected IP once a week and ATMc-treatment was injected one day after the depletion. The depletion schedule is described in the supplementary data (Sup Fig. 1B). The findings confirm that CD8+, not CD4+ T cells, were responsible for tumour eradication upon treatment (Fig. 19A-B). CD4+ and CD8+ T cell depletion was confirmed by blood analysis using FACS on the day of the first ATMc-treatment (data not shown).

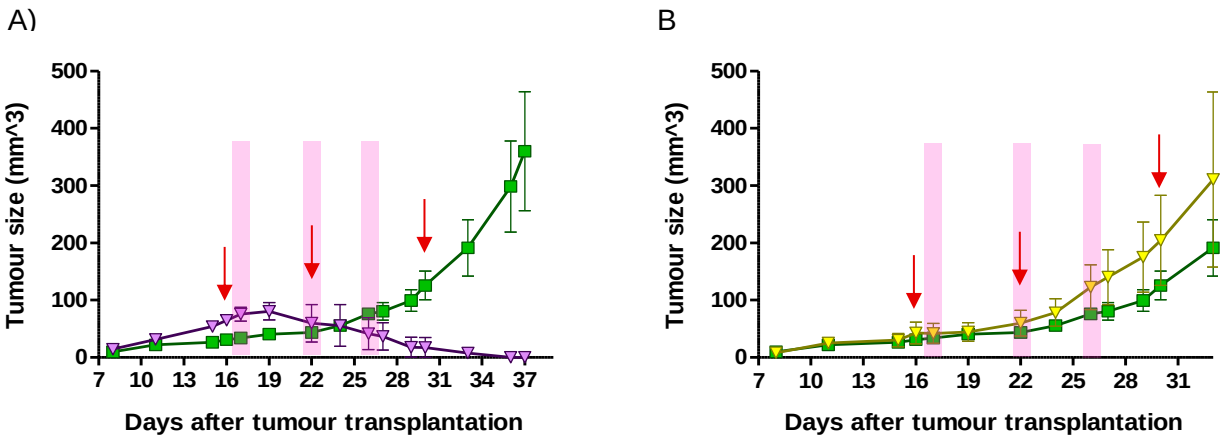


Figure 19: CD8+ T cells, not CD4+ T cells, are necessary for tumour eradication upon TLR3 ligand and anti-CD40 injection (A) E0771 tumour growth in non-treated mice (green square; n=4) or mice treated on days 17, 22 and 26 and depleted with anti-CD4 on days 16, 22, 30 (red arrows) after tumour implantation (violet triangle; n=3). **(B)** E0771 tumour growth in non-treated mice (green square; n=4) or mice treated on days 17, 22 and 26 and depleted with anti-CD8 on days 16, 22, 30 (red arrows) after tumour implantation (yellow triangle; n=4).

Since the treatment does not induce tumour regression when CD8+ T cells were depleted, our data suggest that tumour eradication upon TLR3 ligand plus anti-CD40 treatment is mediated by effector CD8+ T cells.

Discussion

Our studies aimed to induce anti-tumour myeloid cells *in vivo* and characterize their features and mechanism of action(s). For that, we used maturing agents such as TLR ligands and costimulatory receptor agonists, known to induce MC activation. Injection of the ATMc-treatment directly into the tumour (when tumour volume is below 150mm3), consistently led to the complete remission in over 90% of treated animals. Since tumour regression was not obtained when macrophages and CD8+ T cell populations were depleted, our data suggest that tumour eradication upon TLR3 ligand plus anti-CD40 treatment is due to a crosstalk between anti-tumour macrophages and effector CD8+ T cells.

Henceforth in this discussion, we will elaborate on: 1) Experimental limitations of our findings; 2) Possible scenarios of action of treatment on MC that leads to CD8+ T lymphocyte-mediated tumour eradication; 3) Long term goal of our studies; 4) Implications of our findings.

1. Limitations

In the next sections we will discuss the limitations of our model and propose alternative approaches.

1.1. Orthotopic model

The *in vivo* results presented in this thesis were achieved using the E0771 orthotopic triple negative mammary adenocarcinoma model, transplanted into the mammary fat pad of female C57BL/6J mice. Although we were able to mimic the normal progression of cancer cells by injecting directly in their typical environment, our model remains poorly realistic. Therefore, disadvantages like the injection of genetically homogeneous cancer cells, the absence of elimination and equilibrium phase of cancer immunoediting and the rapid growth of tumour cells without chronic inflammation are present in our model (Eruslanov et al. 2017). However, many discoveries now used in the clinic began with experiments in transplantable tumour models, such as the discovery of Ab-mediated CTLA4 and PD-1 blockade (Harding et al. 1992) (Ishida et al. 1992). Nevertheless, undoubtedly oncogene-driven tumour mouse models more accurately reflect tumour development, being highly heterogeneous with respect to their onset, progression, histology and antigen expression (Zitvogel et al. 2016).

