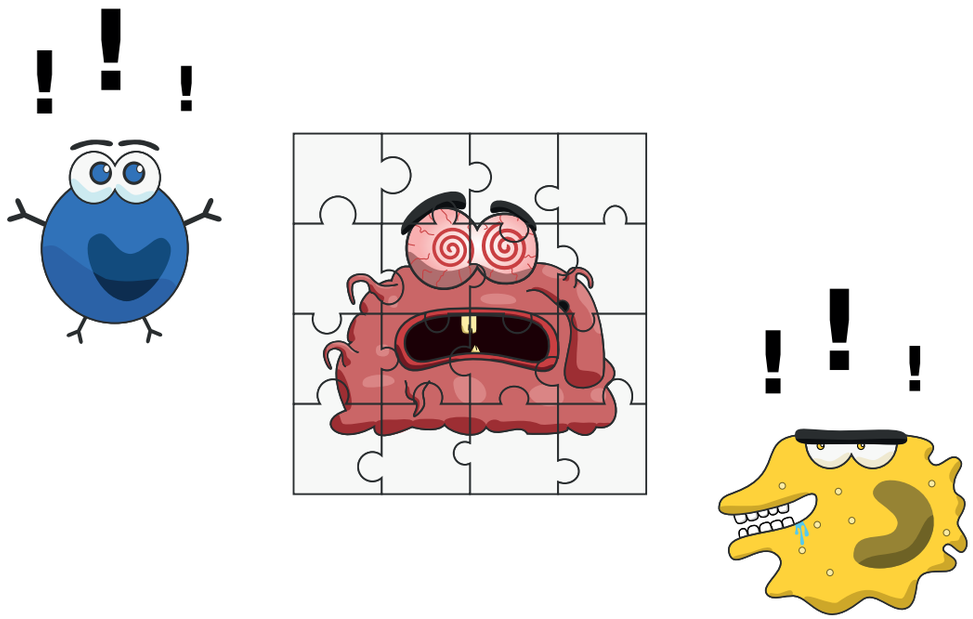

Thesis for doctoral degree (Ph.D.) 2019

Decoding the tumor microenvironment: a B cell and Macrophage perspective

Vanessa F. Boura

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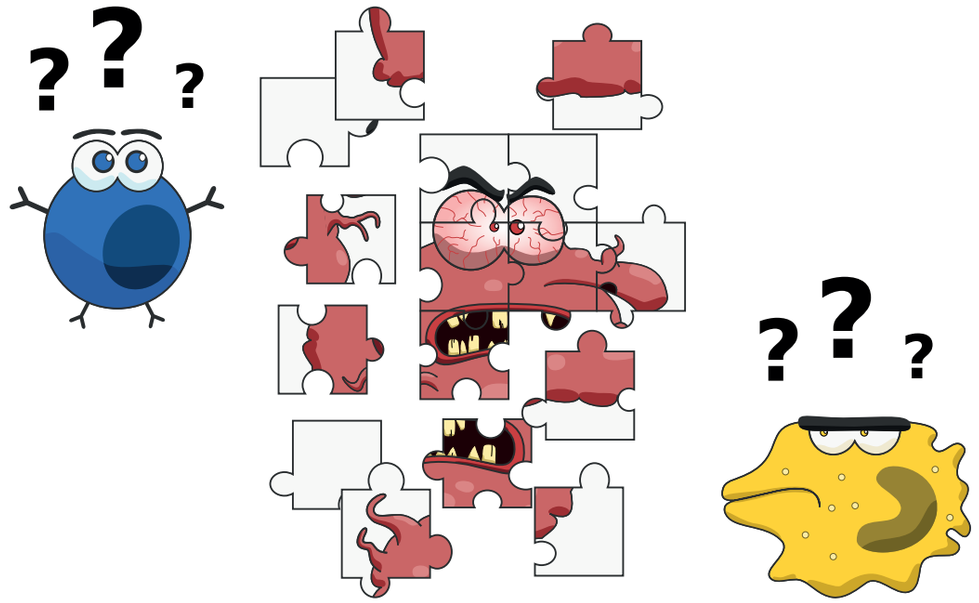

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DECODING THE TUMOR MICROENVIRONMENT:

A B CELL AND MACROPHAGE PERPERCTIVE

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Decoding the tumor microenvironment: a B cell and Macrophage perspective

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my parents

“There is no real ending. It’s just the place where you stop the story.”
— Frank Herbert

ABSTRACT

The immune system plays a role in many different functions in our body, and disease is no exception. Cancer is one of the biggest challenges of the twenty first century and vast efforts have been made to understand this disease. Since the 1900s, we have evidence based that the immune system participates in cancer progression, but it was only some decades ago that we started to investigate it extensively. Currently, we know that the tumor microenvironment is infiltrated by different immune cells and depending on space and time they can participate in tumor initiation, promotion, progression and metastasis. Moreover, it has been shown that certain immune cell can influence tumor resistance to therapy. So far, most of the studies have been directed towards understanding tumor infiltrating T cells. However, in this thesis I focused on the interplay between different immune cells including B cells, T cells, Natural killer cells, macrophages and the tumor cells. Further from this research, we found potential predictive biomarkers associated with the checkpoint therapies, such as anti-CTLA-4, anti-PD-1 and anti-PD-L1.

This thesis can be divided into two major studies investigating two immune cell types infiltrating the tumor, B cells (**Paper I** and **II**) and macrophages (**Paper III** and **IV**). More specifically, **Paper I** touches upon the role of B cells infiltrating two types of tumors, melanoma and breast cancer, studied in mouse models. Here, we could detect the presence of a heterogeneous B cell population that could be further divided by its surface CD5 expression. These subpopulations were phenotypically and functionally distinct. In particular, CD5+ TIBs were found to have an activated phenotype and were able to secrete proinflammatory cytokines, which were induced by the tumor milieu. Additionally, we identified a similar B cell subpopulation in human breast cancer highlighting the importance of this finding. **Paper II** focused on the B cell responses towards melanoma when treated with immune checkpoint therapies anti-PD-1 or anti-PD-L1. This study showed an augmented IgG response against the tumor mice treated with checkpoint antibodies. More importantly, it revealed significant differences in IgG subclasses depending on the checkpoint treatment given.

In the second part of the thesis, **paper III** identifies MARCO, a scavenger receptor, as a novel marker for immunosuppressive TAMs subset present in three different mouse models, namely melanoma, mammary carcinoma and colon cancer. Monoclonal antibody against MARCO was found to successfully decrease the tumor growth and metastasis, while re-polarizing this subpopulation of TAMs to have an anti-tumor phenotype. Moreover, we could observe an enhanced effect of anti-CTLA-4 treatment when combined with anti-MARCO. Finally, MARCO proved to be expressed in human metastatic melanoma and in an aggressive breast cancer subtype. Thus, targeting MARCO could potentially be a combinatory treatment for these cancer types. **Paper IV** further provides evidence on MARCO expression in a

distinct subset of immunosuppressive TAMs, but now in human non-small cell lung cancer. Moreover, this study uncovered the strategical localization of MARCO positive macrophages at the tumor-stroma border. This creates an immunosuppressive barrier that could be potentially targeted with anti-MARCO.

In summary, this thesis contributes to our overall understanding of the tumor microenvironment, specifically of B cells and macrophages. It gives us new possible targets and approaches for cancer therapy as well as potential predictive biomarkers.

LIST OF SCIENTIFIC PAPERS

I. Boura, V.F., Sohn, S., Hoekstra, E., Sarhan, D., Pedersen, G., Erikson, E., McGaha, T., Karlsson Hedestam, G. B., Karlsson, M. C. I.
The tumor microenvironment induces innate cytokine producing B cells
Manuscript

II. Boura, V.F., Sohn, S., Hallgren, Å., Pico de Coaña, Y., Kämpe, O., Kiessling, R, Karlsson, M. C. I.
B cell responses to checkpoint therapy in melanoma
Manuscript

III. Georgoudaki, A.-M., Prokopec, K. E., Boura, V. F., Hellqvist, E., Sohn, S., Östling, J., Dahan R., Harris, R. A., Rantalainen, M., Klevebring, D., Sund, M., Brage, S. E., Fuxe, J., Rohlny, C., Li, F., Ravetch, J. V., Karlsson, M. C. I. (2016).
Reprogramming Tumor-Associated Macrophages by Antibody Targeting Inhibits Cancer Progression and Metastasis.
Cell Reports, 15(9), 2000–2011.

IV. La Fleur, L., Boura, V. F., Alexeyenko, A., Berglund, A., Pontén, V., Mattsson, J. S. M., Djureinovic, D., Persson, J., Brunnström, H., Isaksson, J., Brandén, E., Koyi, H., Micke, P., Karlsson, M. C. I. and Botling, J. (2018)
Expression of scavenger receptor MARCO defines a targetable tumor-associated macrophage subset in non-small cell lung cancer.
International Journal of Cancer, 143(7), pp. 1741–1752.

