A Study of Type 2 Mediated Immune Responses

Thesis

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A STUDY OF TYPE 2-MEDIATED IMMUNE RESPONSES
ABSTRACT

Within tumors, immune responses strongly influence tumor development and stroma architecture, determining cancer progression. Tumor immune responses may be polarized towards a type 1-like “pro-inflammatory” or a type 2-like “alternative” phenotype, the latter mediated by type 2 cytokines (IL-4, IL-5, IL-9 and IL-13). Type 2 responses are generally thought to be associated with enhanced cancer growth. Whilst the effects of single type 2 cytokines in tumor progression have been extensively studied, the overall role of type 2 responses is far from thoroughly understood. In our study we utilised a unique quadruple IL-4/5/9/13 deficient mouse (T2KO), which features complete genetic depletion of type 2 responses. We assessed how the complete lack of type 2 cytokines affects both tumorigenesis and tumor progression.

We showed that the complete lack of type 2 responses reduced tumor growth in a mouse model of transplantable breast carcinoma, correlating with improved T cell tumor infiltration and type 1 polarization. We further demonstrated that T cell depletion abolished the protective effects. Thus we formally demonstrated that type 2 responses promote tumor growth.

Surprisingly, in carcinogen-driven tumor formation, lack of type 2 responses significantly increased tumor incidence. This was associated with a decreased protective fibrotic encapsulation of the carcinogen, matching pioneering studies on the protective effects of foreign body responses in carcinogenesis. The lack of carcinogen encapsulation in T2KO mice led to enhanced carcinogen spreading, thus suggesting that type 2 responses are protective against carcinogenesis.

These findings further suggested a possible protective role against carcinogenesis of enhanced type 2 responses, such as in cases of allergy or asthma. To verify this, we examined two human patient cohorts, in Norway and Italy, and confirmed that, in men, pre-existing type 2 immune responses are significantly associated with reduced incidence of lung cancer.

In summary, our findings suggest a protective role of type 2 responses from carcinogenesis, which may become detrimental in cancer progression. Our data also pose novel questions about the influence of immune responses on tumor formation, tumor growth and on tumor-associated fibrosis, occurring at different stages of the disease.
I would like to express my deep gratitude to Professor Marinos Kallikourdis, my supervisor, for his support, his guidance and his constructive critiques.
ABBREVIATIONS USED IN THIS DISSERTATION (in order of use)

T2KO knock out of type 2 cytokines
WT wild type
ROS reactive oxygen species
IL interleukin
TNF tumor necrosis factor
HMGB1 high mobility group box 1
MMP metalloproteinase
LOX lysyl oxidases
DAMP damage-associated molecular patterns
IFN interferon
TLR Toll-like receptor
NK natural killer
NKT natural killer T
GM-CSF granulocyte-macrophage colony-stimulating factor
M-CSF macrophage colony-stimulating factor
iNOS inducible form of nitric oxide synthases
ROS reactive oxygen species
Cox2 cyclooxygenase
NO nitric oxide
ADCP antibody-dependent cell-mediated phagocytosis
ADCC antibody-dependent cell-mediated cytotoxicity
MPO myeloperoxidase
NOX2 NADPH oxidase
KIR killer-cell immunoglobulin receptor
NKG2D natural killer group 2 member D protein
PRF1 pore-forming protein
GZM granzyme
TNFα tumor necrosis factor
TRAIL tumor necrosis factor-related apoptosis-inducing ligand
BCR B cell receptor
T<sub>H</sub> T helper
Tfh T follicular helper
Treg regulatory T cells
MDSC myeloid derived suppressor cells
TAM tumor associated macrophages
LFA-1 leukocyte function-associated antigen 1
VLA-4 very late antigen
VCAM-1 vascular cell adhesion molecule-1
ICAM-1 intercellular adhesion molecule-1
ECM extracellular matrix
ADAM disintegrin and metalloproteinase
VEGF vascular endothelial growth factor
IDO indoleamine-2,3.dioxygenase
PD-L1 programmed cell death ligand
CTLA-4 cytotoxic T lymphocyte antigen 4
LAG-3 lymphocyte-activation gene 3
TIM-3 T cell immunoglobulin mucin 3
FGF fibroblast growth factor
EGF epidermal growth factor
IGF insulin growth factor
IGF2 insulin-like growth factor II
IGF1R IGF1 receptor
HIF hypoxia inducible factor
PDGF platelet derived growth factor
TGFβ transforming growth factor
Bcl-2 B cell lymphoma 2
MAPK mitogen-activated protein kinase
Erk extracellular signal-regulated kinase
Ig Immunoglobulin
DC dendritic cells
MHC major histocompatibility complex
CD cluster of differentiation
CAF cancer-associated fibroblast
FAP fibroblast activating protein
αSMA α smooth muscle actin
EMT epithelial to mesenchymal transition
CARs chimeric antigen receptors
ILC2 group 2 innate lymphoid cells
3-MCA 3-methylcholantrene
ROI region of interest
SHG second harmonic generation
2-P two photon-excited fluorescence
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1. INTRODUCTION

AN OVERVIEW OF CANCER ASSOCIATED INFLAMMATION AND TUMOR PROGRESSION

1.1 Cancer initiation and progression

Cancer is a pathological condition characterised by sustained abnormal proliferative capabilities and resistance to death of cells that progressively grow and metastasise. More than 200 types of cancers have been classified on the basis of the tissue of origin. Recently the improvement and integration of epigenetic, sequencing and ‘omics’ technologies (such as epigenomics, transcriptomics and proteomics) has allowed deeper understanding of cancer cell biology and consequently the sub-classification of cancer types on a molecular basis (Palucka and Coussens 2016).

The multistep process that drives cancer initiation originates from extrinsic (environmental agents, viral infections) and intrinsic factors (genetic predisposition) causing cell genomic alterations. Ongoing genome instability triggers tumor progression by increasing the chance of acquiring successive neoplastic capabilities, such as growth capability and replicative immortality, the loss of contact inhibition, plasticity and resistance to senescence and cell death (Hanahan and Weinberg 2011). Clonal expansion of neoplastic cells drives tumor mass growth and tissue invasion. While genetic lesions are necessary for tumorigenesis, sustained tumor growth depends on reciprocal interactions between cancer cells and stromal cells (such as vascular endothelial cells, adipocytes, fibroblasts, pericytes and bone marrow derived mesenchymal cells). Indeed, vascular endothelial cells contribute to carcinogenesis through vascularisation (Hanahan and Coussens 2012).

Malignancies are characterised by dynamic reorganisation of matrices, the formation of endothelial vessels and increased lymphatic vessel density, that favours dissemination and tumor metastasis (Lu, Weaver et al. 2012).

Moreover cancer development is reflected on its associated immune response. Chronic inflammation itself (as e.g. in for colorectal cancer resulting from ulcerative colitis), unresolved pathogen driven inflammation (as for hepatocellular carcinoma and
hepatitis) or environment induced non-resolving inflammation increase the risk of developing cancer in specific tissues, especially gastrointestinal tract, liver, lung and skin (Balkwill and Mantovani 2012). Reactive oxygen species (ROS) and reactive nitrogen intermediates associated with inflammation against pathogens could drive the induction of DNA mutations through oxidative damage and nitration of DNA bases. Indeed persistent damage and tissue regeneration enhance the risk of interactions between DNA and reactive species in proliferating epithelial cells, which drives permanent mutations (Hussain, Hofseth et al. 2003). Inflammation is a constant component of solid cancers. In immunocompetent hosts, the immune system can recognise and eliminate neoplastic cells. Immunosurveillance occurs in presence of high immunogenic cancers. The so-called hot tumors display high mutational load and a wide range of neo-antigens on cell surface. These drive strong antigen-specific immune system responses against cancer cells. However some tumors could present a low immunogenic phenotype or be refractive to Immunosurveillance. Indeed, during cancer progression, neoplastic cells evade the immunosurveillance because of downregulation of antigen presentation, altered or inefficient immune responses and rise of suppression pathways (Shankaran, Ikeda et al. 2001).