1.2. Calliper tumour measurement

The measure of tumour volume *in vivo* is performed using a calliper and calculated as: (length x width x width)/2 mm3. Although this calculation gives us a 3D aspect of the tumour, it merely represents an approximation of the exact tumour volume present in the animal. The deepness of the tumour is not accessible with the calliper so it is only documented the visible or palpable tumour. Live animal imaging could be another option to accurately quantify the animal tumour burden over time. Prior to the tumour cell injection, tumour cells could be virally transduced to express luciferase and images of the tumour can be taken using a charge-coupled device camera 10min after D-luciferin injection. Luciferase could be a good addition in the early stages of tumour growth, corroborating our measurements with the calliper. However, when the tumour size is increasing, luciferase could not reach the interior or the necrotic areas of the tumour, being very unprecise (laboratory experience).

2. Possible scenarios of ATMc-treatment action on MC, leading to CD8+ T lymphocyte mediated tumour eradication.

As expected, we observed MC accumulation in the blood of tumour-bearing mice, in late stages of tumour progression. This is consistent with the extensively reported accumulation of MDSC in the circulation of cancer patients (Xu et al. 2016) (Eruslanov et al. 2012) (Gabitass et al. 2011) (Zhang et al. 2013). These circulating MDSC have been proposed to infiltrate the tumour and potentially give rise to TAN or TAM with pro-tumour functions (Kusmartsev et al. 2005). ATMc-treatment injected directly in the tumour induced an alteration in the myeloid cell infiltrate, increasing the frequency of neutrophils (from 10% to 50%) and decreasing the frequency of F4/80+Ly6C+ macrophages (2 and 3 days after treatment). Using Ab-dependent depletion we ruled out neutrophils that, though recruited, were not essential for tumour remission. By contrast, we found that macrophage populations were required for the ATMc-treatment-induced tumour elimination. Therefore, we set out to test if ATMc-treatment induced these macrophages to acquire anti-tumour effector features by assessing their phenotype and functions. We found that upon IT injection of TLR3 ligand plus anti-CD40, the frequency of PD-L1high myeloid cells infiltrating the tumour of treated animals decreased compared to non-treated mice.

In addition, although the main MC subset that accumulated in the tumour was neutrophils, they did not change their cytokine expression profile. Strikingly, we observed that two macrophage populations, CD11b+F4/80+Ly6C+ and CD11b+F4/80+Ly6C- cells,

produced high amounts of the anti-tumour cytokines TNF-α and IL-1β. CD11b+F4/80+Ly6C+ macrophages represented 90% of the double producing TNF-α and IL-1β cells, while CD11b+F4/80+Ly6C- cells also increased the production of the anti-tumour enzyme iNOS. After tumour eradication, mice subjected to a second challenge of tumour cells did not develop tumour, suggesting the presence of an adaptive immune response and memory T cells that rapidly respond against E0771 tumour cells. This was consistent with further analysis indicating that, 10 days after treatment, the frequency of PD-1+Lag-3+ dysfunctional and IFN-γ+TNF-α+ effector tumour-infiltrating CD8+ T lymphocytes were reduced and increased, respectively.

We will discuss how TLR3 ligand and anti-CD40 can act on responding MC subsets and attempt to determine: i) the pathway(s) responsible for ATMa activation and, ii) their potential crosstalk with IFN-γ +TNF-α+ CD8+ T effectors that would, directly or indirectly, promote anti-cancer immunity.

2.1 TLR3 ligand signalling pathway responsible for anti-tumour macrophage activation.

It is likely that TLR3 ligand plus anti-CD40 treatment is acting directly on macrophages that are present or infiltrating the tumour. Diverse studies suggest different pathways associated with TLR3 ligand signalling. Some propose that F4/80+ macrophages produce pro-inflammatory cytokines, including TNF-α, in a toll-like receptor adaptor molecule 1 (TICAM-1) dependent manner (Shime et al. 2012). Others believe that TLR3 ligand injection could activate the NLRP3 inflammasome in macrophages, allowing for the production of TNF-α and IL-1β in a TLR3 independent manner (Franchi et al. 2014).

In our study, it is still unclear if the expression of these cytokines is due to the TLR3 or NLRP3 activation, the synergistic action of both or other signalling pathway(s). In the future, to find out the role of NLRP3 in mediating the treatment efficacy we will use an NLRP3 inhibitor - a small molecule MCC950 that specifically inhibits NLRP3 but not AIM2, NLRC4 or NLRP1 inflammasomes (Coll et al. 2015).

2.2 Macrophage production of TNF-α, iNOS and IL-1β can promote CD8+ T lymphocyte activation.

a) By direct acting on CD8+ T cells.

Some evidence support a direct effect of anti-tumour features of the macrophages, such as the production of TNF-α, iNOS and IL-1β, in CD8+ T cell activation (Fig. 20).