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cells
Arg1	arginase-1
B16 mOVA	B16 membrane-bound Ovalbumin
Bcl6	B cell lymphoma 6
BCR	B cell receptor
Be	B effector
BMDM	Bone-marrow derived macrophages
Breg	B regulatory cells
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CSF1	Colony stimulating factor-1
CSR	Class switch recombination
CTLA-4	Cytotoxic- T lymphocyte antigen 4
CXCL	C-X-C motif chemokine ligand
DAMP	Damage-associated molecular patterns
DC	Dendritic cells
dLN	draining lymph node
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
FcγR	Fc-gamma receptor
FDC	Follicular dendritic cell
FoB	Follicular B cells
GC	Germinal center
GM-CSF	Granulocyte-macrophage colony stimulating factor
HDL-R	High-density lipoprotein receptors

IFNγ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
iNOS	inducible Nitric oxide synthase
IRA	Innate response activator
IRAE	Immune-related adverse events
LDL-R	Low-density lipoprotein receptors
LPS	Lipopolysaccharide
LT	Lymphotoxin
MAM	Metastasis-associated macrophages
MARCO	Macrophage receptor with collagenous structure
MART-1	Melanoma-associated antigen recognized by T cells-1
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MMP	Matrix metalloproteases
MPS	Mononuclear phagocyte system
MSR1	Macrophage scavenger receptor 1
MZ	Marginal zone
MZB	Marginal zone B cells
MZP	Marginal zone progenitors
NK	Natural killer
NKT	Natural killer T cells
NLR	NOD-like receptors
NO	Nitric oxide
NSCLC	Non-small cell lung cancer
NY-ESO-1	New York-esophageal squamous cell carcinoma-1
OVA	Ovalbumin
PAMP	Pathogen-associated molecular patterns

PD-1	Programmed cell death Protein 1
PDGF	Pallet-derived growth factor
PD-L1	Programmed cell death-ligand 1
PF4	Platelet factor 4
PRR	Pathogen recognition receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SHM	Somatic hypermutation
T.C.M.	Tumor conditioned medium
T1	Transitional type 1
T2	Transitional type 2
TAM	Tumor-associated macrophages
TCR	T cell receptor
TD	T cell dependent
Tfh	T follicular helper cell
Tfreg	T follicular regulatory cells
TGFβ	Transforming growth factor beta
Th	T helper
TI	T cell independent
Tie2	angiopoietin 2 receptor
TLR	Toll-like receptor
TLS	Tertiary lymphoid structures
TMA	Tissue microarrays
TNF	Tumor necrosis factor
Treg	T regulatory cell
VEGF	Vascular endothelial growth factor
VISTA	V-domain Ig suppressor of T cell activation

1 INTRODUCTION

1.1 BRIEF OVERVIEW OF THE IMMUNE SYSTEM

The immune system´s main physiological function is to provide host defense against invading pathogens, such as virus, bacteria and fungi. It is comprised by a complex network of immune cells, organs and specialized molecules, which work together to prevent or eradicate potential threats and protect us from disease. This is possible due to unique capacity of the immune cells to discriminate between self- and non-self-molecules [1]. Deriving from hematopoietic stem cells precursors in the bone marrow through a process called hematopoiesis, the immune cells can commit to either to lymphoid or myeloid lineage. While the lymphoid lineage gives rise to B cells, T cells , Natural Killer (NK) cells and innate lymphoid cells (ILC), the myeloid progenitors develop into granulocytes (basophils, eosinophils and neutrophils) and monocytes (later differentiating into macrophages and dendritic cells (DCs) [1]. These different types of immune cells participate in and have defined functions during an effector response. Classically, the immune response can be divided into two branches, the innate and the adaptive immune system. They diverge mainly in the timing and duration of the response, but also in the immune cells involved and specificity [2]. More importantly, they differ in the mechanisms through which they recognize antigens.

The innate or "natural" immune system can be found in all multicellular organisms, being phylogenetically an older defense system. The pathogen recognition is therefore based on receptors encoded in the germline[3]. These receptors are called pattern recognition receptors (PRRs) and are able to identify structures shared by pathogens and not present on normal cells, named pathogen-associated molecular patterns (PAMPs) [1]. Examples of PPRs are for instance the toll-like receptor (TLR), which can bind to a wide range of molecules, including lipopolysaccharides (LPS), bacterial DNA and viral RNA. Additionally, they can also recognize endogenous damage-associated molecular patterns (DAMPs), which are released in damaged or necrotic cells [1]. The innate immune system is characterized by the ability to mount a fast effector response when encountering a pathogen for the first time. It occurs within minutes or hours of infection and therefore, is responsible for the first line of host defense. The innate immune system is constituted by different defensive barriers. First, anatomical (e.g. skin and musical membranes) and chemical barriers (e.g. low pH and antimicrobial peptides) protect the organism from most pathogens and when breached, internal barriers such as the complement system, phagocytic cells and inflammation come into play [2]. Macrophages and neutrophils are phagocytic cells that have a distinct ability to engulf microbes via PPRs. Besides these immune cells, NK cells, DCs and ILCs also participate in the effector function of innate immunity. In particular, DCs have the important function of antigen-presentation to T cells through the major histocompatibility complex (MHC), bridging the innate with adaptive immune system [1]. Likewise, inflammation has a

vital role in establishing an effector immune response. It is initiated by cells present in the damaged/infected tissue, for example macrophages, DCs and mast cells, which are able to release chemical factors responsible for the inflammatory response. Two other hallmarks of the innate immune system are the absence of antigen specificity and immunological memory. However, new studies have started to challenge the later concept by the discovery of memory-like NK cells in mice [4][5][6], which we presently name "trained immunity"[7].

On other hand, the adaptive immune system is a more specific response against pathogens. It is additionally a slower immune response, peaking only days after the immune recognition of the pathogen by the innate immune system. The adaptive immune effector function is mediated by B and T cells, two immune cells of lymphoid origin [1]. They are characterized by the expression of specialized receptors named B cell receptor (BCR) and T cell receptor (TCR), which undergo gene rearrangements (somatic recombination) during development [1]. This results in a wide diversity of antigen binding capacity of these receptors, which is an import hallmark of adaptive immunity. Moreover, both receptors differ in what they can bind. Whereas the BCR can bind directly to free antigen, taking into consideration for instance its conformational structure, the TCR can only bind to peptides presented on MHC molecules on the surface of antigen-presenting cells (APCs). In an ongoing response, T and B cells get activated upon antigen recognition and can undergo clonal selection, to ultimately eradicate the threat. Adaptive immunity is characterized by immunological memory, allowing faster responses towards a second encounter of the pathogen and thus, conferring to the host a lifelong protection [1].

In general, the immune system plays a central role in pathogen protection, but it is also involved in clearance of dead cells and initiation of tissue repair. However, when improperly regulated contributes to disease initiation and progression.

1.2 B CELLS

In all vertebrates, the adaptive immune system is fundamentally organized by T and B cells. The separation between these two cell types was first described by Max Cooper and Robert Good, in 1965[8]. In chicken models, they discovered that B cells were developed in the bursa of Fabricius (equivalent to bone marrow in humans and mice) and the T cells in the thymus and more importantly, that both had different functions in adaptive immunity[8][9]. T cells were responsible for a delayed-type hypersensitivity response (later, T cell mediated immunity), while the B cells were accountable for the production of antibodies (humoral immunity). Since then, B cells have been studied and found to be a heterogeneous population capable of not only antibody production, but also act as APCs and as cytokine producers [10]. During the last years, B cells have come forward as important players in autoimmunity diseases and cancer malignancies. In this chapter, I will focus on the major subsets of B cell populations and their effector functions.

1.2.1 B cell subsets

Continuously produced throughout life, B cells leave the bone marrow, where they assemble their B cell receptor and enter circulation as transitional B cells. These cells have a BCR (specifically IgM) capable of recognizing specific antigenic epitopes and as described by Carsetti et al, can be subdivided into two groups, transitional type 1 (T1) and transitional type 2 (T2) B cells [11]. T1 are characterized by their surface expression of IgM and upon further differentiation, they gain increased IgD expression through alternative splicing and become T2 B cells [12]. As transitional B cells have the ability to go to the periphery, they migrate to the spleen to become mature B cells.

The mature lineages of B cells can be divided in two: B1 and B2 cells [13][14]. B2 cells originate from the bone marrow and can be further divided into follicular B cells (FoB) and marginal zone B cells (MZB). FoBs are the most predominant B cell subpopulation in the spleen and lymph nodes. They are circulating cells and as the name indicates, they home mainly to B cell follicles of secondary lymphoid organs, in close proximity to T cell zones [15]. Functionally, they are better suited to respond to protein antigens, in a T cell-dependent manner. Once activated FoBs and T cells meet in the T-B border, germinal center (GC) B cells are formed, differentiating finally, into antibody producing plasma cells [15][16]. Most of FoBs exhibit high levels of IgD, low levels of IgM and are positive for CD23 and CD21 on their cell surface [15]. In contrast to FoBs, murine MZBs are fairly non-circulating and reside in the marginal zone of the spleen[14]. MZBs are considered to be innate-like B cells and their localization in the spleen provides access to blood-borne pathogens, enabling them to mount rapidly a T-independent response [14][15][17][18]. MZBs are characterized by expression of high levels of CD21 and CD1d [14][15]. These markers are important for the effector function of MZBs. While CD21 can help the MZBs to mediate the transport of immune complexes to the B cell follicles, the high levels of CD1d permits MZBs to present

lipid antigens to natural killer T cells (NKT) [14][15]. MZBs are thought to be a vital source of lipid specific antibodies [14][15].

Lastly, murine B1 cells are also considered to be innate-like B cells, due to their effector functions. Unlike the B2 cells, they derive primarily from the fetal liver and occupy mainly the pleural and peritoneal cavities [14][17]. However, they can also be encountered in secondary lymphoid organs (e.g. spleen) and mucosal sites [14][17]. B1 cells can traffic and home to these sites continuously, and this migration is believed to be through the omentum and CXC-chemokine ligand 13 (CXCL13) dependent [14][19]. Moreover, B1 cells renewal has been suggested to take place in the spleen. This was proposed due to splenectomized mice experiments, which resulted in depletion of the B1 cells in the peritoneum, but not of the B2 cells[19][20]. Phenotypically, they are characterized by being $CD19^{high}$, $B220^{low}$, CD43+, CD23- and IgM^{high}. B1 cells can be further divided into B1a and B1b by the expression of the surface marker CD5 on the B1a cells[19]. Functionally, B1 cells are easily activated and the main producers of natural antibodies. Highly crossreative, these antibodies are present in steady state and are important in host defense against pathogens by direct neutralization [19][21]. In this regard, MZBs also contribute to the pool of natural antibodies[18]. Giving a rapid and early immune response, B1 cells can differentiate into IgM secreting short-lived plasma cells, but also switch into IgA secreting cells in a T cell independent manner[14][17].

Besides these B cells, we can also have other subsets depending on their different effector functions. Examples include B regulatory cells capable of secreting the cytokine IL-10 (B regulatory cells, Breg)[22] and the innate response activator (IRA) B cells responsible for producing granulocyte-macrophage colony stimulating factor (GM-CSF)[23][24].