The reciprocal influence of immune responses, stromal cell activity and the evolving phenotype of neoplastic cells determine the continuous evolution of cancers. The paracrine factors at the interplay between immune cells, stromal cells and cancer cells mainly promote the acquisition of malignant traits. Among those, growth factors (insulin like growth factor (IGF) and epidermal growth factor (EGF), cytokines (interleukin 6 (IL-6), tumor necrosis factor (TNF)-α) and chemokines are mainly involved in neoplastic cell growth; alarmins (e.g. High mobility group box 1 (HMGB1), chemokines (CCL2, CCL3, CCL5, CCL9, CXCL10, CXCL12), enzymes (metalloproteinases (MMPs), cathepsins and lysyl oxidases (LOXs)) are involved in dissemination (Gijsbers, Gouwy et al. 2005; Bertran, Caja et al. 2009; Singh, Nannuru et al. 2009); chemokines (CCL25) and metabolic products (fatty acids, lactate and ketone bodies) are involved in chemoresistance (Marcucci, Bellone et al. 2014).

1.2 Early Tumor associated Immune responses
At early stages of tumorigenesis, neoplastic cells generate signals of danger that are sensed by immune cells. Early anti-tumoral immune responses are characterized by the enhanced presence of myeloid cells (including neutrophils, macrophages, mast cells, dendritic cells cells and myeloid derived suppressor cells) and lymphoid cells (natural killer (NK) cells, γδT cells (a minority of T lymphocytes bearing T-cell receptor composed of γ and δ chains instead of traditional α and β chains) natural killer T (NKT) cells and T lymphocytes and B lymphocytes) (Scapini, Laudanna et al. 2001; Chiba, Ikushima et al. 2014), (Bauer, Groh et al. 1999; Vetter, Groh et al. 2002; Gatault, Legrand et al. 2012), (Fan, Yu et al. 2006).

The recruitment of immune cells follows the release of danger signal by cancer cells and the expression of tumor-specific antigens. Damage-associated molecular patterns (DAMPs) include (i) intracellular molecules, released by apoptotic cancer cells, such as uric acid, heat shock proteins, adenosine triphosphate and; (ii) pro-inflammatory molecules, such as type I interferons (IFNs), IL-1α and IL-33 released by neoplastic cells; (iii) extracellular matrix fragments, as hyaluronic acid, fibronectin and surfactant proteins (Garg, Krysko et al. 2012), (Garg, Martin et al. 2014), (Dunn, Old et al. 2004). Toll-like receptors (TLRs) expressed by innate immune cells are the main pattern recognition receptors able to sense DAMP signalling. DAMPs can also activate neutrophils, NK cells and γδT cells (Garg, Vandenberk et al. 2017). DAMP signals together with cancer antigen exposure lead to the maturation of antigen presenting cells (including dendritic cells, macrophages and B cells) and consequently priming and activation of tumor-specific T cells (Garg, Martin et al. 2014). Cancer cells, stromal cells and immune cells themselves finely regulate the trafficking of the different subsets of immune cells within tumors (Nagarsheth, Wicha et al. 2017).

1.2.1 The Myeloid Cells

Myeloid cells are the major component of tumor infiltrating immune cells at early cancer stages (Lavin, Kobayashi et al. 2017). Release of soluble factors, as IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) by neoplastic cells, attracts immune cells and favours immature myeloid cell proliferation. Tumor myeloid cells include mononuclear phagocytes and granulocytes at different stages of differentiation. In response to tissue injury they perform their functions of cytotoxicity
induction, damaged cell clearance, tissue repairing and activation of adaptive effector cells (Gabrilovich, Ostrand-Rosenberg et al. 2012; Alaeddine, Prat et al. 2019).

Among mononuclear cells, macrophages contribute to early anti-tumor responses through phagocytosis, antigen degradation and induction of pro-inflammatory responses. Classically macrophages have been described as plastic cells able to acquire two distinct phenotypes. Macrophages with “M1-like” pro-inflammatory phenotype play a defensive role via free radical and inflammatory cytokine production; while macrophages with “M2-like” are thought to be not promoting type 1 inflammation and play a role in remodelling and restoration of tissue homeostasis (Murray and Wynn 2011). At initial stages of tumor development M1-like macrophages infiltrate tumors (Wang, Li et al. 2011). Their anti-tumor roles have been demonstrated in vivo, in murine models of hepatocellular carcinoma, fibrosarcoma, glioma and neurofibroma. In those studies early macrophage depletion significantly increase tumor growth. (Prada, Jousma et al. 2013). They present up-regulation of pro-inflammatory cytokines IL1b, IL6, IL12b, and potential tumouricidal agents, as inducible form of nitric oxide synthases (iNOS), reactive oxygen species (ROS) and cyclooxygenase (Cox2), (Movahedi, Laoui et al. 2010; Kim, Koh et al. 2019). The anti-tumor effector function of macrophages can rely on their expression of TNFα. The up-regulation of TNFα may promote monocyte attractive chemokine CCL2 production by monocytes and cancer cells (Alleva, Askew et al. 1993; Villeneuve, Tremblay et al. 2005). CCL2 triggers monocyte recruitment to the tumor site, creating a self-enhancing loop. Direct monocyte/macrophage killing of cancer cells may occur through the action of immunoglobulins and Fc receptors. M1-like macrophages present higher FcγR expression and more antibody-dependent cell-mediated phagocytosis (ADCP) potential than M2-like macrophages (Grugan, McCabe et al. 2012). Recently it has been demonstrated in vivo that the cross-linking between Immunoglobulin (Ig) E-coated tumor cells and Fce-R expressed on macrophage surface could trigger cancer cell lysis via antibody-dependent cell-mediated cytotoxicity (ADCC), or antibody-dependent cell-mediated phagocytosis (Josephs, Bax et al. 2017).

Among myeloid cells, dendritic cells (DCs) are the main mediators of antigen presentation and activation of T cell mediated adaptive immune responses (Merad, Sathe et al. 2013). Tumor antigen capture by DC can happen at the tumor site or at the
draining lymph nodes, where soluble antigens or cancer cells traffic via lymphatic vessels. In lymphoid tissues, naïve T cells encounter tumor antigen-charged DCs and differentiate into tumor specific effector T cells. DCs, as well as B cells and macrophages are antigen presenting cells, thus they can present tumor antigenic peptides in the context of major histocompatibility complex (MHC) class I or class II, and lipid antigens in the context of non-classical MHC class I-related glycoprotein CD1d. Antigen presentation trough MHCs allows selection of tumor specific antigen T lymphocytes both CD4+ and CD8+ T cells. Effective T cell activation requires tumor derived-antigen peptide presentation, co-stimulation through surface molecules (such as CD80, CD70 and CD137), and through cytokine release, such as IL-15, IL-12 and IFNs (Thery and Amigorena 2001; You, Zhang et al. 2017) (Steinman and Dhodapkar 2001). Cancer lipidic antigen presentation in CD1d context allows selection of anti-tumor NK T cells, which are T lymphocytes with innate effector functions (Smyth, Crowe et al. 2002; Swann, Crowe et al. 2004). Several subsets of DCs contribute to early anti-tumor responses: plasmacytoid DCs (pDCs), conventional DC (cDCs) and inflammatory DC (infDCs). For example, in melanomas, pDC, a bone marrow-derived population exhibiting plasma cell morphology, inhibit cancer cell growth by production of IFN-α (Wenzel, Bekisch et al. 2005; Veglia and Gabrilovich 2017). Again in melanomas, the presence of cDC CD141+ correlates with better prognosis through improved cross-priming ability in T cell stimulation (Sluijter, van den Hout et al. 2015). Similarly another subset of cDC, CD103+ dendritic cells expressing chemokine receptor CCR7, required for lymph node homing, has the unique ability to carry intact tumor antigens to draining lymph nodes, thus controlling cytotoxic CD8+ T cell activation and antitumor adaptive immune responses (Roberts, Broz et al. 2016; Salmon, Idoyaga et al. 2016). Finally several studies have characterised infDC anti-tumor roles both in animal models and in cancer patients. Their frequency positively correlates with CD8+ T cells and activity with an effective anti-cancer immune response. They protect from cancer progression via TNFα and NO production. (Marigo, Zilio et al. 2016).