TNF-α is extensively documented has being implicated in the induction of anti-tumour responses, being able to block the expression of M2 like genes in TAM (Kratochvill et al. 2015) and to induce a massive haemorrhagic tumour necrosis essential for tumour eradication (Guiducci et al. 2005). However, TNF-α secretion and haemorrhagic necrosis in tumours was not enough to produce complete tumour eradication in the majority of tumours treated with immunotherapeutic agents (Jassar et al. 2005). A study showed that the use of DMXAA, a vascular disrupting molecule, efficiently activated TAM to release anti-tumour cytokines including TNF-α (Jassar et al. 2005). Interestingly and accordingly to our results, CD8+ T cells were required for anti-tumour efficacy. Finally, TNF-α was also described to act on multiple stromal cells to improve tumour perfusion, leukocyte extravasation and immune stimulation, stabilizing blood vessels and potentiating immunotherapy (Johansson et al. 2012). Altogether, these studies support our findings, showing that the activation of TNF-α producing macrophages could be crucial to generate CD8+ T lymphocyte anti-tumour responses.

Alongside TNF-α, iNOS production by macrophages has been reported in having cytotoxic effects on tumour cells (Stuehr & Nathan, 1989). Animals treated with TLR9 ligand plus anti-IL-10 receptor antibody had thrice more iNOS production than control animals (Guiducci et al. 2005). Thus, iNOS is described as an important enzyme that could be associated with the presence of an anti-tumour phenotype in macrophages. Interestingly, it was also proposed that iNOS+ macrophages are not only required but sufficient to mediate effector T cell recruitment into tumours, successfully inducing tumour rejection (Klug et al. 2013). iNOS production by macrophages, upon low-dose gamma irradiation, induced endothelial activation and expression of TH1 chemokines, suppressing the production of angiogenic, immunosuppressive, and tumour growth factors.

Interestingly, IL-1β is also highly produced by macrophages in treated animals when compared to non-treated. However, the role of IL-1β in cancer is highly controversial as already described. Although mainly reported as a pro-tumour cytokine associated with angiogenesis promotion, IL-1β has also been shown to promote CD8+ T cell anti-tumour

functions (Ghiringhelli et al. 2009). In anthracycline-treated tumours, the NLRP3 inflammasome is activated, stimulating IL-1β production and subsequently, activation of IFN-γ producing CD8+ T cells. In addition, one other study suggests that perforin production by CTLs is required for NLRP3 inflammasome activation in APCs that produce IL-1β, contributing to the induction of antigen-specific anti-tumour immunity (Yao et al. 2017). This article suggests that IL-1β is the consequence of CTL activation and not the cause, showing a positive feedback loop of adaptive immunity to promote innate immunity, amplifying anti-tumour immunity. Both studies show that IL-1β production is due to NLRP3 inflammasome activation, potentiating the fact that TLR3 ligand could activate macrophages via the activation of the inflammasome. In other studies, pro-inflammatory cytokines have been associated with lymphocyte extravasation into tumours. IL-1β is associated with the activation of endothelial cells that, through the production of TNF-α-induced protein 2, allow lymphocyte trans-endothelial migration (Barzilai et al. 2016).

In sum, TNF-α, iNOS and IL-1β can be responsible for CD8+ T cell-mediated tumour eradication via several ways. This strongly suggests that ATMc-treatment induced a shift in TAM into ATMa. Therefore, to make sure all of them are, or which cytokine is, essential for the induction of tumour remission, we will assess if clodronate-liposome lead to the disappearance of TNF-α+ and/or iNOS+ and/or IL-1β+ macrophages, using FACS. To further investigate if IL-1β mediates CD8+ T cell activation, we will take advantage of IL-1β-deficient and IL-1R-deficient mice, already in our possession, as recipients for E0771 tumours, and subject them to ATMc-treatment.