1.2.2 B cell activation

Activation of B cells has a central role for the generation of plasma cells and memory cells, which are important for host defense and ultimately a hallmark for humoral activity. Their activation, depending on the nature of the antigen and T cell help, can be divided into T cell independent (TI) and T cell dependent (TD) activation [1]. As the name indicates, TI responses do not require further signaling from T cells and can be subdivided into TI type I or type II. Whereas in TI type I responses B cells activation occurs through TLR signaling independently of BCR engagement, in type II responses it requires BCR antigen binding, specifically the crosslinking of multiples BCRs to an antigen with similar antigenic sites. Antigens such as LPS or bacterial DNA are a typical TI type I antigens, while long polysaccharides with highly repetitive binding sites are examples of TI type II antigens. In general, TI B cell activation is characterized by being a rapid response and resulting in the generation of low affinity antibodies. Additionally, TI responses might require a second signal from other immune cells besides T cells, such as NK cells, which can contribute for secretion of cytokines. As mentioned above, innate-like B cells are more likely to respond to

TI antigens and rapidly differentiate into short-lived plasma cells and memory cells in extrafollicular areas [14][25].

Contrary to TI responses, TD B cell activation is reliant on BCR ligation to protein antigens and T cell help. Normally, TD antigens are recognized by FoBs and upon binding to the BCR, the antigens get endocytosed, degraded into peptides and presented on MHC class II molecules on the surface of these B cells. Alongside, activated B cells upregulate C-C chemokine receptor type 7, allowing them to migrate to T-B cell border and meet cognate T cells. Here, T cells recognizing the antigen-MHC complex presented on B cells, will provide the needed signals for B cell proliferation and differentiation. T cell help comprises of co-stimulatory molecules (e.g. CD40L) and cytokines (e.g. interleukin -4 (IL-4) and IL-21) [1]. At this point, activated B cells can enter into two different pathways, named extrafollicular or follicular pathway. In the extrafollicular pathway, B cells can develop into short-lived plasma cells and early-memory B cells outside lymphoid follicles [26]. In the follicular pathway, some activated B cells will upregulate the transcription factor B cell lymphoma 6 (Bcl6) and return to the follicles to form GC B cells, ultimately resulting in the formation and selection of high affinity antibodies [26]. GCs are lymphoid microstructures found in secondary lymphoid organs, formed at T-B borders [27]. In the GC, B cells will induce the expression of activation-induced cytidine deaminase (AID), an enzyme vital for somatic hypermutation (SHM) and class switch recombination (CSR) [28]. On one hand, SHM results in enhanced antibody affinity due to the introduction of random point mutations in the antigen-binding site of the BCR by AID [27][29]. On the other hand, CSR results in isotype switching of the BCR, enabling for different effector functions of antibodies without altering their antigen specificity [29]. Within the GC, B cells receive support from specialized CD4+ T cells, named T follicular helper cells (Tfh), by the secretion of cytokines (especially IL-21) and costimulatory molecules [30]. Tfh are essential for the formation and maintenance of GCs, sustaining extensive B cell proliferation, SHM and CSR [30]. Similarly, follicular dendritic cells (FDCs) facilitate these processes and present complement-fixed antigens on their cell surface to B cells, allowing the selection of high affinity BCRs [31]. Importantly, FDCs are stromal cells and not DCs, and they are found in B cell follicles. Finally, B cells that undergo GC formation will be selected for their high affinity to the antigen and differentiate into plasma cells or long-lived memory B cells [26][27]. B cells that fail this selection process will undertake apoptosis and be phagocyte by macrophages [27][32].

1.2.3 B cell effector functions

B cells are normally associated with production of antibodies, but also are able to secrete cytokines and can present antigens to T cells (**Figure 1**). Briefly, B cells when activated are capable of differentiating into antibody secreting cells. Antibodies, also called immunoglobulins (Ig), are an important effector function of B cells. Structurally, they consist of two identical light chains and two identical heavy chains, which are associated to each

other by disulfide bonds to form a "Y" shape [26]. Functionally, there are two main regions in an antibody, the variable or the N-terminus region and the constant or C-terminus region. While the variable region of the heavy and light chains is responsible for binding to the antigen, the constant region of the heavy chains is essential for the effector functions of the antibody. Based on the C-terminus regions of the heavy chains, five isotypes of antibodies can be defined, IgM, IgD, IgG, IgA and IgE [1][26]. Depending on the isotype and binding affinities, different effector functions can be exerted [1][26]. The main ones are: 1) neutralization of pathogens or toxins, which prevent the infection of healthy cells; 2) opsonization, which is a process where the targets are tagged with antibodies to facilitate their phagocytosis; 3) complement activation (IgM is a good activator of the classical pathway of complement system); and 4) antibody-dependent cell-mediated cytotoxicity (ADCC), where for example NK cells bind to IgG coated cells and provoke the lysis of the targets [1][26]. Ultimately, antibodies provide us with immunity against infections, but if self-reactive can also participate in disease pathologies (e.g. autoimmune diseases).

Another function of B cells is their ability to present antigens to T cells. As any other APC, B cells express the molecule MHC class II on their surface. Stepwise, once the BCR binds to the antigen on B cells, it gets internalized, processed and presented on a MHC class II molecule. This allows B cells to present antigen peptides to $CD4^+$ T cells [26]. Though, DCs are more effective APCs than B cells, it has been demonstrated that B cells are also important for proliferation and differentiation of T cells [33][34][35][36], especially when the antigen is limited [37][38][39]. Additionally, B cells can act as APCs to cognate CD4+ T cells with high efficiency, in order to gain help for production of high affinity antibodies[36]. Similarly to DCs, B cells also present co-stimulatory molecules, such as CD80, CD86 and CD40, which are important for the activation and proliferation of T cells [26][38].

Finally, B cells can act as immunomodulators by the secretion of cytokines. Long known for this ability, only recently has this topic been closely studied, motivated mostly by B cell depletion experiments with rituximab (anti-CD20 antibody) for treatment of autoimmune diseases. Interestingly, many patients that received rituximab showed improved symptoms of the disease without a corresponding decrease in autoantibodies levels in the serum [40][41]. Taken together, this suggests that B cells might have a role in the disease progression in an antibody-independent manner [41]. The cellular origins of these cytokine-producing B cells is still in debate, but B cells upon TLR binding, T cell priming or both are able to secrete cytokines and chemokines [38][40][41]. Lund and Harris were the first to divide B cells depending on their cytokine profile and effect on the T helper cells (Th) polarization [42]. Therefore, B effector (Be) 1 would produce Th1 like cytokines, including interferon-γ (IFNγ), IL-12p40 and tumor necrosis factor (TNF), while Be2 would secrete Th2 like cytokines, IL-2, IL-4, IL-13, IL-6 and TNF [41][42]. Moreover, in later studies, it was found that Be1 cells IFNγ secretion was dependent on the activation of IFNγ receptor and the T-box transcription factor, T-bet, on B cells [41][43]. In contrast, Be2 "commitment" was observed to be controlled by the activation of IL-4 receptor α on B cells and Th2 cells [41][44]. Hence, B cells are able to produce a broad variety of cytokines, not only the Be1 and Be2, but also pro-

inflammatory cytokines IL-1, IFN-α and lymphotoxin (LT) [40][45]; the hematopoietic growth factor GM-CSF [23][24]; and C-C chemokines ligand 5 (CCL5) and CCL7 [40][46][47]. Functionally more studies are needed, but it has been reported that TNF promotes the expansion of CD4+ T cells [48] and differentiation of Th1 together with CCL3 [45][49], in different infection models. IFNγ is suggested to promote Th1 responses and macrophage activation [40][45][50]. And LTαβ is responsible for the development of secondary and tertiary lymphoid organs [40][41][45]. Additionally, B regulatory cells are considered a cytokine-producing B cell subset of its own. Responsible for producing anti-inflammatory cytokine IL-10, they have a regulatory function in the immune system [51]. Until now, it was reported different subsets within the Breg population: T2-MZP (marginal zone progenitors) [52], MZBs [53], B10 cells (CD5+ CD1d^{high}) [54], plasma cells [55] and plasmablasts [56].

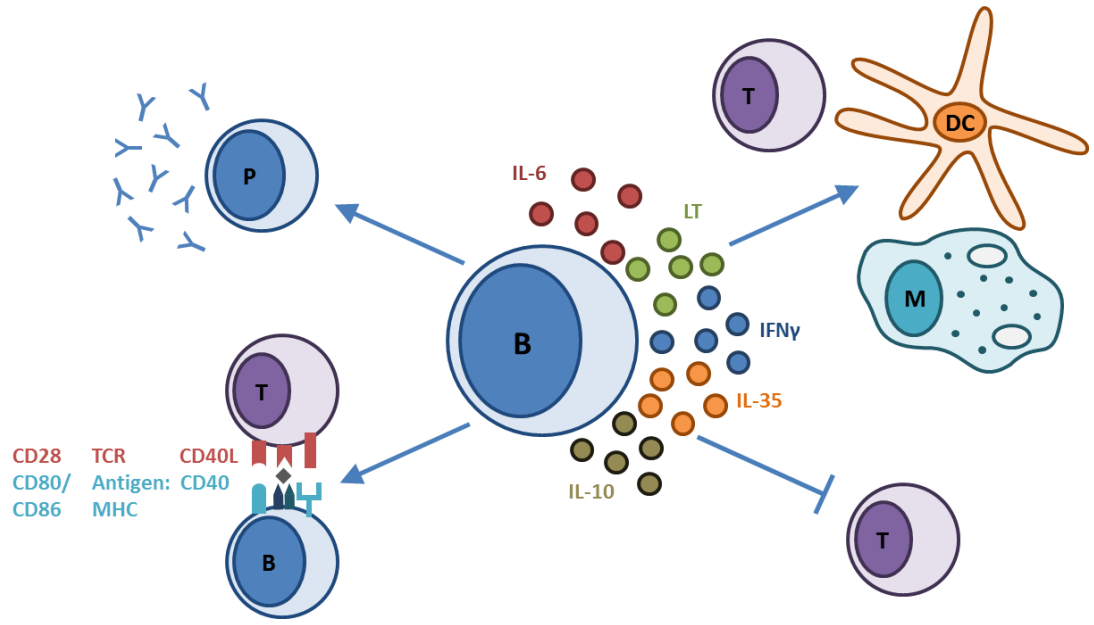

Figure 1. Depiction of B cell effector functions; including antibody production by plasma cells (P); antigen presentation to T cells (T) and respective interactions; and examples of cytokine secretion, such IL-6, LT and IFNγ and possible target cells (T cells, dendritic cells (DC) and macrophages (M)) and the immunosuppressive IL-10 and IL-35 (mentioned section 1.4.1) blocking T cells.