Among myeloid cells, granulocytes display efficient cytotoxic activity trough release of soluble toxic and inflammatory molecules stored in granules. Mast cell degranulation
and eosinophils degranulation, further studied in asthma and allergic diseases (Reichman, Karo-Atar et al. 2016), mediate potent cytotoxic effects on cancer cells and tumor tissues damage. Granules contain a plethora of cytotoxic proteins, destructive enzymes, pro-inflammatory cytokines and chemokines and vasoactive molecules (Tartour, Fossiez et al. 1999). **Mast cells** recruitment at early steps of carcinogenesis is a rapid response, reflecting the role of mast cell as resident cells involved in immediate responses to pathogens (Galli, Maurer et al. 1999). Their direct cytotoxic effect on cancer cells depends on release of pre-stored granules containing the destructive enzymes tryptase and cathepsin (Dalton and Noelle 2012) (Oldford, Haidl et al. 2010) (Shikotra, Ohri et al. 2016). Moreover mast cells are essential to the initial angiogenesis, lymphangioiogenesis and remodelling of extracellular matrix via release of vascular endothelial growth factors (VEGF-A and VEGF-B) and MMPs. These initial stroma changes favour immune cell recruitment to the cancer site (Lund, Medler et al. 2016). Similarly, **eosinophil** recruitment to the tumor site occurs in different type of cancers, such as melanoma, colorectal, gastric, esophageal, breast, lung and ovary carcinomas. Eosinophil accumulation mostly relies on chemoattractant factor IL-5, released by cancer cells and immune cells, and cognate receptor CCR3 expressed on eosinophil surface (Cormier, Taranova et al. 2006) (Reichman, Karo-Atar et al. 2016). Their localisation near tumor necrotic areas could be associated to their potent antitumor activity via degranulation. Eosinophil granules contain eosinophil cationic protein and major basic proteins, able to disrupt the integrity of lipid bilayers of neoplastic cells. Moreover, eosinophil granules may contain eosinophil-derived neurotoxin or eosinophil peroxidase, which can catalyse NO oxidation promoting oxidative stress and cancer cell death (Gleich 2000) (Legrand, Driss et al. 2010). In addition, they can exert indirect antitumor activity releasing CCL5, CXCL9 and CXCL10 chemokines that attract tumor-specific cytotoxic T lymphocytes (Carretero, Sektio glu et al. 2015). Recruited polymorphonuclear **neutrophils** (PMNs) can exert antitumor activity, as well, at early phases of tumor development (Eruslanov, Bhoj nagarwala et al. 2014; Blaisdell, Crequer et al. 2015). Neutrophil anti-tumor effects on cancer cells mainly reflect the mechanisms that these myeloid cells use in antimicrobial host defence (Singel and Segal 2016). They can directly inhibit tumor growth via cytolytic effects of antibody-dependent cellular cytotoxicity (ADCC) or via
cytotoxic mediators ROS, membrane-perforating agents, proteases. Upon LFA-1 recognition on target cells, neutrophil can carry out cytolytic reactions via release of myeloperoxidase (MPO), a heme-containing peroxide that generates cytotoxic hypochlorous acid, which mediates tumor cell lysis. In tumor surveillance, MPO activation together with NADPH oxidase (NOX2) convert superoxide into cytotoxic hypohalous acid. Additionally NOX2 activates cytotoxic cationic proteins of granules (Clark, Olsson et al. 1976). Indeed neutrophil degranulation can orchestrate reactive oxidant attack to cancer cells. Interestingly, release of granule proteases and induction of ROS also have an effect on matrix remodelling and basement membrane detachment of cancer cells (Blaisdell, Crequer et al. 2015). Moreover neutrophil azurophil granules contain high amount of defensins that have shown cytotoxicity against cancer cells in several types of carcinomas (Lichtenstein, Ganz et al. 1988; Mittendorf, Alatrash et al. 2012). Neutrophils can also indirectly sustain anti-tumor immune responses via release of soluble mediators activating cytotoxic cells, such as IFNs and TNF-α (Otten, Rudolph et al. 2005) (Reeves, Lu et al. 2002). Recently, a strong association has been reported between NK cell presence and neutrophil activity. NK cells may regulate neutrophil recruitment and function, favouring their survival and activation, via release of IFNγ (Costantini, Micheletti et al. 2010; Molgora, Supino et al. 2018). NK cell depletion in a transplantable model of murine sarcoma abolished neutrophil antitumor activity, while worsening their pro-tumor and pro-angiogenic functions (Ogura, Sato-Matsushita et al. 2018).

Thus far, all these findings highlight the capability of each subset of the innate immune response to promptly combat, via different antitumor mechanisms, cancer growth and progression.

1.2.2 The lymphoid cells: T cells, B cells, NK cells and other populations

Lymphoid cells, such as CD4+ T cells, CD8+ T cells, NK cells, NKT cells and γδ T cells, are able to specifically recognise and eliminate cancer cells. Effector functions of CD8+ T cells are activated only upon recognition of unique antigenic molecules on target cells. Following antigen (antibody generator) recognition, costimulation via
interactions between surface molecules and the action of soluble factors activates the effector cytotoxic functions.

Among lymphoid cells, NK cells, NKT cells and γδ T cells share features with innate immune cells. Preferential homing into peripheral tissues rather than lymphoid tissues allows them to immediately respond to stress and to damage releasing high amounts of cytotoxic cytokines, in synchrony with innate responses. Meanwhile they feature the ability to direct effector functions against target cells through specific recognition. NK cells can interact with tumors through killer-cell immunoglobulin receptors (KIR) and C-type lectin CD94/NKG2 family receptors. Additionally activation of NK via natural cytotoxicity receptors (NCRs) and IL-2 costimulation can trigger specific effector functions against cancer cells, in an MHC independent manner (Farag, Fehniger et al. 2002). Differently, T cell receptor (TCR) express on NKT cells recognise tumor lipid antigens in the context of non-polymorphic MHC class I like molecule CD1d (Metelitsa, Naidenko et al. 2001) (Godfrey, Uldrich et al. 2015). The antitumor activity of unconventional γδ T cells against cancer cells is carried out by the major subset of Vγ9Vδ2 T cells. Activation of Vγ9Vδ2 relies on T cell receptors and natural killer receptors expressed on their cell surface (Gober, Kistowska et al. 2003). Signals of cell stress by cancer cells could activate natural killer receptors and toll-like receptors. Natural killer receptors and toll like receptors provide co-stimulatory signals to Vγ9Vδ2 TCRs, which can recognise tumoral non-peptidic phosphoantigens. Additionally, natural killer group 2 member D protein (NKG2D) can recognise tumor antigens by binding to non-classical MHC molecules of the MHC class I chain related molecules (MIC) express on cancer cells. Upon ligand binding, NKG2D promotes IL2α receptor (CD25) expression on γδ T cells, improving their cytolytic function (Bauer, Groh et al. 1999; Wrobel, Shojaei et al. 2007) (Girlanda, Fortis et al. 2005).