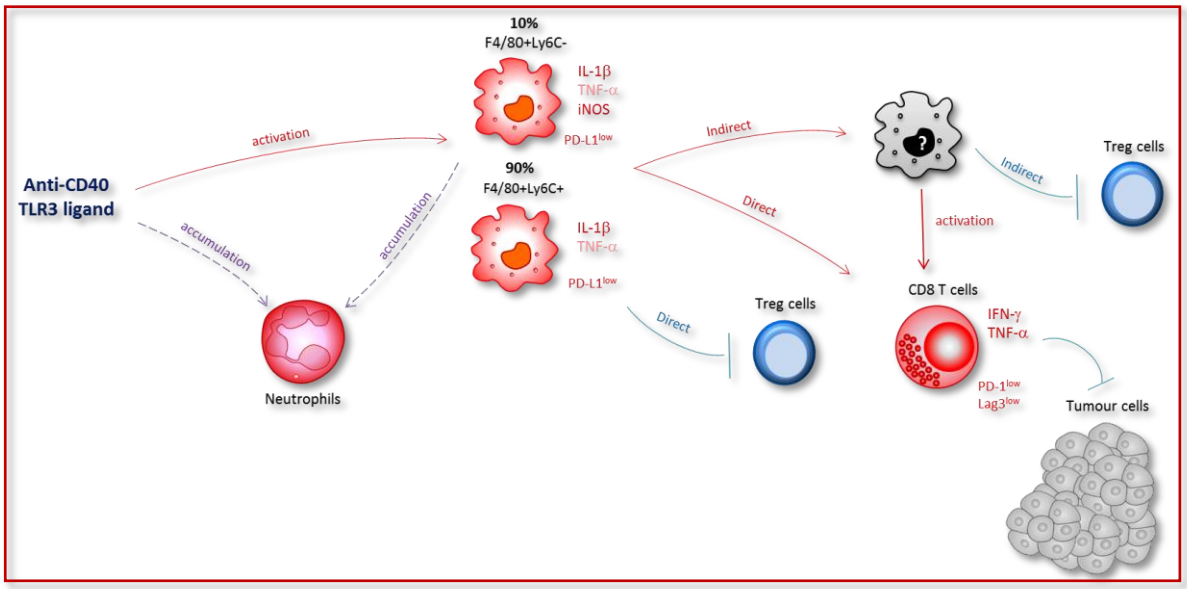


Figure 20: Representation of our proposed working model

b) By indirectly promoting CD8+ T cell activation.

It is also plausible that macrophages are the first to respond to ATMc-treatment, promoting the activation of other myeloid cell subsets (Fig. 20) For instance, in our model ATMc-treatment induced a recruitment of neutrophils to the tumour, despite the fact tht TLR3 is supposedly not expressed on neutrophils (Hayashi et al. 2003). In a model of poxvirus infection, it was shown that TLR3 ligand induced neutrophil recruitment in the liver (Jenne et al. 2013). Interestingly, the depletion of macrophages, in mice deficient in TLR3, completely abolished neutrophil recruitment after TLR3 ligand treatment. Thus, it is possible that ATMc-treatment induced the activation of macrophages that, in turn, promoted neutrophil accumulation. We confirmed that neutrophils were not involved in tumour regression upon ATMc-treatment but other myeloid cell subsets could participate in the induction of CD8+ T cell-mediated anti-tumour response.

DCs, for instance, could be a possible intermediate myeloid cell subset, emerging recently as key effector cells in priming anti-tumour T cells in the tumour (Zelenay et al. 2015) (Ruffell et al. 2014). However, we found no difference in APC function (measure via MHCII expression) on macrophages and DCs between treated and non-treated animal tumours. Likewise, there were also no differences in tumour infiltrating DCs number, frequency and anti-tumour cytokine production after ATMc-treatment. However, macrophages could induce DC migration into the draining lymph nodes, where DCs would be able to perform APC functions and activate anti-tumour CD8+ T cells. Indeed, TNF-α production by macrophages was able to create a massive haemorrhagic tumour necrosis that induced DC migration to the lymph nodes for subsequent CTL priming and clearing of all tumour cells (Guiducci et al. 2005).

Clodronate-containing liposomes are described to be ingested and digested by macrophages, followed by an intracellular release and accumulation of clodronate that induces their apoptosis (Van Rooijen et al. 1996). However, we can not rule out that DCs were not affected by the clodronate-liposomes, since they also have strong phagocytic capabilities. To clarify this point we will use fluorescent liposomes and assess whether these are up taken by DCs in the tumour and/or in the draining lymph nodes of treated and control animals. In parallel experiments we will assess APC functions and anti-tumour cytokine production by macrophages and DCs in the draining lymph nodes using FACS. DCs expressing CD103 have been shown to be particularly efficient at promoting anti-tumour responses (Zelenay et al. 2015) (Ruffell et al. 2014). Thus we will also asses the the expression of CD103 by DCs in the tumour and draining lymph node.

In conclusion and as exposed above, further experiments are required to dissect the scenario that operates upon TLR3 ligand and anti-CD40 treatment.

2.3 Other findings that could be exploited

To determine if TLR3 ligand anti-CD40 would operate in different tumour models and distinct mouse backgrounds we used 3 distinct tumour models. In a transplantable colon cancer (CT26) tumour model in BALB/c, repetitive injections of ATMc-treatment induced tumour complete eradication in 2 mice, a tumour growth arrest in 2 mice, and a delay in tumour growth in 3 mice out of 7 treated animals (Sup Fig. 2). In a melanoma model (B16F0) in C57BL/6J and a triple negative breast cancer (4T1) in BALB/c, repetitive injections of treatment (with 2/3 days of interval) did not induce tumour remission but a delay in tumour growth (Sup Fig. 2). In sum, the treatment induced regression in E0771 and in some CT26 tumours from two different mouse backgrounds, C57BL6 and BALB/c, respectively, but only delayed the tumour growth in B16 and 4T1 tumours.