1.3 MACROPHAGES

Macrophages are specialized myeloid immune cells characterized by their ability to phagocytose foreign material, debris and dead cells. They were first described by Ilya Metchnikoff, the father of cellular immunology, almost 200 years ago, referring to them as "the big eaters" from the Greek makros `large´ and phagein `eat´ [57]. Since then, macrophages were found to participate in diverse functions in the organism and to have an essential role in innate and adaptive immune system. This is enabled by their remarkable plasticity, which allows macrophages to respond to different environment cues. In this section, I will go more in depth on their origins, populations and functions.

1.3.1 Origin and classifications

In adult mammals, macrophages are found all over the body, where they display cellular and functional diversity [58]. Originally, tissue-resident macrophages were thought to differentiate from circulating monocytes, which originate from hematopoietic stem cells precursors in the bone marrow [58]. This concept arises from the initial macrophage classification, where they are considered part of the mononuclear phagocyte system (MPS) [59]. A system that encompasses all phagocytic cells with hematopoietic cell linage derived from progenitor cells in the bone marrow [60]. However, reports from monocytopenic human patients and mouse models showed practically unaffected tissue-resident macrophages populations [59][61]. Such observations suggested a different origin for these cells during ontogeny and with the support of fate-mapping studies the concept of MPS was revised [59][58]. Now, it is accepted that there are three different lineages for macrophages in the mouse: yolk sac-derived and fetal liver-derived during embryogenesis and bone marrow HSC-derived in adulthood [58][62]. During embryogenesis, yolk sac progenitors give rise to most of the tissue-resident macrophages of the skin, brain, lung, liver, pancreas and spleen. They are defined as F4/80 bright and are characterized as long-lived and self-renewed, persisting into adulthood [58][59]. In parallel, the fetal-liver has been shown to contribute to this group of macrophages. In tissues, such as the lung, it was found that macrophages can have a chimeric origin from yolk sac and fetal liver [58]. During adulthood, bone marrow HSC-derived circulating Ly6C+ monocytes can differentiate into short-lived and non-self-renewing F4/80low tissue-resident macrophages [58][59]. They can be found in peripheral tissue with ongoing homeostatic inflammatory conditions, for example the intestine and mammary gland [59][58]. In sum, tissue-resident macrophages from healthy tissue are normally establish prenatally, while macrophages associated with homeostatic inflammation are typically derived from tissue-infiltrating monocytes developed in adulthood [59].

Tissue-resident macrophages have distinct gene expression and epigenetic profiles. They are integral components of the host tissues and can take different forms. For example, in the spleen, there exist marginal zone macrophages, metallophilic macrophages (MMM) and red

pulp macrophages; in the peritoneum, the peritoneal macrophages; in the liver, kupffer cells; in the brain, microglia and in the bone, osteoclasts [59].

Macrophages can also be classified based on their activation or "polarization" state in response to different cues under non-homeostatic conditions [62]. Traditionally, macrophages were simply categorized into two extreme states of activation, named as classical activated macrophages (M1) and alternatively activated (M2) macrophages [63]. These terms reflected the different effects of specific cytokines in inducing distinct Th immune responses. Specifically, whereas the inflammatory cytokine IFNγ was able to polarize macrophages to M1 phenotype and participate in Th1 cell responses, the M2-polarized macrophages were induced by IL-4 and/or IL-13, cytokines characteristic of a Th2 driven immune response [63][64][65][66]. However, this binary classification was not able to reflect the complexity and diversity of macrophages found *in vivo*, lacking granularity [64][67]. Thereby, a more complete view/classification was set to account for transcriptional, morphological and secretory profile changes together with their functions. Thinking in a more continuous spectrum of macrophages instead of a linear one, macrophages were then grouped depending on their functions, namely host defense, wound healing and immune regulation [68]. This was depicted as a color wheel, where the three primary colors represented the three main macrophages and the merging colors/secondary colors signified the different-shades of macrophages yet to be identified, sharing functions with two out of the three types of macrophages [68]. The M1 or classically activated macrophages are responsible for the host defense and are induced mainly by IFNγ and TLR stimulation (e.g. LPS, but also TNF secreted by other immune cells. This signal combination results in a macrophage able to produce high amounts of pro-inflammatory cytokines IL-1β, IL-6 and IL-23 and chemokines CCL2, CCL15, CXCL9 and CXCL10 [68]. M1 macrophages are also characterized by the upregulation of *Nos2*, responsible for encoding the enzyme inducible nitric oxide synthase (iNOS), which stimulates the synthesis of nitric oxide (NO) from L-arginine [66]. Together with increased production of NO, M1 macrophages increase their production of reactive oxygen species (ROS), resulting in potent cytotoxic activity against pathogens [69]. In general, classically activated macrophages cytokine profile amplifies the Th1 immune response. Moreover, IFNγ and TNF increase the expression of MHC class II and co-stimulatory molecules (e.g. CD86) on M1 macrophages [1]. These features augment the capacity of antigen presentation by macrophages and further support T cells responses.

On the contrary, M2 wound healing macrophages are polarized by anti-inflammatory cytokines IL-4 and IL-13 [66]. These cytokines are produced by basophils, mast cells and other granulocytes and important drivers of Th2 immune responses. Interestingly, both cytokines can also be secreted by Th2 T cells, which in turn support the development and maintenance of these type macrophages [67][70]. M2 wound healing macrophages are characterized by the upregulation of arginase-1 (*Arg1)*, responsible for encoding the protein arginase, an enzyme involved in the production of extracellular matrix, important for wound healing [67]. Other markers such as Fizz1 (*Retnla*), Ym1 (*Chi3l3*), IL-4 receptor (IL4ra) and

chemokines such as CCL17, CCL18 and CCL22 help to further define these macrophages [67][68][71].

Lastly, regulatory macrophages are characterized by a strong secretion of the immunosuppressive cytokine IL-10 and abrogation of IL-12 [68]. Several factors have been implicated with the differentiation of regulatory macrophages, including TLR agonists, immune complexes, apoptotic cells, prostaglandins, dopamine, histamine and the IL-10 itself [68]. Different combinations of these stimuli will give rise to different regulatory subpopulations of macrophages. Nevertheless, all regulatory macrophages are characterized by the necessity of two stimuli for their anti-inflammatory activity (first signal being for example immune complexes or apoptotic cells and the second signal a TLR ligand) and the downregulation of IL-12 followed by the upregulation of IL-10. These macrophages are important inhibitors of inflammation and differ from wound healing macrophages, since do not contribute for the assembly of extracellular matrix [68].

In summary, all different macrophage polarization states can be influenced by both innate and adaptive signals and if not properly regulated can lead to potential participation in disease. Of note, it is important to mention that classification of macrophages is still not fully known and accepted as they are very plastic cells.

1.3.2 Macrophage functions

The heterogeneity of macrophage populations in responding to different environmental stimuli allows them to participating in a wide range of functions. Their most known ability is phagocytosis, the capacity to uptake and clear off foreign bodies as well as intracellular endogenous debris. Macrophages can recognize these pathogens and modified self-ligands by a family of receptors named PPRs, as mentioned in the first chapter. They can be found membrane-bound and in the cytosol at intracellular compartments. There are different classes of PPRs, including TLRs, NOD-like receptors (NLR), C-type lectin receptors and scavenger receptors [1]. In particular, TLRs can bind to wide range of PAMPs and can be expressed in the cell membrane and intracellular compartments. For instance, extracellular TLR4 binds to LPS and intracellular TLR9 can sense methylated DNA. Likewise, scavenger receptors can bind to different ligands of pathogens, apoptotic cells and modified lipids and as the name suggests, scavenge and phagocyte these threats [72][73]. Scavenger receptors are a broad family with at least eight classes (called A-H). For the purpose of this thesis, I will focus on one type of scavenger receptor named macrophage receptor with collagenous structure (MARCO). This class A scavenger receptor is mainly found on splenic marginal zone macrophages, peritoneal macrophages and the medullary cords of lymph nodes [74]. In the spleen, they strategically locate in the marginal zone (MZ) where the blood flows slowly, granting them access to blood-borne antigens [75]. Moreover, they interact with MZBs that are in close proximity and MARCO expression has been associated with retaining these cells in the marginal zone [76]. MARCO structurally has a short intracellular domain without any

signaling motif, suggesting that it needs a signaling partner for downstream signaling [74]. However, the molecular mechanisms mediating MARCO signal transduction remain unknown. Nevertheless, MARCO has an important role in inflammation and apoptotic cell clearance [75][77][78]. It is important to mention that PPRs are differentially expressed in different polarized macrophages, participating also in wound healing and immune regulation.

Macrophages not only participate in immunosurveillance, but they can also contribute to other physiological functions. For example, microglia have been shown to participate in brain development, more specifically in a process called synaptic pruning, consisting in the clearance of defective or immature neuronal synapses [58][79][80]. Similarly, macrophages in the mammary gland have been observed to play a role in the growth and branching of the ductal structure during pregnancy. Additionally, osteoclasts (tissue-resident macrophages of the bone) have been shown to influence bone generation through the secretion of molecules promoting or inhibiting bone formation, such as cardiotrophin-1 or semaphoring 4D respectively [79][81]. More recently, macrophages have been linked to maintain metabolic homeostasis in the adipose tissue, pancreas and liver. Regarding the adipose-tissue macrophages, in healthy conditions, they are able to sustain insulin sensitivity in adipocytes partly through the secretion and action of IL-10 [58][79]. In the liver, kupffer cells are involved in cholesterol recycling in the plasma and express high-density lipoprotein receptors (HDL-R) as well as low-density lipoprotein receptors (LDL-R) [59][82]. Since macrophages have wide range of functions, it is natural that they play an important part in disease initiation, promotion and progression. This will be discussed in the chapter 1.4.2 in regards of cancer.