Upon activation, lymphoid cells can promote cytolysis of cancer cells directly, through apoptosis induction, or by sensitising target cells to apoptosis. Cytotoxic cells release preformed cytolytic granules toward cancer cells, in an intercellular space delimited by temporary cellular junctions: the immunological synapse. Preformed granules mainly contain cytolytic enzymes, pore-forming protein (PRF1), able to form pores in the target cell membrane and granzymes (GZMA and GZMB) that are serine proteases.
Upon delivery in a target cytosol, granzymes cleave cellular proteins that control cell death and survival, inducing apoptotic death (Lopez, Susanto et al. 2013). Alternatively, the granules may contain cytolitic granulysin, a member of saposin-like protein family. Granulysin elicits apoptosis inducing the loss of mitochondrial electrostatic potential that causes release of cytochrome c and subsequent activation of caspase-3 apoptotic pathway (Kishi, Takamori et al. 2002) (Pardo, Perez-Galan et al. 2001). Cell-cells interaction is another mechanism through which cytotoxic lymphoid cells can induce apoptosis. Upon activation, they can express death ligands on their cell surface, such as tumor necrosis factors (TNFα), Fas ligand (FasL or CD95) or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). The binding of death ligands to cognate receptors expressed on cancer cell surface triggers activation of the caspase-8 cascade and apoptosis. Moreover, activated NK cells, NKT cells and γδ T cells release pro-inflammatory cytokines, such as IL-2, IL-12 and IFNγ, which attract, polarise and sustain activation of cytotoxic effectors. IFNγ binding to its cognate receptor activates signal transduction and final expression of molecules associated with antitumor effects, such as MHC class I, CD95 and caspase 1 (Martinez-Lostao, Anel et al. 2015).

Unlike NK cells, NKT cells and γδ T cells, B lymphocytes and T lymphocytes, CD4+ and CD8+ αβ T cells, specifically recognise antigens and respond respectively through immunoglobulin production (for B cells), cytokine release and costimulation of B cells (for CD4+ T cells), and or cell-mediated killing (for CD8+ T cells). This is due to the variability of their rearranged B cell antigen receptors (BCR) or T cell antigen receptor (TCR), respectively (Emerson, Sherwood et al. 2013; Kirsch, Vignali et al. 2015). At early stages of carcinogenesis antitumor B cells sustain effector humoral immune responses via antigen-driven clonal expansion, antibody class switching and affinity maturation (Nzula, Going et al. 2003). Tumor associated antibodies recognise tumor specific neoantigens or self antigens (autoantibodies) released by dead tumor cells (Joseph, Darrah et al. 2014). Antibodies that recognise antigens on cancer cell surface and alter target cell functions may mediate a direct antitumor activity of B cells. Antibodies may also act indirectly, upon binding on cancer cells, by being recognised by cytotoxic cells, leading to ADCC, or by activating the complement cascade.
Furthermore, B cells may elicit antitumor responses by acting as antigen presenting cells for CD4+ T cells (Yuen, Demissie et al. 2016).

Tumor CD4+ T cells orchestrate adaptive immune responses by providing help to cytotoxic T cells and to B cells, thus they are called “T helper” (T_h), via cytokine release (Claman, Chaperon et al. 1966; Cassell and Forman 1988). Classically CD4+ T_h1 cells help CD8+ T cells in responses against intracellular pathogens. CD4+ T_h1 cells express the transcription factor T-bet and release monocyte/macrophages chemotactic CCL2 and CCL3, pro-inflammatory cytokines IL-2, IFNγ and TNFα. CD4+ T_h2 cells help B cells in responses against extracellular parasites. They express the transcription factor GATA-3 and release cytokines IL-4, -5, -9 and -13. CD4+ T_h2 cells promote isotype switching and affinity maturation of B cells and can boost innate immune responses via recruitment and activation of basophils, eosinophils (trough IL-4 and IL-5), mast cells (trough IL-9) and macrophages (IL-4 and IL-13). Among CD4+ T cells, Th1 and Th2 populations were the first described with potential antitumoral functions. More recently, other Th subsets have been described with anti-cancer functions, such as Th9 and Th17 cells, even if their role in tumor progression is still debate. IL-9 derived from Th9 cells has antitumor properties in solid tumors, such as melanoma and adenocarcinoma, through the triggering of innate and adaptive antitumor-immunity (You, Zhang et al. 2017). Th17 adoptive transfer in murine model of melanoma have shown improved tumor surveillance, via IFNγ induction (Muranski, Boni et al. 2008).

At the tumor site CD4+ T cells may recognize cancer antigens, self antigens and viral antigens responsible for neoplastic transformation, exposed in the context of MHC class I and class II (Horna, Cuenca et al. 2006) (Perez-Diez, Joncker et al. 2007) (Ayyoub, Pignon et al. 2013). At early stages of tumor development, activated CD4+ T cells can release IL-4 and enhance eosinophil infiltration, promoting antitumor effects (Tepper, Coffman et al. 1992). Furthermore in secondary lymphoid tissues, tumor activated CD4+ T cells may acquire a follicular helper phenotype (Tfh) and trigger B cell differentiation into immunoglobulin secreting cells (Bindea, Mlecnik et al. 2013). However the most notable antitumor effect of tumor antigen-activated CD4+ T cells is direct killing of cancer cells. The presence of tumor infiltrating CD4+ T cell correlates with good prognosis and with improved cytotoxic T cell trafficking in lung cancer and in esophageal carcinoma. Indeed tumor specific CD4+ T cells can produce lytic
enzymes, perforin and granzyme; enhance cytotoxic responses through IL-2 and IFNγ secretion. IL-2 mediates clonal expansion of cytotoxic CD8+ T cells, as well as NK cells. IFNγ enhances the expression of CD8+ T cell chemoattractants, such as CCL2, CCL3, CCL5, CXCL9 and CXCL10, favouring an antitumor inflammation positive feedback loop (Manici, Sturniolo et al. 1999) (Hombach, Kohler et al. 2006).

Activation of T cells relies on TCR recognition of tumor antigens presented on MHC complexes. In lymphoid tissues, naïve CD4+ T cells and naïve CD8+ T cells may recognise cancer peptides on antigen presenting cells. In the presence of co-stimulatory signals, antigen recognition triggers T cell activation. Following effective antigen encounter, T cells acquire effector phenotypes, rapidly undergo clonal expansion and migrate to peripheral tissues, where they display effector functions. Among antigen-primed T cells, the persistence of progeny lymphocytes occurs through antigen specific central memory T cells. Central memory T cells recirculate in lymphoid tissues via CCR7 expression and present higher antitumor response capabilities through rapid clonal expansion and effector functions when they re-encounter tumor-specific antigens (Klebanoff, Gattinoni et al. 2005). On the other hand effector memory CD4+ T cells and CD8+ T cells down-regulate chemokine receptor CCR7 (required for lymphoid tissue homing) and start to express different chemokine receptors. These enable T cells to migrate into tumor tissues expressing cognate chemokine ligands (van der Woude, Gorris et al. 2017).

Once at tumor site, antigen experienced CD8+ T cells recognise target cells by recognising antigenic peptides on MHC complexes, traditionally MHC class I complexes. In the presence of costimulation, antigenic stimuli trigger effector memory CD8+ T cell activation. Cytotoxic responses consist of release of lytic enzymes (perforins and granzymes), production of cytotoxic molecules (TRAIL, CD95) and pro-inflammatory cytokines (IL-2, IFNγ and TNFα) (Rosenberg, Dyer et al. 2013). Recently, a subset of tumor infiltrating T cells expressing integrin αEβ7 (CD103+ CD8+ T cells, also called tissue resident memory CD8+ T cells), have been shown to be highly protective in a tumor context. Enhanced presence of tissue resident memory CD8+ T cells (CD8+ TRM cells) in tumors positively correlates with good prognosis in head and neck tumors, lung tumors and triple negative breast cancer (Ganesan, Clarke et al. 2017)
Interestingly IFNγ-expressing TH1 cells could mediate not only killing of tumor cells, but also inhibit angiogenesis, thus preventing rapid tumor growth (Qin, Schwartzkopff et al. 2003).