We believe that the different effects of TLR3 ligand and anti-CD40 combination in different tumour models are likely due to the different immunogenicity between the tumour cell lines. It is plausible that the intensity of immunosuppression in the tumour microenvironment varies according to the tumour type. Thus, when tumour cells induced a strong immunosuppressive microenvironment, the ATMc-treatment failed to convert MC to anti-tumour effectors, which consequently did not promote anti-tumour T cells. One way we found to get information about tumour cell line immunogenicity was by looking in the literature for the response of these tumour models to immune checkpoint therapies (Table 2). E0771 and CT26 responded better to immune checkpoint therapy than B16F0 and 4T1 tumour models, suggesting that they could be more efficient at inducing a host anti-tumour immune response. Consistent with this, MHCI background level expression was higher in E0771 and CT26 than B16F0 and 4T1 cell lines rendering that the first two tumour cell lines were more susceptible to cytotoxic CD8+ T cells. Thus, in addition to the resistance to ATMc-treatment, the low MHCI expression could limit CD8+ T cell recognition of B16F0 and 4T1 tumour cells (Sup Fig. 5). These results suggest that converting MC *in situ* in poorly immunogenic cancers might reveal a difficult task to achieve.

Table 2: Reference search on tumour cell line immunogenicity in response to immune checkpoint therapy.

Cell lines	Responses to anti-CTLA4 therapy	Responses to anti-PD-1 therapy	Responses to anti-CTLA4 + anti-PD-1 therapy	References
E0771 C57BL6	Not tested	45% overall survival	Not tested	(Gray et al. 2016)
CT26 BALB/c	60% overall survival	0 - 20% overall survival	100% overall survival	(Kim et al. 2014), (Sagiv-Barfi et al. 2015)
B16 C57BL6	0 - 25% overall survival	0 - 10% overall survival	12,5 - 45% overall survival	(Curran et al. 2010)
4T1 BALB/c	0% overall survival	0% overall survival	30% overall survival	(Kim et al. 2014), (Sagiv-Barfi et al. 2015)

3. Long term goal of our studies

The first goal is to design novel ways to reprogram myeloid cells, unleashing their full potential as anti-tumour effector cells and CD8+ T cell activators. For that, we will assess if other maturing agents have the capacity to “re-program” AT macrophages and/or other lineages of MC effectors, particularly neutrophils. We aim at efficiently assess neutrophil anti-tumour functions and find out if, like macrophages, ATNe can lead to tumour remission upon treatment. For that, we will treat E0771 tumours with other TLR ligands known to be expressed by neutrophils, such as TLR2, TLR4, TLR7 and TLR9 agonists (Hayashi et al. 2003). Some articles report a synergy between GM-CSF and TLR2, showing that neutrophils enhance their expression of TLR2 and TLR9 following GM-CSF treatment (Hayashi et al. 2003). Therefore, we will use TLR ligands plus anti-CD40, in the presence or not of GM-CSF, treatment and inject directly into the tumour to induce ATNe *in vivo* and, consequently, assess their ability to promote anti-tumour responses.

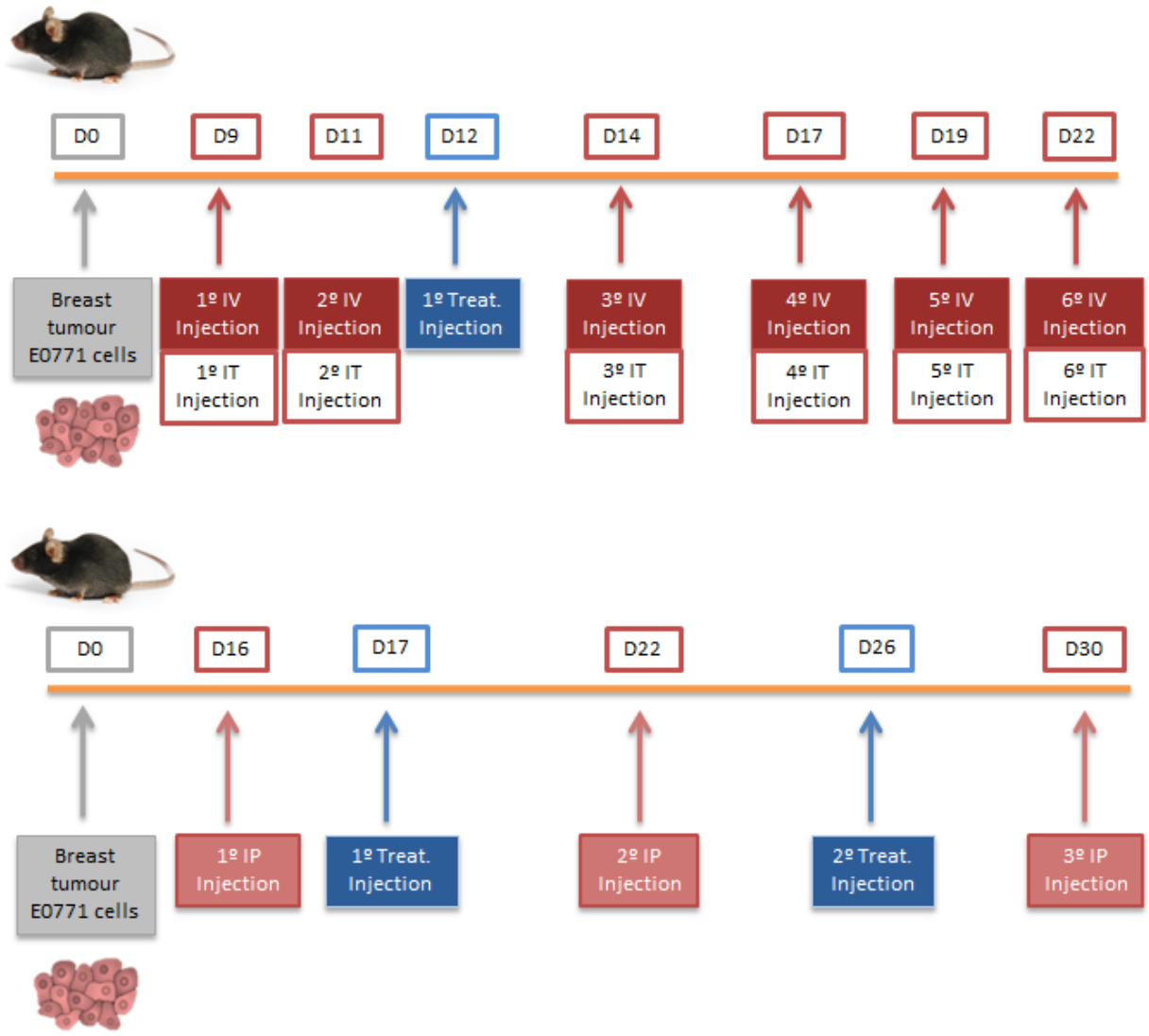
The last and long-term goal is to decipher the transcriptional program that controls the differentiation of ATMc. By defining the transcriptional signatures associated with other anti-tumour MC subsets, our study may reveal novel therapeutics to target MC against cancer.