1.4 THE TUMOR MICROENVIRONMENT

Cancer is a complex disease where transformed cells attain throughout time hyperproliferation, invasiveness and survival abilities [83]. However, a tumor is not only comprised of cancer cells. Tumor microenvironment contains a wide range of cells, such as pericytes, cancer-associated fibroblasts, adipocytes, endothelial cells and immune cells (**Figure 2**) [83][84]. All these cells together create an intricate network that supports tumor growth and metastasis. Within the tumor infiltrating immune cells, we can find T and B cells, NK cells, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), dendritic cells, neutrophils and eosinophils [84].

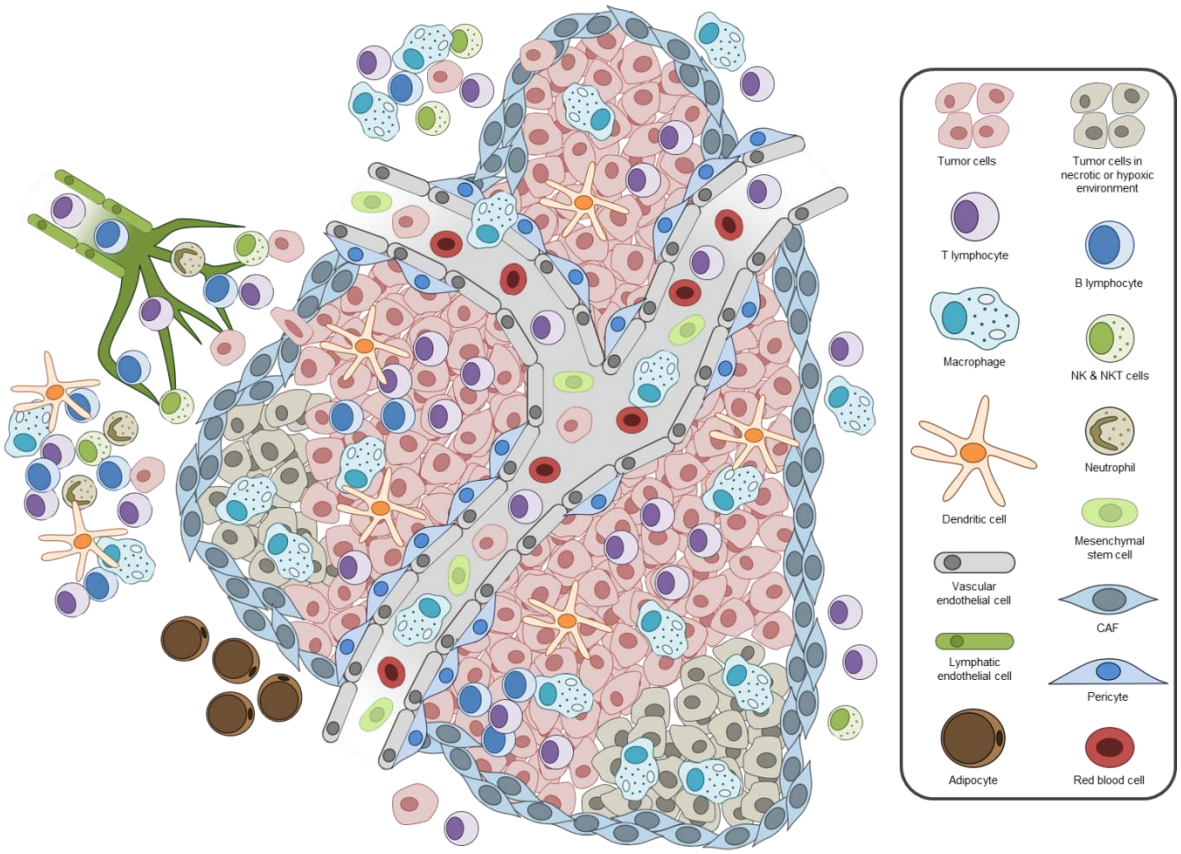

Figure 2. Representation of the tumor microenvironment, including the tumor cells (hypoxic or not), blood vessels, lymphatic vessels, adipocytes, pericytes, cancer-associated fibroblasts (CAFs), mesenchymal stem cells and immune cells (T and B cells, dendritic cells, macrophages, NK and NKT cells and neutrophils).

Crosstalk between tumor, immune cells and different environment cues is a complex process that will dictate the fate for anti-tumor or pro-tumor immunity. In 1957, Thomas and Burnet postulated that the immune system can recognize and eliminate transformed cells, in their immunosurveillance theory [85][86][87][88]. It was only until decades later, with the

improvement of genetic mouse models for cancer and blocking monoclonal antibody technology that this concept was accepted. Currently, it is known and has been demonstrated that the immune system plays a dual role in cancer development. In a dynamic process, the immune system not only can protect the host against tumor growth, but also can actively participate in promoting its progression. This gave rise to a new concept named cancer immunoediting, which consists in three steps: elimination, equilibrium and escape[89]. Briefly, the elimination phase, as described before, encompasses the concept of immunosurveillance. This is followed by an equilibrium stage, where there is a dynamic balance between immune cells and the remaining tumor cells that have survived the elimination phase. The mechanisms of this step are still not fully understood, nevertheless it is recognized that some tumor cells acquire genetic and epigenetic changes during this phase, which in turn will enable them resistant to immune recognition [86][90]. Additionally, this phase is also characterized by a balance between pro-tumor (e.g. IL-10) and anti-tumor (e.g. IL-12) cytokines [90]. Immune escape represents the final phase of immunoediting, where selected tumor cells with immune escape advantage take over and begin to expand progressively and uncontrollably. The tumor can avoid immune recognition through different mechanisms, such as loss of tumor antigens, downregulation of the antigen presentation molecule MHC class I and the upregulation of inhibitory surface proteins (e.g. PD-L1) [85][86]. As a consequence, this will lead to absence of T cell recognition of the tumor and inhibition of T cell activation. Moreover, tumor can establish an immunosuppressive microenvironment through the production of IL-6, tumor necrosis factor beta (TGFβ), CSF1 and vascular endothelial growth factor (VEGF) for example, which in turn will skew the immune cells towards a pro-tumor phenotype [86]. Increasing evidence on immune escape controls were recently recognized by being part of the ´Hallmarks of Cancer´, along with tumor-promoting inflammation, which is intricately connected with immune system and has been largely associated with tumorigenesis [86][91]. For this thesis, I will only focus on two types of immune cells within the tumor microenvironment: B cells and tumor-associated macrophages.

1.4.1 B cells in solid cancers

The tumor immunology field has been so far focused on the study of T cells and TAMs, mostly because they are predominant tumor-infiltrating immune cells [92]. However, B cells are too known to infiltrate human tumors, such as in melanoma, breast, colorectal, prostate, ovarian and small lung cancer [93][94][95][96][97]. Mostly from correlative studies, tumor-infiltrating B cells have been suggested to have either a supportive or suppressive role in tumorigenesis. In 1970s, tumor-infiltrating B cells (TIBs) were for the first time reported to be protumorogenic. By using anti-IgM to deplete B cells in a murine tumor model, they found slower tumor growth and a decrease rate of spontaneous metastasis in the lung [98]. In later studies, the formation of IgG immune complexes was correlated with poor prognosis [99][100][101][102][103]. Mechanistically, these can deposit in the tumor stroma, resulting

in the recruitment of immunosuppressive myeloid cells via the engagement Fcγ receptors (FcγR) [101][102]. With the discovery of Bregs, it was found that TIBs could exert their tumor immunosuppressive function by the production of IL-10 and TGFβ. More specifically, Bregs can differentiate naïve $CD4^+$ T cells into T regulatory cells, which can lead to cancer metastasis [104]. Recently, IL-35 secreting TIBs were associated to facilitate tumor cell proliferation in pancreatic cancer [105]. Additionally, TIBs might have a potential role in cancer therapy efficiency. Shalapour *et al*. showed that tumor-infiltrating plasma cells, expressing IgA and producing IL-10, can negatively modulate the chemotherapy response in three different mouse models for prostate cancer. These plasma cells , when present in the tumor, inhibited the cytotoxic function of T cells induced by the chemotherapy [106].

In contrast to this tumor supportive role, B cells might act as APCs to autologous T cells, promoting T cell proliferation and hence, affecting the tumor progression [107]. Moreover, CD20+ B cells were detected to be in close proximity to CD8+ T cells in the tumor stroma, which correlated to favorable prognosis in ovarian cancer [108]. Studies in a melanoma mouse model further demonstrated the importance of B cells in the CD4+ and CD8+ T cells responses towards the tumor [109]. In this study, depletion of B cells led to impaired T cell response and ultimately, increase in tumor volume and metastasis [109]. Additional evidence that TIBs might play a role in anti-tumor immunity comes from their ability to secrete lymphotoxin. This cytokine, as mentioned in 1.2.3 subchapter, facilitates the development of secondary and tertiary lymphoid structures (TLS) and in the tumor, enables the formation of ectopic TLS [92][102][103]. Likewise, in human cancers, the number of TLS is associated with a positive outcome [92][102][110].