1.2.3 T cell immunosurveillance

Several studies identified cytotoxic T cells as the main player in antitumor immune responses in primary solid tumors (e.g. breast and lung carcinoma) and secondary tumors (colorectal metastases) (Pages, Berger et al. 2005). Indeed, in cancer patients, favourable prognosis seems to correlate with increased frequencies of CD8+ T cells, sustained by the helper function of CD4+ T cells and antigen presentation of mature DC (Church, Jensen et al. 2014) (Bos and Sherman 2010; Goc, Germain et al. 2014). In vivo studies have shown how CD8+ T cells are attracted into tumors (Deguine, Breart et al. 2010). CD8+ activation and clonal expansion are triggered by recognition of tumor associated antigens along with proper costimulation, e.g. with CD28 and CD27 activation, member of tumor necrosis factor. Transcriptomic analyses confirmed that memory cytotoxic T cells with a type 1 phenotype (CD8+CD45RO–CCR7–, expressing Tbet, perforin, granulysin and granzyme B) in tumors is associated with longer disease-free survival, without metastatic progression (Pages, Berger et al. 2005). This means that, as for pathogen infections, memory CD8 T cells may confer long lasting protection and rapid, stronger cytotoxic responses against cancer (Xiang, Lode et al. 1999) (Klebanoff, Gattinoni et al. 2005) (Brummelman, Mazza et al. 2018). Immunostaining for CD3, CD8, CD45RO and granzyme B confirmed that a higher density of T cells, memory T cells and cytotoxic T cells at the tumor core, rather then at invasive margin, correlates with better clinical outcomes in patients with colorectal cancer. Thus immunosurveillance relies not only on effector memory T cell activity, but also on their density and localisation within tumors (Kirilovsky, Marliot et al. 2016). Adhesion molecules and chemokines expressed on endothelial cells or secreted by tumor stromal cells and myeloid cells regulate T cell trafficking into the tumor core. Initial rolling of T cells within tumor vessels requires the interaction between adhesion molecules, such as E/P selectins, and their ligand on T cells, such as the integrin leukocyte function-
associated antigen 1 (LFA-1) or very late antigen 4 (VLA-4), binding respectively to endothelial receptor vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Upon adhesion to endothelial cells, lymphocyte extravasation requires the arrest of tumor infiltrating lymphocytes via involvement of chemokine/chemokine receptor axis (Mikucki, Fisher et al. 2015). Clinical studies highlighted a positive correlation between chemokine expression at the tumor site, T cell infiltration and favourable clinical outcomes. Several chemokines, such as CCL2 and cognate receptor CCR2 on T cells, as well as CCL3, CCL4, and CCL5 and cognate receptor CCR5 are involved in T cell trafficking in tumors and are associated with improved clinical outcomes (Harlin, Meng et al. 2009) (Mlecnik, Tosolini et al. 2010). Similarly in Ewing sarcoma, melanoma and colorectal carcinoma patient expression of CXCL9 and CXCL10 at tumor site correlates with enhanced trafficking of CXCR3+ cytotoxic T cells and with efficacy of antitumor immune responses and favourable prognosis. Interactions between chemokine/chemokine receptors are required for intra-tumor trafficking and also support the extravasation. In melanoma cancer, CXCR3 expression on CD8+ T cells is required for T cell crossing into intravascular space. (Berghuis, Santos et al. 2011) (Harlin, Meng et al. 2009) (Mlecnik, Tosolini et al. 2010). Once T cells reach their target cells, integrins, such as LFA-1, are fundamental initiators of immunological synapse formation. Lymphocyte activation induces conformational changes in LFA-1, exposing the ligand binding site. Activated LFA-1 can bind to its ligand (ICAM) expressed on the target cell surface, favouring T cell mediated killing via cytotoxic granule polarization (Anikeeva, Somersalo et al. 2005) (Petit, Demotte et al. 2016).

In summary, effective T cell immunosurveillance requires i) appropriate specific antitumor cytotoxic T cell activation and proliferation, ii) cytokine mediated T helper support and functional persistence, iii) establishment of tumor-specific memory featuring strong antitumor effector functions and capable of long-term manteinence, iv) appropriate chemotaxis and localisation within the tumors.

In summary, the crosstalk between innate immune cells and lymphocytes occurring at the tumor site, as well as the pro-inflammatory cytokine-enriched milieu, regulate early antitumor responses, via functional activation of cytotoxic immune mediators (such as NK cells, neutrophils, T cells).
1.2.4 The stromal compartment

Dynamic stroma remodelling is concomitant to early pro-inflammatory responses triggered by cancer cells. Initial cancer growth requires the remodelling of host tissue architecture. A plethora of soluble signals from neoplastic cells induce the activation of the stromal compartment. The early evolution of stroma includes (i) dynamic changes in extracellular matrix (ECM) components, (ii) mesenchymal cell activation and (iii) reorganisation of endothelial vessel networks. In normal tissues, the extracellular matrix is a relaxed network of fibrous proteins (collagens, elastins, fibronectins and laminins), proteoglycans (chondroitin sulfates and keratin sulphates) and hyaluronic acid. The association of fibers and proteoglycans creates extracellular structures that compose the interstitial matrix. The proteoglycan component in the interstitial matrix confers a gel-like texture, required for cell hydration, tensile stress resistance, binding and storage of growth factors. Collagen sustains cell adhesion, chemotaxis and trafficking (Jarvelainen, Sainio et al. 2009). The basement membrane is a thin specialised ECM that constitutes a barrier to connective tissue (stroma) and a supportive structure for adherent endothelial cells and epithelial cells. The basement membrane is mainly composed of collagen IV and glycoprotein (such as perlecans and laminins). The tight junctions of epithelial cells on the basement membrane regulate tissue permeability (LeBleu, Macdonald et al. 2007).

Fibroblasts continuously regulate ECM homeostasis via reorganisation and secretion of fibrous proteins (e.g. collagen I, collagen III, elastin, fibronectin etc), fibroblast metalloproteinases and metalloproteinase inhibitors (Calcinoatto, Brevi et al. 2018).

At early stages of tumorigenesis, activated fibroblasts alter the composition of ECM, enhancing matrix degradation, in order to create a permissive stroma for cancer cell growth. Local extracellular matrix degradation is carried out by matrix metalloproteinases, disintegrin and metalloproteinases (ADAM). Initial stroma remodelling resembles the wound-healing processes. Indeed cancer-induced desmoplasia is characterised not only by fibroblast enrichment, enhanced collagen deposition and improved dynamic extracellular matrix remodelling, but also by increased density of dilated and enlarged capillaries (Brown, Guidi et al. 1999).