4. Implications of our findings

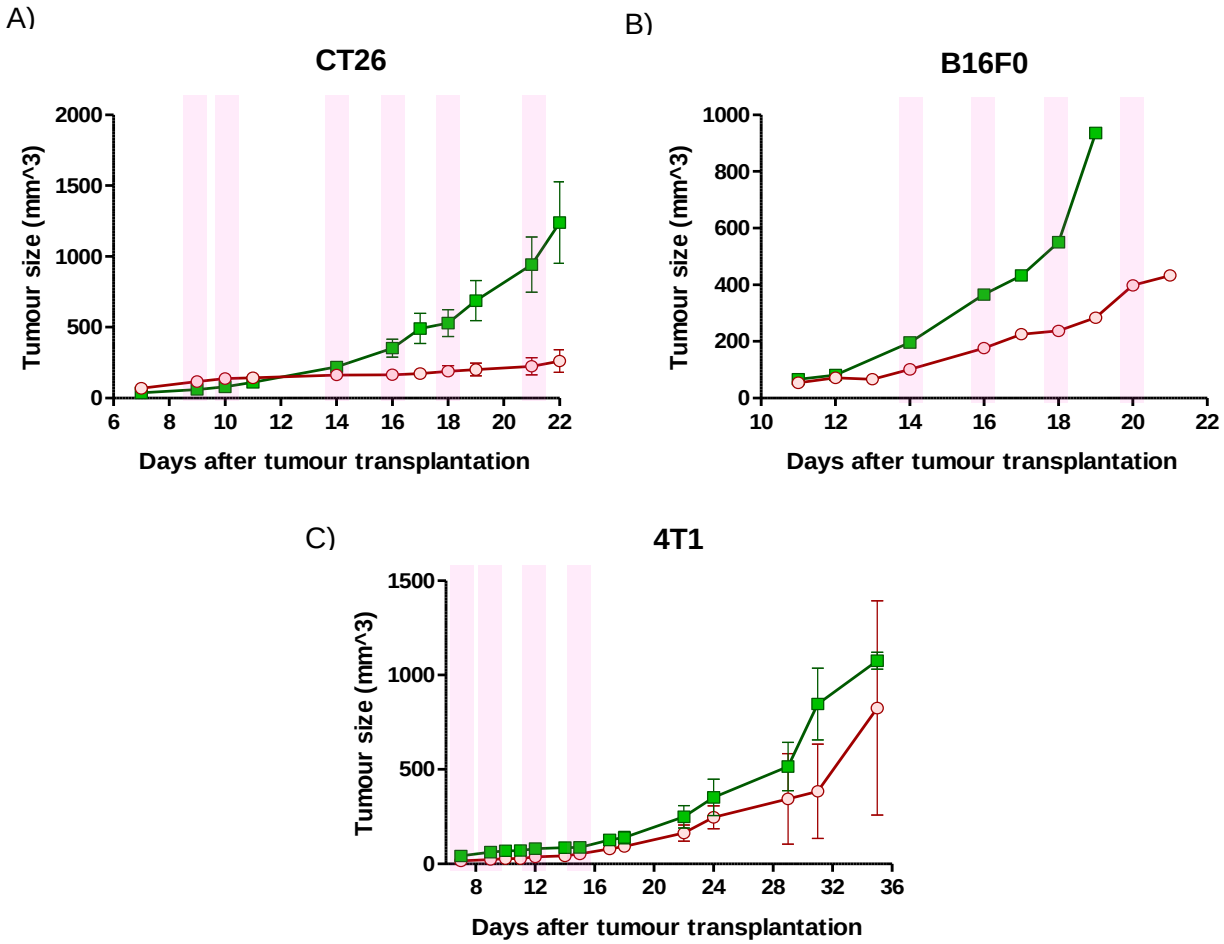
In this report we successfully demonstrated that myeloid cells can have anti-tumour functions upon stimulation *in vivo*. Upon IT injection of agonist TLR3 ligand dsRNA and anti-CD40 mAb, macrophages were able to produce anti-tumour cytokines and enzymes. We can define anti-tumour macrophages as CD11b+F4/80+Ly6C(+/-) cells that co-produce TNF-α and IL-1β. We further propose a possible crosstalk between macrophages and CD8+ T cells, promoting potent anti-tumour immune responses and leading to tumour complete eradication *in vivo*. However, translation of these results into clinical applications is not straightforward and the promise held by TLR ligands in the clinic is still to be verified. Many side effects are associated with the usage of TLR, in particular TLR-induced tolerance (Kaczanowska et al. 2013). Nevertheless, combinations of TLR ligands with other immunotherapeutic approaches that revolutionized treatments against cancers, such as immune checkpoint inhibitors, might have positive outcomes, promoting tumour regression and long-term survival in otherwise unresponsive patients.