1.4.2 Tumor associated macrophages

Macrophages are found to infiltrate a wide range of tumors, representing in some cases up to 50% of the total tumor mass in both mouse and human cancers [111][112][113]. Notably, TAMs are normally correlated with poor prognosis and can participate in all stages of carcinogenesis, from initiation to metastasis [114][115][116][117]. In response to an inflammatory tumor microenvironment, TAMs are recruited to the tumor and start to secrete IL-6, IFNγ and TNF, further supporting inflammation. This step is thought to be crucial to sustain chronic inflammation in the tumor microenvironment, creating a mutagenic milieu and thereby supporting tumor initiation and promotion [114]. Once the cancer is established, TAMs are educated to develop a more pro-tumoral phenotype [114][118][119]. This is accompanied by a shift in T cell responses, from a Th1 to Th2 type immunity, resulting in polarization of macrophages to M2-like. Cytokines such IL-4 and IL-13 are known to promote this M2-like phenotype, as well as increase production of growth factors by the tumor, including colony stimulating factor-1 (CSF1) and GM-CSF [114]. One of the mechanisms where TAMs can promote tumor progression is by supporting an immunosuppressive environment through secretion of cytokines and chemokines. For

instance, TAMs can induce CD4+ T cells to become T regulatory cells (Tregs) thought the secretion of IL-10 and TGFβ. Both cytokines were found to induce this T cell phenotype by upregulating the transcription factor Foxp3 in CD4+ T cells [114][120][121]. Additionally, IL-10 and TGFβ inhibit cytotoxic T cells as well as Th1 and Th2 CD4+ helper functions [114][122][123]. TAMs are also able to recruit Tregs to the tumor microenvironment, indirectly suppressing effector cells. Chemokines CCL20 and CCL22 synthetized by TAMs recruit CCR6+ Tregs in human ovarian adenocarcinoma and CCR4+ Tregs in colorectal cancer, respectively [124][125]. Additionally, TAMs can secrete the enzyme arginase 1 and metabolize L-arginine, which plays an important role in the re-expression of CD3ζ chain after downregulation of TCR upon antigen stimulation [114][126][127]. Expression of cell surface markers, CTLA-4 (Cytotoxic- T lymphocyte antigen 4), PD-1 (Programmed cell death protein 1) and PD-L1 (Programmed cell death-ligand 1) on macrophages can also contribute to the inhibition of cytotoxic T cell functions when engaging with their respective ligands (CD80/CD86; PD-L1/PD-L2 and PD-1, respectively) [114]. In the particular case of PD-L1, TAMs have been shown to upregulate this molecule as consequence of hypoxia-inducible factor-1 alpha (HIF-1α) signaling in hypoxic regions of the tumor [128].

Angiogenesis, or formation of new blood vessels, is a necessary step for tumor growth [91]. Stepwise, for the creation of new vessels, the recruitment of endothelial cells needs to be preceded by the destruction of the basement membrane and extracellular matrix (ECM) [111]. This process is enabled by TAMs by the production of proteolytic enzymes that degrade ECM, such as matrix metalloproteases-2 and -9 (MMP-2 and MMP-9) and the release of pro-angiogenic factors, including TNF, TGFβ, vascular endothelial growth factor (VEGF) and pallet-derived growth factor (PDGF) [111]. VEGF besides increasing vascular permeability can also promote migration of TAMs to the tumor and in hypoxia conditions it is upregulated by macrophages along with the expression of angiopoietin 2 receptor (Tie2) [111][129][130].

Lastly, TAMs participate in tumor cell invasion and metastasis in different tumors [116]. Tumor cells can secrete CCL2 and recruit CCR2+ Ly6C+ monocytes to the site, where they differentiate in metastasis-associated macrophages (MAMs) [131]. Phenotypically, these macrophages are characterized by the expression of CCR2, VEGF receptor and F4/80 and promote cell extravasation partly via expression of VEGF [114][131]. When depleted this MAM population, results in inhibition of metastatic seeding and consequent growth [111][131][132].

It is important to mention that some TAMs (proinflammatory M1-like) can also have a tumoricidal function. They can identify altered glycosylation patterns of cell surface molecules in tumor cells via binding of lectin-like receptors and lyse tumor cells by generation of ROS and NO [111]. Moreover, they can inhibit tumor metastasis directly and indirectly through the production of certain factors, including interferons and platelet factor 4 (PF4 or CXCL4) [111][133]. However, the tumor microenvironment promotes a more

immunosuppressive phenotype as described before, which can be further stimulated through the release of TGFβ and prostaglandin E_2 by tumor cells [111].

In summary, TAMs secrete a variety of cytokines, chemokines, enzymes, growth factors and express specific cell surface molecules that directly or indirectly support tumor initiation, promotion, progression and metastasis. Nevertheless, further investigation is needed in regards to the different subpopulations of TAMs and their distribution in human cancers.

1.5 CANCER IMMUNOTHERAPY

Cancer immunotherapy it is based on the principle that enhancing the immune response and restoring its reactivity may neutralize or eliminate cancer [134]. This is achieved by using components or mechanisms to stimulate the immune system [134]. Cancer immunotherapy can be further divided into active and passive. Active immunotherapy attempts to induce a specific and long-lasting anti-tumor immune response [135]. In other words, vaccination is used as an active method to stimulate the immune system. Tumor vaccination can go from using tumor-associated proteins and peptides as immunogens to dendritic cell vaccination [134][135]. In contrast, passive immunotherapy does not provoke a memory response and uses monoclonal antibodies or the transfer of donor cytotoxic T cells to directly target the cancer cells or activate the host adaptive immune system [135]. One type of monoclonal antibodies that has changed the current cancer treatments is checkpoint blockade antibodies [136]. This strategy aims to reverse the immunosuppression present in the tumor microenvironment by blocking the inhibitory receptors present on T cells and consequently, stimulate them to exert their anti-tumor functions [137]. This method has shown success in the clinic, as anti-CTLA-4, anti-PD-1 and anti-PD-L1 therapies are FDA approved [137]. CTLA-4 is inhibitory receptor that is expressed transiently on T cells and constitutively on T regulatory cells [137]. T cells, when they get activated by CD28 binding to CD80 or CD86 on APCs, CTLA-4 gets expressed on the cell surface, competing for the same ligands. CTLA-4 engagement results in T cell activation dampening and acts as a control mechanism of the immune system [1][137]. However, when the same happens in a cancer setting, T cells cannot perform their anti-tumor activity and therefore, the need of CTLA-4 blockade antibodies. In 1996, James P. Allison was able to demonstrate this in a murine model, where treated mice led to the rejection of the tumors[138]. PD-1 is also an inhibitory receptor expressed on T cells and similarly to CTLA-4, suppresses the T cell function[137]. It binds to PD-L1 and PD-L2, which are expressed on the surface of APCs and tumor cells. In this case, cancer cells can immune escape the cytotoxic function of T cells by binding to PD-1[137]. In some cancers, the expression of this ligand is normally associated with poor prognosis[137]. Checkpoint therapies, however, can incite certain side effects connected to overall activation of T cells[135].

2 AIMS

Paper I. To investigate the role of tumor infiltrating B cells in a mouse model for melanoma and breast cancer

Paper II. To study the effect of checkpoint therapies, anti-PD-1 and anti-PD-L1, on the B cell responses towards melanoma antigens in a murine model and human cancer

Paper III. To investigate the scavenger receptor MARCO expression on TAMs and inhibit tumor progression by reprogramming immunosuppressive TAMs using a monoclonal antibody against MARCO

Paper IV. To examine the expression of MARCO on TAMs in non-small cell lung cancer

3 RESULTS AND DISCUSSION

3.1 THE TUMOR MICROENVIRONMENT INDUCES INNATE CYTOKINE PRODUCING B CELLS (PAPER I)

The study of the tumor microenvironment, especially the crosstalk between cancer cells and immune cells, has enabled us to better understanding of tumor initiation, progression, metastasis and resistance to therapy. Nevertheless, many questions continue to be unanswered, including which B cells subpopulations infiltrate the tumor and their respective function. This immune cell type has long been known for their role in humoral immunity, but they are also important antigen-presenting cells and immunomodulators through cytokine production. Recent studies have found that TIBs are part of the tumor milieu and they may play either a supportive or suppressive function in tumor growth. In paper I, we sought to clarify the role of TIBs, specially the phenotype and functions, in two mouse models for melanoma and breast cancer.

Using a B16 mouse model modified to express membrane-bound ovalbumin (B16 mOVA), we confirmed by immunofluorescence the presence of B220+ TIB cells nearby the tumor blood/lymphatic vessels and/or the tumor capsule. Notably, these regions were enriched by infiltrating CD3+ T cells and NK1.1 NK cells. The presence of B cells in the tumor milieu was further proven using flow cytometry. Here, we could detect CD19+ TIB cells and two major TIB subpopulations identified by CD5 expression or lack of it on the cell surface. Similarly, an orthotopic model for breast cancer (EO77.1) showed the same TIB subpopulations within the tumor. Expression of CD5 is normally associated to a specific subset of B cells in mice, named B1a cells [139]. They are considered to be innate-like B cells and can mount immune responses in a T independent manner, thus contributing to the first line of the host defense. The presence of the surface marker CD5 on CD19+ TIBs then suggested that these cells might be innate-like B1. Using a genetic mouse model lacking B1 B cells (*Bumble* mice) [140], we could detect a significant reduction of CD5+ TIBs in the B16 mOVA model when compared to littermate controls. Strengthening this finding, the B1 B cell surface marker CD43 was markedly expressed on CD5+ TIB subpopulation [141]. Taken together, it indicates that CD5+ B cell subset infiltrating the tumor might be an innate-like B1 cell.

We further assessed the phenotype of these two TIB subpopulations by using flow cytometry. In both mouse models, we observed higher expression levels of the activation markers CD69 and CD86 on CD5+ TIB cells compared to their CD5- counterparts. Functionally, we looked into the ability of TIBs to secrete cytokines in the tumor microenvironment. Notably, no IL-10 expression was detected in neither of TIB subpopulations. Instead, CD19+ TIBs secreted proinflammatory cytokines TNF and IFNγ, specially the CD5+ fraction. Altogether, CD5+ TIBs have a more activated and a proinflammatory profile compared to the CD5- TIB faction.