ECM degradation allows the release of growth factors, including vascular endothelial growth factors (in particular VEGFA), which enhance the permeability of blood vessels and of neo-capillary formation. Additionally, cancer associated fibroblasts can express pro-angiogenic factors during carcinogenesis, such as VEGF and fibroblast growth factor (FGF) (Erez, Truitt et al. 2010). Endothelial cells, pericytes and smooth muscle cells are involved in endothelial vessel neo-formation. As in wound healing, immune cell recruitment into the tumor site is favoured by newly-formed endothelial and lymphatic vessels. (Fukumura, Xavier et al. 1998) (Hutchings, Ortega et al. 2003) (Brown, Guidi et al. 1999). The structural fibers of the tumor stroma function as a source of chemokines and cytokines that can attract and sustain immune cell infiltration (Mueller, Goumas et al. 2007). Tumor imaging has shown that ECM architecture with thin collagen fibers favours T cell trafficking into tumors. Within tumors, T cell migration follows parallel-organised fibres that surround blood vessels (Salmon, Franciszkiewicz et al. 2012). Moreover, in premalignant dysplasia in squamous skin melanoma and in pancreatic ductal carcinoma, activated fibroblasts themselves express pro-inflammatory gene products, such as cytokines IL-1β, TNF-α, IFN-β and IL-6, and chemoattractive molecules CCL2, CCL5, CXCL1, CXCL2 and CXCL10. Pro-inflammatory gene signatures in cancer-associated fibroblasts (CAF) seem to be directly induced by immune cells and could boost antitumor responses (Erez, Truitt et al. 2010). Hence tumor-induced extracellular matrix remodelling could restrict tumor progression trough regulation of antitumor immune cell migration, guiding immune cell trafficking and localisation trough low affinity interactions between cells and collagen, and sustaining activation of effector cells (Friedl, Entschladen et al. 1998) (Mrass, Takano et al. 2006).

On the other hand, enhanced release of pro-angiogenic cytokines, such as VEGF, and of soluble molecules inducing cancer-associated fibroblast differentiation, such as TGFβ and FGF, dramatically impact vascular and stromal structures, leading to the establishment of a milieu permissive to cancer (Brown, Guidi et al. 1999; Akashi, Minami et al. 2005). These issues are addressed in more detail in section 1.3.2.

1.3 Chronic inflammation and alteration of anti-tumor Immune responses
Impaired or misled immune responses may be unable to orchestrate protective antitumor responses. Lack of protective T cell immunity and continuous infiltration and activation of tumor leukocytes (such as mast cells, macrophages, and granulocytes) may result in chronically inflamed tissues. Chronicity relies on the cytokines and chemokines produced by the tumor infiltrating immune cells that induce chemokine synthesis in neoplastic and stroma cells, establishing a continuous recruitment of inflammatory cells and potentiating the activation of stroma cells (Grivennikov, Greten et al. 2010). Chronic unresolved immune responses are a hallmark of cancer progression. Several mechanisms have been described to account for cancer immune escape. Persistent inflammatory responses subject tumor cells to selective pressure. “Immunoeediting” refers to the immune system sculpting malignant cells: neoplastic clones with low antigenicity will escape immune detection. T cells are thus determinant players of immunosurveillance but also of selection of low immunogenic neoplastic cells (Klein, Sjogren et al. 1960; Shankaran, Ikeda et al. 2001; Matsushita, 2012 #149; Malladi, Macalinao et al. 2016).

Tumor specific antigen recognition by effector cells can be impaired not only by low antigenicity but also by defects in antigen presentation. Immuno-inhibitory molecules, functional impairment of cytotoxic effector cells and immunosuppressive cell activity contribute to worsening anti-tumor immunity (Campoli and Ferrone 2008) (Gastl, Abrams et al. 1993) (Baitsch, Baumgaertner et al. 2011). Chronically inflamed tissues present an enrichment of immune suppressors, e.g. myeloid derived suppressor cells (MDSC), suppressive T cells and B cells and IL-10 producing macrophages, which damp on antitumor responses.

Persistence of infiltrating immune cells in turn can also favour malignancy through the building up of a protumor milieu. Availability of soluble immune-mediator molecules can directly sustain cancer cell survival and proliferation and indirectly stimulate the activation of angiogenesis, supporting tumor growth (Fouad and Aanei 2017). Inflammatory mediators can induce expression of genes regulating epithelial to mesenchymal transition in cancer cells. Extracellular matrix modifying enzymes released by tumor associated macrophages and immune-activated stromal cells can disrupt tissue architecture promoting cancer metastasis, in concomitance with epithelial
mesenchymal transition and angiogenesis. Thus unresolved inflammation, mainly through type 2 cytokine-activated myeloid cells, continuously triggers tissue remodelling, which improves availability of mediators supporting tumor mass growth (Hanahan and Coussens 2012).

1.3.1 Tumor escape from immune surveillance

Several mechanisms, including modifications mediated by cancer cells, as well as immune-mediated stromal changes, drive to cancer tolerance. Genomic modifications can impair antigen presentation on cancer cells through down-regulation of MHC class I complex on neoplastic cell surface and through defects on antigen processing. The impairment of antigen presentation impacts recognition by tumor-specific CD8+ T cells and consequently may lead to a lack of immunosurveillance (Restifo, Esquivel et al. 1993) (Schreiber, Old et al. 2011). Several types of cancer, including melanoma, breast, renal, bladder and lung carcinoma display downregulation of MHC class I complex (Campoli and Ferrone 2008). Furthermore, immunosuppressive signals can occur within tumors. Neoplastic cells up-regulate the production of inhibitory molecules, such as IL-10. Additional sources of IL-10 are IL-10 producing tumor associated macrophages and CD4+ regulatory T cells (Tregs). Release of anti-inflammatory IL-10 triggers IL-10 receptor activation on DCs and macrophages. Intracellular signal transduction downstream IL-10 receptor drives NF-κB inhibition and downregulation of pro-inflammatory cytokines, impairing antigen presentation. Additional immunosuppressive signals are induced by upregulation of immunoregulatory molecules on the cancer cell surface, such as indoleamine-2,3-dioxygenase (IDO) or programmed cell death Ligand (PD-L1). IDO expression in tumor tissues acts through degradation of tryptophan, an amino acid indispensable for T cell activation, proliferation and effector function (Uyttenhove, Pilotte et al. 2003). The expression of PD-L1 on cancer cells plays a major role in immune escape (Iwai, Ishida et al. 2002). The PD-L1/PD-1 interactions keep T cells from carrying out anti-tumor cytotoxicity, inducing a phenotype switch to induced T regulatory cells or activation-induced cell death programs (Tsushima, Yao et al. 2007) (Wang, Pino-Lagos et al. 2008).
In addition to PD-1, chronic inflammation-driven activation per se can induce immune checkpoint receptor expression on the surface of T lymphocytes, e.g. cytotoxic T lymphocyte antigen 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3) or T cell immunoglobulin mucin 3 (TIM-3) (Grosso, Goldberg et al. 2009). Immune checkpoint receptors exert negative regulation of antitumor activity. In several tumors, the expression of checkpoint receptors negatively correlates with infiltrating lymphocyte antitumor activity (Matsuzaki, Gnjatic et al. 2010) (Fourcade, Sun et al. 2010). Finally, another mechanism of evasion is cancer cell downregulation of adhesion molecules, which results in the impairment of antitumor T cell trafficking into tumors. In breast cancer, in squamous carcinoma and in melanoma, downregulation of the adhesion molecules ICAM, E-selectin and P-selectin is associated with reduced T cell infiltration and consequent lack of immunosurveillance (Clark, Olsson et al. 1976; Madhavan, Srinivas et al. 2002; Weishaupt, Munoz et al. 2007). All these mechanisms can prevent cytotoxic T cell antigen recognition and killing of cancer cells, establishing cancer immune tolerance.