Accordingly to the data described in this thesis, a possible combination of TLR3 ligand plus anti-CD40 injected IT with anti-PD-1 and/or anti-PD-L1 therapy could improve CD8+ T cell activation. This would lead to a faster and stronger anti-tumour response, since these PD-1 and PD-L1 markers are upregulated in CD8+ T cells and MC from non-treated animals, respectively. If other TLR could target and activate other MC populations, such as neutrophils, a synergic affect could be exploited where not only ATMa are induced but also ATNe and other myeloid cell subsets. Answering these questions might help in designing improved immunotherapies capable of manipulating MC in a hostile immunosuppressive microenvironment, encouraging new studies and ways to increase the efficacy of anti-tumour therapy.

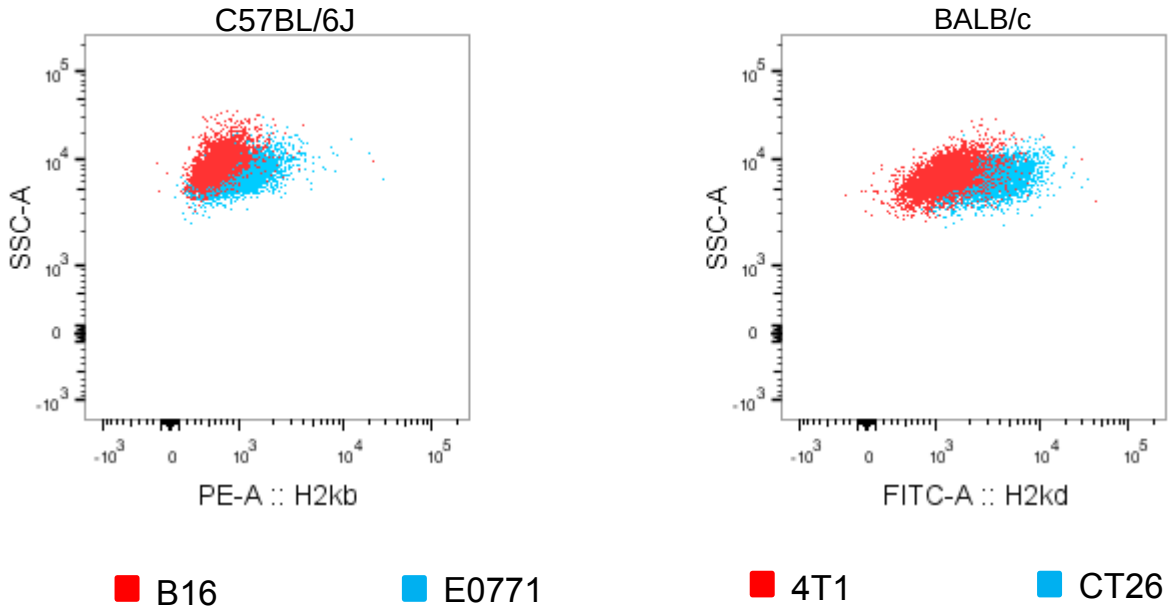
Supplementary Figures



Sup Figure 1: Schematic representation of the schedule for the tumour injection (grey), ATMc-treatment injection (blue) and depletion (red) of macrophages (top diagram) and lymphocytes (bottom diagram).