To explore if the tumor milieu had any impact on the cytokine production of TIBs, we *in vitro* stimulated naïve peritoneal B cells with tumor conditioned medium ('T.C.M.') from B16 mOVA. This ´T.C.M.´ was collected after *ex vivo* culture of 10 to 14 days bulk tumors, thus mimicking the microenvironment that B cells might encounter when infiltrating the tumor. This had especially a focus on the molecules produced by the cells within this milieu. In line with the *in vivo* data, we detected that ´T.C.M.´ conditioned peritoneal CD5+ B cells acquired a proinflammatory phenotype. More specifically, these B cells secreted more TNF and IFNγ and less IL-10 when compared to control (medium cultured B cells), particularly when further stimulated with LPS.

In the particular case of IFNγ, it is known that two pathways are responsible for the secretion of this cytokine by B cells, a T cell dependent and independent way. Whereas, for the T cell dependent way, IFNγ secretion by CD4+ T cells directly promotes its production in B cells [42][43], for the independent pathway, it requires normally the cytokines IL-12 and IL-18 [43][142]. *In vivo*, we observed very few IFNγ+ CD4+ T cells infiltrating the B16 mOVA tumors, suggesting that these cells might not be the responsible inducers of IFNγ secretion in CD5+ TIB cells. For the independent pathway, we could detect the presence of IL-12 in ´T.C.M.´, but undetectable IL-18 protein levels. Focusing then on the IL-12, isolated *ex vivo* tumor cells and the cell line itself were proven to be not responsible for its production. TAMs activated in a M1-like state are known to produce IL-12 in these conditions [143][144]. *In vitro* cultured F4/80+ TAMs isolated from the tumor were capable of secreting IL-12 and in similar levels to those detected in ´T.C.M.´. Therefore, these results show that TAMs might be accountable for the IFNγ production in CD5+ TIB cells. Nevertheless, we cannot entirely exclude the participation of CD4+ T cells.

Next, we examined the potential effect of TIBs on other immune cells in the tumor microenvironment. Using anti-CD20 antibody *in vivo*, we observed a complete depletion of B cells in the B16 mOVA tumors. This was accompanied by a significant decrease in IFNγ+ CD8+ T cells and CD3- NK1.1+ NK cells. We further examined these results in an *in vitro* co-culture system. Notably, CD8+ T cells when co-cultured with ´T.C.M.´ conditioned B cells showed a decrease in IFNγ+ CD8+T cells, which revealed to have an exhausted phenotype, with higher expression of LAG3 and Tim-3 compared to control. In contrast, tumor conditioned B cells were found to increase IFNγ+ and CD107+ cytotoxic NK cells. Thus, our data suggest that intratumoral B cells affect both cytotoxic T and NK cells, leading to exhaustion of CD8+T cells and an increase in IFNγ+ NK cells.

Additionally, B cell depletion allowed us to observe a possible effect on the tumor growth. However, no significant difference in tumor volume was observed during the experimental time. Interestingly, when using the *Bumble* mice, which lack B 1 cells, we could detect an increased tumor growth compared to littermate controls. Moreover, we could show a negative correlation between CD5+TIBs and tumor volume. Altogether, this might indicate a possible CD5+TIB subpopulation with antitumoral activities.

Finally, we confirmed the presence of CD19+ TIBs in the tumor of human breast cancer tissue by flow cytometry. Along with the mouse *in vivo* data, we could identify TIBs expressing either CD43 or CD5 and secreting the proinflammatory cytokines TNF and IFNγ. Using The Cancer Immunome Atlas, we further observed that melanoma and breast cancer patients had better overall survival when high number of activating B cells were present in the tumor.

In summary, we could identify an innate-like B1 cells infiltrating the tumor of murine melanoma and breast cancer models. This subpopulation was characterized by having an activated phenotype and a proinflammatory profile, which was further associated with a direct effect of the tumor milieu. Likewise, human breast cancer was also found to be infiltrated by similar B cells and thus, giving us a better understanding of the several B cell subpopulations infiltrating the tumor and their possible effector functions.

3.2 B CELL RESPONSES TO CHECKPOINT THERAPY IN MELANOMA (PAPER II)

The overall clinical success of immune checkpoint therapies brought a promising cure for a wide range of cancers. Nevertheless, within the treated patients there is still quite heterogeneity in the outcome, ranging from complete remission to non-responders and in some cases, accompanied with immune-related adverse events (IRAEs). A recent study on patients receiving combination checkpoint therapy reported the importance of changes in circulating B cells subsets in predicting IRAEs, acting as biomarkers [145]. This study was the first of its own nature and in line with our paper II that tries to answer how the B cell responses are affected by the checkpoint therapies, in particular the anti-PD-1 and anti-PD-L1.

Using the same mouse melanoma model as in paper I, we treated tumor bearing mice with the checkpoint inhibitors, anti-PD-1 and anti-PD-L1, and PBS as control. As expected, after three treatment injections a significant reduction of the tumor size was observed, being nearly gone in some cases. Interestingly, we could detect an increase in OVA specific IgG antibodies, on days 14 and 21, in serum of mice treated with checkpoint therapies. This was accompanied by no significant differences in GC B cells in both spleen and draining lymph nodes (dLN). Notably, depending on the treatment given, differences in the IgG subclasses were observed in the serum of tumor bearing mice. Whereas for anti-PD-1 treatment, we could notice an increase in OVA specific IgG1 and IgG3; for anti-PD-L1, anti-OVA IgG2b and IgG2c levels were significantly increased compared to control. It is important to mention that this effect was specific to the tumor, since OVA immunization in Alum followed by checkpoint therapy did not result in a change of IgG subclasses in the serum.

In an attempt to explain the differences in antibody responses observed in the serum of treated mice, we examined the Tfh and T follicular regulatory (Tfreg) populations present in

the secondary lymphoid organs. Tfh cells are important CD4+ T cells present in the B cell follicles and responsible to deliver survival signals to GC B cells and are connected to B cell SHM and CSR [30][146]. In the opposite side, CD4+ Tfreg cells, characterized by FOXP3 expression, were observed to suppress Tfh and GC B cell proliferation [147][148]. In this study, Tfh were identified by the double expression of surface CXCR5 and PD-1; and Tfreg with the additional expression of the intracellular transcription factor FOXP3. Notably, we could observe, in dLN of tumor-bearing mice treated with anti-PD-L1, a significant increase of Tfh cells when compared to control. Likewise, splenic Tfh were markedly increased along with decrease in splenic Tfreg cells. However, for anti-PD-1 treatment no significant changes were observed for both T follicular cell subpopulations in dLN, with the exception of an increase in splenic Tfreg cells. For anti-PD-1 therapy, we were hypothesizing greater variations on these cells, since Tfh and Tfregs are characterized by a high expression of PD-1 and thus, being natural targets for this treatment. Nevertheless, a further characterization is needed of these cells to clarify the present results as well as a closer examination of the GC kinetics.

Class switching is also greatly influenced by the cytokine milieu present in an immune response [149]. Thus, we explored the potential role of cytokines present in the serum of treated mice. So far, we could identify increased levels of IFNγ in mice receiving anti-PD-1 compared to control. This result partially explain the differences in IgG subclasses found in anti-PD-1 treated mice, as previously reported, IFNγ alone is known to increase IgG3 levels, but also decrease IgG1 [149][150]. As such, a more detailed cytokine profiling in the serum of these mice is needed to fully comprehend these results.

Next, we investigated if variation in melanoma specific antibodies was also detected in the serum of patients undergoing anti-PD-1 therapy. Notably, only three patients had an increase in IgG antibodies towards the neo-antigen New York-esophageal squamous cell carcinoma-1 (NY-ESO-1), when comparing before and after treatment. Moreover, we were not able to detect any differences in IgG levels for melanoma-associated antigen recognized by T cells-1 (MART-1) as well as for changes in the BCR of circulating B cells. Altogether, these results did not translate what was observed in the mouse setting for the melanoma model. Further experiments are required to fully clarify if there is no difference in B cell responses in a human context. Nevertheless, it is noteworthy to mention the distinct antibody response found in a melanoma mouse model when using different immune checkpoint therapies.

3.3 REPROGRAMMING TUMOR ASSOCIATED MACROPHAGES BY ANTIBODY TARGETING INHIBITS CANCER PROGRESSION AND METASTASIS (PAPER III)

TAMs are heterogeneous myeloid population present in the tumor microenvironment capable of a wide range of functions, from tumor initiation to metastasis. In paper III, we aimed to investigate MARCO expression on TAMs and to potentially reprogram them to inhibit cancer progression.

The scavenger receptor MARCO has a restricted expression on certain tissue-resident macrophages and on activated DCs [74][151]. To test whether MARCO was also present on TAMs, we screened three different cancer mouse models using immunofluorescence, namely mammary carcinoma (4T1), melanoma (B16) and colon carcinoma (MC38). Here, we found MARCO to be co-expressed on F4/80+ macrophages close to the tumor capsule. Importantly, no other myeloid or lymphocytes expressed MARCO in the tumor microenvironment, indicating its unique expression on TAMs infiltrating B16 tumors. Furthermore, MARCO expression was not present in all F4/80+ TAMs, suggesting that this receptor defines a distinct subset of TAMs. Using flow cytometry and qPCR, we could show that MARCO was expressed on a specific subpopulation of TAMs phenotypically characterized by CD11b+ Ly6G- $Ly6C^{low}$ and $MHCII^{low}$. This subset is considered to have a M2-like polarization, which we observed by the gene expression of *Cx3cr1*, *Arg1* and *Retnla*, along with MARCO presence. Additionally, this subpopulation of TAMs exhibited low expression of M1 markers (*H2-Ab1* and *Nos2*) confirming MARCO expression on macrophages with a M2-like gene signature in the tumor microenvironment.

Next, we explored the possible factors behind the upregulation of MARCO on this subset of TAMs. Using bone-marrow-derived macrophages (BMDMs) *in vitro* polarization system, we observed upregulation of MARCO on M2-polarized and tumor supernatant stimulated macrophages. Taking into account that cytokines such as IL-10 and TGFβ are associated with an immunosuppressive environment in the tumor and can promote M2-like polarization, we hypothesized that these cytokines might drive MARCO expression. Stimulation of BMDMs with either IL-10 or TGFβ resulted in upregulation of MARCO on these cells. Nevertheless, we failed to further prove it by using blocking antibodies against either cytokines *in vitro*, possible due to the participation of other factor in the tumor supernatant.