1.3.2 Tumor-promoting chronic inflammation

Soluble molecules and cell-cell interactions between cancer cells, immune cells and stromal cells are the main influencers of the balance towards antitumor versus protumor responses. Prevalence of protumoral inflammation favours continuous proliferation of neoplastic cells. Growth mediators secreted by immune and stromal cells, such as fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin growth factor (IGF), transforming growth factor (TGFβ), and mitogen cytokines (including IL-1, IL-6, IL-11, IL-17 and IL-22) may activate transcription factors AP-1, NF-KB and STAT3 in neoplastic cells. These transcription factors enhance the expression of genes associated with cell survival and cell proliferation, worsening malignancy (Pikarsky, Porat et al. 2004; Grivennikov, Karin et al. 2009). Among immune mediators, chemokines can also promote cancer cell survival and proliferation. On the one hand, stimulation of chemokine receptors can induce a protein expression shift from pro-apoptotic to anti-apoptotic, favouring cancer cell survival, e.g. through upregulation of survivins, inhibition of caspases and downregulation of regulating cell death protein B-
cell lymphoma 2 (Bcl-2) (Roca, Varsos et al. 2008; Song, Park et al. 2012). On the other hand, chemokine receptor activation can trigger stimulation of mitogen-activated protein kinase (MAPK) and associated extracellular signal-regulated kinase (Erk) pathways, with consequent expression of growth related genes, such as cyclins (Wani, Nasser et al. 2014). In several cancers, e.g. melanoma, pancreatic and non-small-cell lung cancers, signalling via CXCR2 can promote cell proliferation. Similarly in pancreatic carcinoma the overexpression of CCL20 stimulates cancer cell growth. The persistence of immune mediators can indirectly drive dramatic changes in the tumor stroma that sustains cancer growth. Inflammation driven angiogenesis via chemokine and pro-angiogenic factor release contributes to enhanced blood supply, required for oxygen and nutrient supply to neoplastic cells. Immune mediators (e.g. TGFβ, PDGF and FGF2) can activate fibroblasts. Immune-activated CAF mediate ECM degradation, subsequently uncaging bioactive mitogens, which in turn promote cancer cell growth (Lu, Takai et al. 2011). Activated fibroblasts can also enhance collagen production and matrix remodelling via the release of proteolitic enzymes (cysteine, serine and metallo-proteinases) and of lysyl oxidase enzymes (LOX and LOXL), responsible for fiber crosslinking (Lucero and Kagan 2006). Consequent ECM stiffening improves integrin and focal adhesion molecule expression on cancer cells that can thus acquire a more aggressive cancer phenotype (Levental, Yu et al. 2009). Enhanced matrix tension results in reorganisation and alignment of collagen fibers in compact structures in peritumoral area: capsule formation. Tumor capsule has been shown to physically impede antitumor immune cell access into solid tumors (Salmon, Franciszkiewicz et al. 2012).

**Pro-tumor activities of myeloid cells**

In cancer associated chronic responses, myeloid cells can modulate key protumor activities, such as escape from immunosurveillance, cancer cell survival, angiogenesis, lymphangiogenesis, matrix remodelling and tumor invasion. Several clinical studies correlated high levels of tumor infiltrating myeloid derived suppressor cells (MDSC), tumor associated macrophages, monocytes, neutrophils, and mast cells with poor prognosis (Parker, Beury et al. 2015).
**Myeloid derived suppressor cells** (MDSC) consist of a heterogeneous group of cells, including myeloid progenitors, monocyte and granulocyte undifferentiated precursors. They principally exert suppressor functions (Almand, Clark et al. 2001). MDSC inhibition of tumor specific cytotoxic T cells results from the release of soluble immune suppressor molecules, such as nitric oxygen (NO), ROS, peroxynitrite, programmed cell death ligand 1 (PD-L1), arginase 1 and TGFβ. Upon release, ROS and peroxynitrite can impede TCR/MHC complex interaction, PD-L1 inhibits T cell activation, Arginase 1 impedes T cell differentiation and expansion through arginine degradation and consequent metabolic inhibition, while TGFβ suppresses T cell antitumor functions (Mazzoni, Bronte et al. 2002; Nagaraj, 2007 #184) (Kuang, Zhao et al. 2009) (Young, Wright et al. 1996) (Zea, Rodriguez et al. 2005). Among myeloid cells, an additional contribution to immunosuppression derives from **tumor associated macrophages** (TAM) via production of immunosuppressive cytokines (IL-10 and TGFβ), enzymes (arginase), and metabolites (IDO and prostaglandins), or via expression of surface inhibitory checkpoints PD-L1 and 2 and B7-H4, which trigger respectively inhibitory PD-1 and CTLA4 pathways in T cells. In addition to immunosuppressive functions, TAMs play a central role in supporting tumor growth, via secretion of mitogenic cytokines and growth factors (e.g. IL-6, TNF, EGF). Moreover TAMs can promote angiogenesis, via release of pro-angiogenic agents, such as VEGFA, VEGFC, VEGFF, IL-1β, IL-6 and IL-8 and proteases MMP-2, MMP-7, MMP-9 and MMP-12. A subset of tumor infiltrating monocytes/macrophages, Tie2 expressing monocytes, is recruited by CXCL12 and angiopoietin 2 (the ligand of Tie2 receptor) in perivascular areas, where they directly support angiogenesis thanks to significantly overexpression of VEGF (Harney, Arwert et al. 2015) (Lewis, Landers et al. 2000). Macrophage released metalloproteinases, together with cathepsins, could favour migration of tumor cells via ECM degradation and stroma remodelling (Kessenbrock, Plaks et al. 2010). Classically all these effector functions have been associated to an M2-like phenotype. In growing tumors, several stimuli, such as cytokines IL-4, IL-13, IL-10, TGFβ, chemokine CSF-1 and glucocorticoids, have been seen to enable macrophages to switch to pro-tumor phenotype (DeNardo, Barreto et al. 2009) (Ruffell, Chang-Strachan et al. 2014). The overall effect of myeloid cells is weighted toward driving vascularisation and lymphangiogenesis of growing tumors. Both neutrophils and mast cells display high
expression of VEGFs and proteolytic enzymes (MMP-9, tryptases and chymases). 
(Coussens, Raymond et al. 1999) (Melillo, Guarino et al. 2010) (Zitvogel and Kroemer 2014) (Bergers, Brekken et al. 2000) (Zitvogel and Kroemer 2014). The plasticity of myeloid cells allows them to acquire a wild spectrum of functions, depending on a plethora of soluble signals and cytokines released by cancer cells (e.g. TGFβ, IL-10 or CSF-1), by stromal cells (IL-1) and by other immune cells (IL-4 and IL13 secreted by CD4+ Th2 cells and eosinophils; immune complexes by B cells) at tumor sites. Thus there is growing evidence that the dual role of innate immune cells, via direct antitumor activity or indirect protumor activity, can be driven by local signals, at the crosstalk between cancer cells, immune cells and stromal cells.

Pro-tumor activites of lymphoid cells

CD4+ T lymphocytes can direct immune responses via cytokine release. Classically, tumor evasion from immune responses has been associated with immunesuppression mediated by IL-10 producing regulatory T cells, (Zhu 2016). Protumoral immune inflammation and cancer progression have been associated with Th2 responses (mediated by IL-4, IL-5, IL-9 and IL-13 cytokines). In fact IL-4 can induce STAT6-dependent M2-like phenotype of TAMs, which are involved in cancer associated angiogenesis and tissue remodelling (De Monte 2011; Pernot 2014; Gabitass 2011). Recently, another subset of T cells have been described with a phenotype between Treg and Th2, so called Th2-like Treg cells, presenting immunosuppressive and protumor activities. Th2-like Tregs are enriched in melanoma and in colorectal carcinoma and are able to release significantly high levels of IL-4, IL-5, IL-13 and IL-10 (Halim, Romano et al. 2017).