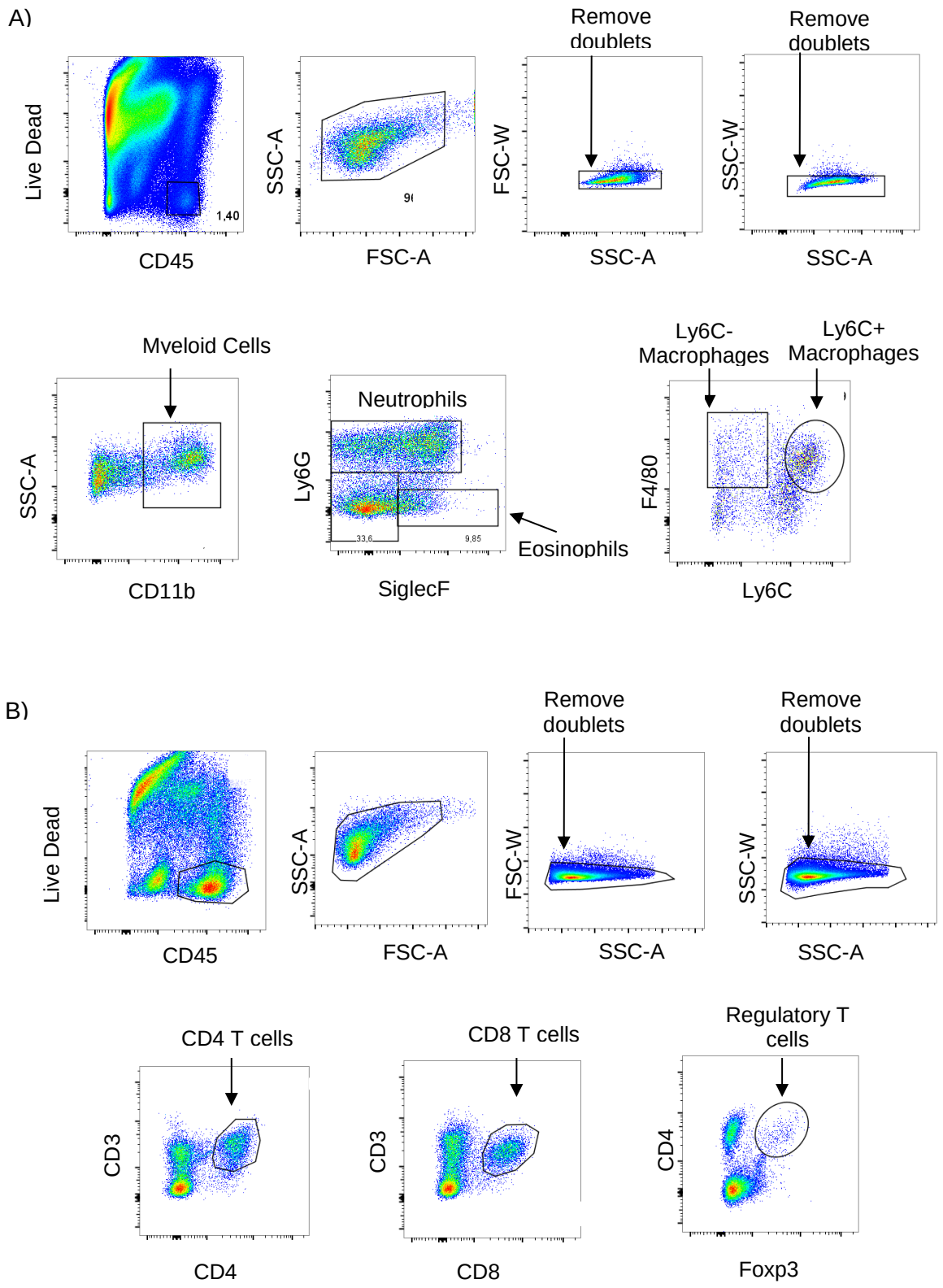


<u>Sup Figure 2</u>: TLR3 ligand and anti-CD40 intra-tumour injection induced tumour remission in CT26 and delayed the tumour growth in B16 and 4T1 tumour cells. (A) CT26 tumour growth in non-treated mice (green square; n=3) or mice treated (red circular; n=7). On day 9, 10, 14, 16, 18, 21 (highlighted in the graphic), mice were injected with anti-CD40 + TLR3 ligand intra-tumour. **(B)** B16 tumour growth in non-treated mice (green square; n=1) or mice treated (red circular; n=2). On day 14, 16, 18, 20 (highlighted in the graphic), mice were injected with anti-CD40 + TLR3 ligand intra-tumour. **(C)** 4T1 tumour growth in non-treated mice (green square; n=4) or mice treated (red circular; n=6). On day 7, 9, 12, 15 (highlighted in the graphic), mice were injected with anti-CD40 + TLR3 ligand intra-tumour.



Sup Figure 3: Background level of MHCI expression on E0771, B16F0, CT26 and 4T1 tumour cell lines.

Antibodies	Clone	Manufacturer
H-2Kb	AF6.88.3	Biolegend
H-2Kd	SF1-1.1	BD Bioscience



<u>Sup Figure 4</u>: Flow cytometry gating strategy of (A) myeloid cell and (B) lymphocyte populations infiltrating the tumour.

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