Since MARCO has a restricted expression on immunosuppressive TAMs, it gave us the opportunity to use a monoclonal antibody against MARCO as a potential target for cancer immunotherapy. Notably, both melanoma and breast cancer models showed a decrease in tumor growth when treated with anti-MARCO antibody. In the particular case of breast cancer, this was observed together with a significant reduction in metastasis. Moreover, flow cytometry analysis on 4T1 tumors revealed a shift from M2- to M1-like TAMs in the tumor microenvironment. Likewise, gene expression of TAMs from the melanoma model showed an upregulation of M1 related genes (*Il1b*) and a downregulation of *Il10*, a M2 marker. We could not identify any additional differences in other tumor infiltrating immune cells, with the

exception of an increase in CD4+/Tregs ratio and OVA-specific CD8+ T cells in the B16 tumors. Mechanistically, anti-MARCO effect might be attributed to the inhibitory function of FcγRIIb, since its effect was not observed in deficient mice for this receptor.

In the melanoma model, combination of anti-MARCO treatment with anti-CTLA-4 immune checkpoint antibody also proved to further decrease tumor growth and increased survival compared to anti-CTLA-4 alone. Similarly, we could observe an increase of efficiency of anti-MARCO in combination with anti-CTLA-4 when treating MC38 tumors. Thus, these results show the potential effect of anti-MARCO on current immunotherapies.

Finally, immunofluorescence stainings on human breast cancer and metastatic melanoma showed the presence of MARCO on a subset of CD68+ TAMs together with M2 marker CD163. Moreover, TCGA and KI/Clinseq datasets on human breast cancer revealed high expression of MARCO in the triple negative subgroup of patients compared to LumA, LumB and Her2 positive subgroups. This subset of patients are normally diagnosed with poor prognosis and have very limited treatment options, thus anti-MARCO could be a potential treatment for these patients.

In summary, scavenger receptor MARCO defines a subset of TAMs with an immunosuppressive phenotype and when targeted with a monoclonal antibody results in a decrease tumor growth and metastasis. Moreover, anti-MARCO in combination with checkpoint therapy anti-CTLA-4 antibody enhances its effect on decrease tumor growth and increased survival. Clinical data additionally confirmed the presence of MARCO on protumor TAMs in human breast cancer and metastatic melanoma. Altogether, repolarization of TAMs with anti-MARCO gives light to a novel and promising approach for cancer treatments.

3.4 EXPRESSION OF SCAVENGER RECEPTOR MARCO DEFINES A TARGETABLE TUMOR-ASSOCIATED MACROPHAGE SUBSET IN NON-SMALL CELL LUNG CANCER (PAPER IV)

As described on Paper III, MARCO was found to be expressed on TAMs and when targeted with a monoclonal antibody was observed to suppress tumor growth and metastasis. In Paper IV, we aimed to characterize MARCO expression on TAMs in non-small cell lung cancer (NSCLC) cohort and its relation to other macrophage markers.

To investigate the presence of TAMs in NSCLC patients, tissue microarrays (TMA) were stained for macrophage pan marker CD68 and protumor tumor markers CD163, macrophage scavenger receptor 1 (MSR1) and MARCO [152][153]. Notably, we could observe a patient heterogeneity when it came to the infiltration of macrophages in the different compartments of the tumor, namely lumen, stroma and tumor. Macrophage density differed from low to high depending on the case and additionally, patients with squamous cell carcinomas had in average a higher density of CD68+ TAMs compared to adenocarcinomas. Interestingly, we

could observe a strong correlation between mean and distribution of CD163 scores to the ones for CD68. This indicates that most of TAMs present in the tumor have an immunosuppressive phenotype. Moreover, MARCO expression on TAMs was lower compared to other markers, suggesting that MARCO defines a distinct subpopulation of TAMs in NSCLC. Likewise, we could identify similar pattern for MSR1, although not has pronounced as MARCO.

To further investigate this MARCO+ TAM subpopulation, we used immunohistochemistry and immunofluorescence analysis on TAMs. We could detect a fraction of MARCO+ macrophages co-staining with CD68 and CD163, demonstrating the immunosuppressive nature of MARCO+ TAMs. Similarly to before, different patients had a large variation on the density of MARCO+ macrophages infiltrating the tumor. Notably, in several patients MARCO+ TAMs tended to situate at the tumor-stroma border, in close proximity to tumor islets.

Given this localization and the possibility of targeting this immunosuppressive barrier with current immunotherapy, we extended the characterization of MARCO+ TAMs subpopulation. The checkpoint inhibitor PD-L1 was found to be co-express with MARCO on CD68+TAMs located in and around the tumor cell islets. Gene expression analysis confirmed the positive correlation between MARCO and PD-L1, but also to other checkpoint genes, such as PD-1, CTLA-4 and V-domain Ig suppressor of T cell activation (VISTA).

Additionally, clinical data on the adenocarcinoma patients showed a tendency for worse overall survival when tumors were highly infiltrated by macrophages, though it was not significant.

Overall, this study follows up on the findings from paper III and reports a distinct TAM subpopulation infiltrating NSCLC based on MARCO expression. Moreover, their localization in the tumor, close to cell nests, and co-expression with PD-L1, makes MARCO+ TAMs an interesting target for cancer immunotherapy.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis brings insight on our understanding of two major immune cells in the tumor microenvironment. More specifically, it focuses on: the role of B cells in the tumor microenvironment and their importance in checkpoint therapies; and the detection of an immunosuppressive subpopulation of TAMs in different mouse and human cancers, with the possibility of modulating them with a monoclonal antibody to an anti-tumor phenotype. The main findings are the following:

I. Tumor-infiltrating innate-like B1 cells have a proinflammatory cytokine profile in the tumor microenvironment of melanoma and breast cancer.

Paper I contributed for our general understanding of the role of B cells in the tumor microenvironment. We revealed that mouse models for melanoma and breast cancer are infiltrated by a heterogeneous B cell population, which are phenotypically and functionally different. This suggested the importance for future studies to differentiate the CD19 positive B cell population into different subsets (as done with T cells) and not representing them simply as bulk. In particular, we demonstrated the presence of a tumor-induced proinflammatory CD5+ TIB subset, with an activated state, in both mouse and human cancers. And although, we could not conclude about their active participation in tumor initiation and progression, we did observe a reverse correlation between frequency of CD5+ TIBs and tumor volume. Moreover, mice lacking CD5+ B cells showed increased tumor growth compared to littermate controls. Altogether, this data suggested an anti-tumor phenotype of CD5+ TIBs, but further experiments are required. Additionally, we observed the potential effect of TIBs on cytotoxic T and NK cells, leading to exhaustion and activation respectively. To conclude this paper, we should further explore the different TIB subsets responsible for this result and the mechanisms behind it. Moreover, it would be interesting to know the origin of these B cells in the tumor microenvironment and expand this research to other tumors. Finally, the increase overall survival of melanoma and breast cancer patients with high infiltration of activated B cells makes these cells potential predictive biomarkers.

II. Melanoma specific antibody responses depend on the immune checkpoint therapy given.

In similar line with Paper I, we attempted to extend our knowledge of checkpoint therapies, namely anti-PD-1 and anti-PD-L1, in a B cell response perspective. Our major finding was the preferential tumor specific IgG subclasses depending on the treatment given to the tumor bearing mice. And although, we could not observe a substantial increase in tumor specific IgG serum levels in melanoma patients receiving anti-PD-1, it does not exclude the importance of this finding in the melanoma mouse model. In fact, it raises important

questions, such as: “How different IgG isotypes play a role in anti-tumor immunity?”; “Does this effect occur in other tumor models?” and “Does it have a predictive value in response to therapy?”. Answering these questions are a good basis for future experiments, but for this paper we still need to explore more the cytokine influence on B cell antibody responses as well as the germinal center response kinetics. For the human melanoma cohort, it would be also worth to further investigate the different IgG subclasses in serum of patients and the T cell responses towards the melanoma antigens reported in this paper.

III. Targeting MARCO positive tumor associated macrophages with a monoclonal antibody results in hinder tumor growth and metastasis.

In paper II, we found a novel marker defining a subpopulation of immunosuppressive TAMs in mouse and human cancers. This was especially important, since MARCO was observed to be highly expressed in the triple negative breast cancer patients, a very aggressive tumor with poor prognosis. Further investigations are needed, but MARCO could potentially be a novel biomarker for this subset of breast cancer. Moreover, it would be interesting to expand this research to various types of cancers. We could also find a new strategy for cancer immunotherapy by modulating TAMs polarization with a monoclonal antibody. So far, this approach has only been focused and successful by blocking CSF1, an important growth factor to sustain M2-like TAMs in the tumor microenvironment. For the future, we still need to further clarify the mechanism by which anti-MARCO exerts its effector function in the tumor. Moreover, we should further characterize the TAM polarization towards M1, when targeted with anti-MARCO antibody (e.g. metabolic changes). In addition, it would be interesting to identify the set of factors, such as cytokines, responsible for inducing MARCO expression on TAMs, since IL-10 and TGFβ blocking experiments did not result in a downregulation of this receptor.

IV. MARCO is a novel marker for a subset of immunosuppressive TAMs in non-small cell lung cancer.

In paper IV, we mainly found that MARCO is expressed in a subset of immunosuppressive TAMs in NSCLC patients, strategically localized between the stroma and tumor cell islets. This immunosuppressive barrier makes these cells a possible target for cancer immunotherapy. Additionally, it could improve tumor progression blockade effect for NSCLC in combination with current checkpoint treatments, since MARCO+TAMs express also surface PD-L1. For future experiments, an effort to know the localization of MARCO+ TAMs in regards to other tumor -infiltrating immune cells should be performed. As MARCO+ TAMs are found at the stoma-tumor border, it would be interesting to observe if these cells hinder the entrance of cytotoxic lymphocytes into the tumor nests. Finally, we

should explore the function of MARCO+ TAMs in human cancers to know how they exert their protumor functions.

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