Finally T helper 17 cells (Th17) can also exert protumoral functions. Th17 presence and IL-17 production seem to correlate with worst prognosis in patients with breast, pancreatic, gastric and colorectal cancers (Kim, et al. 2014). The overall effects of IL17 has been shown on cancer cells via IL17R signalling (Tartour et al., 1999) or via enhanced angiogenesis due to induction of angiogenic molecule production (such as VEGF) in cancer cells (Liu, Duan et al. 2011), or via innate cell synergizing
mechanisms, such as with eosinophils and their pro-tumoral activity (Calcino
tto, Brevi et al. 2018)

Pro-tumorigenic functions of Stromal Cells

Advanced stages of carcinoma are characterised by strong activation and cooperation
between stroma and immune compartments. Immune-activated stromal cells directly
sustain tumor growth, through paracrine growth factors, or indirectly reorganise host
tissue architecture favouring cancer progression. Myoepithelial cells and cancer-
associated fibroblasts release several mitogen factors, such as growth factors (FGFs,
HGF, TGF-β) and chemokines (CXCL12, also known as stromal derived factor 1
(SDF1), and CXCL14) that could stimulate cancer cell growth and proliferation.
Moreover CAF production of fibroblast-derived insulin-like growth factor II (IGF2)
and IGF1 receptor (IGF1R) signalling in cancer stem cells may support stem-like
phenotypes in cancer cells. Co-injection of CAF and cancer cells in murine tumor
models activates cancer cell proliferation programs, favouring tumor growth (Orimo,

Thus stroma cells can sustain tumor growth through several mechanisms, promoting
the development of new blood and lymphatic vessels and inducing profound changes
in tumor matrix.

Extracellular matrix dynamics in cancer progression

The evolution from early lesions and controlled tumors to malignant carcinomas is
typically accompanied by an angiogenic switch. Indeed uncontrolled tumor growth
requires enhanced blood supply, to fulfil the oxygen demand and metabolic activities
of malignant proliferating cells. Thus in carcinomas, robust angiogenic changes form
aberrant truncated blood vessels, presenting excessive branching and dead ends. This
abnormal tumor vascularisation together with an increased distance from the vessels,
impair cancer cell oxygen availability, resulting in hypoxia. In a hypoxic milieu, cancer
cells activate intracellular signalling mediated by hypoxia inducible factor (HIF). The
activation of HIF pathways promotes not only a positive pro-angiogenic feedback loop
but also a metabolic cancer cell switch to glycolysis. (Muz, de la Puente et al. 2015) (Nagy, Chang et al. 2009). Hypoxic conditions force cancer cells to use glucose as a metabolic substrate via anaerobic glycolysis. Enhanced concentration of lactate produced by glycolysis and glucose deprivation stabilise VEGF transcription in neoplastic cells, thus promoting angiogenesis. In parallel, HIF-inducible pro-angiogenic growth factors (e.g. VEGFs, PDGF, EGF and bFGF) and cytokines (such as TGFα and TGFβ, TNFα and IL8) produced by cancer cells activate growth signalling and production of MMPs in endothelial cells (LaGory and Giaccia 2016). While pro-angiogenic molecules allow endothelial cell proliferation, MMP activity allows the invasion of the spaces created in the extracellular matrix and in the basement membrane by enzymatic degradation. Additionally, basement membrane degradation itself allows the release of matrix embedded growth factors and VEGF molecules which amplify pro-angiogenic signalling (Kessenbrock, Plaks et al. 2010). Subsequently, endothelial cells are organised into a network of vessels that need to be stabilised by angiotensins, by pericyte distribution surrounding neo-vessels and by new basement membrane formation (Nishida, Yano et al. 2006). Tumor pericytes are characterised by reduced expression of contractile proteins in comparison to normal tissue pericytes, an activated phenotype and acquire a pro-angiogenic role. Indeed their inactivation, via platelet derived growth factor (PDGF) receptor inhibition, impairs tumor angiogenesis (Birbrair, Zhang et al. 2014). Additional help in enhancing angiogenesis is derived from the CAF secretome. Activated CAFs are the main source of proangiogenic VEGFs (Fukumura, Xavier et al. 1998). Other pro-angiogenic factors produced by CAFs are PDGF, FGF and GM-CSF (Orimo, Gupta et al. 2005). Continuous production of pro-angiogenic factors and metalloproteases from stromal cells and tumor infiltrating leukocytes favours vigorous activation of endothelial cells and pericytes, formation of aberrant vessels with truncated terminations, altered branches, and discontinuity of endothelial cells and pericytes, resulting in vascular leakiness and impeded tumor perfusion. Moreover persistence of myeloid derived FGF and VEGF induces downregulation of V-CAM, I-CAM or e-selectin on endothelial cells of blood vessels (Dirkx, Oude Egbrink et al. 2003). Thus the downregulation of trafficking molecule expression could affect antitumor T lymphocyte trafficking into the tumor and favour cancer cell escape from immunosurveillance. Tumor non-permissive vascular vessels
overexpress immune regulatory molecules such as CD95L, which could induce effector T cell death.

Together with tumor angiogenesis, intense fibrosis and stromal remodelling contribute to pro-tumour milieu establishment and tumor invasion (Grivennikov, Greten et al. 2010). Intense ECM remodelling depends on activated cancer-associated fibroblasts, identified by the expression of fibroblast activation protein (FAP) and α-smooth muscle actin (αSMA) (Principe, DeCant et al. 2016); (Paunescu, Bojin et al. 2011). They are responsible for ECM collagen deposition, release of enzymes (MMPs and LOXs) involved in collagen and elastin fiber remodelling and regulation of contractility of the transformed epithelium (De Wever, Demetter et al. 2008) (Levental, Yu et al. 2009). Enhanced contractility and formation of rigid thick fibers further augments ECM stiffness. In breast carcinoma, ECM stiffening induces integrin expression and focal adhesion aggregation at cell membrane, which consequently activate intracellular mechano-transduction and cytoskeletal rearrangement, resulting in malignant phenotype acquisition (Paszek, Zahir et al. 2005) (Madan, Smolkin et al. 2006). In pancreatic cancer, increase of the matrix rigidity could favour malignant cell growth, induce epithelial to mesenchymal transition (EMT) and inhibit differentiation (Rice, Cortes et al. 2017). EMT allows cancer cells to metastasise to secondary tissues. Via EMT, cancer cells acquire motility through the loss of cell polarity and cell adhesion (Thiery, Acloque et al. 2009). Cancer invasion requires both extracellular and intracellular modifications. Extracellular changes include loss of adhesion to ECM, degradation of basement membrane with formation of an area from which neoplastic cells can invade host tissues (Gialeli, Theocharis et al. 2011). Upon detachment, cancer cell must to adapt to resist cell death. Activation of metabolic pathways via IGF and EGF receptor activation and PI3K-AKT signalling promotes anchorage-independent survival (Levental, Yu et al. 2009). At this stage of the disease, metastatic cancer cells start to express chemokine receptors, such as CXCR4, CX3CR1, CCR1, CCR2, CCR4, CCR7 and CCR9, essential for the migration in secondary organs, following specific chemokine gradients (Bonecchi, Galliera et al. 2009).

Once again, activated CAFs as well as tumor infiltrating myeloid cells are involved in the processes of cancer cell migration via release of matrix metalloproteinases, which degrade ECM and create space for cancer cell invasion (Page-McCaw, Ewald et al. 2009).
Increased stiffness and fibre alignment greatly facilitate cancer cell directional migration and enhance intravasation in lymphatic and blood vessels, which precede colonisation of secondary organs (Riching, Cox et al. 2014); (Han, Chen et al. 2016). ECM stiffness around tumors can also constitute a physical barrier for effector cells homing into the tumors, favouring cancer escape from immunosurveillance (Salmon, Franciszkiewicz et al. 2012).

1.4 Immune based anti-cancer therapy

Considering the strong influence of inflammatory components on tumor progression and the importance of strong antitumor responses in controlling cancer growth, in recent years several therapies have been developed to reinforce immune responses against tumor cells. So called “Immunotherapies” are mainly based on inhibitors of protumor immune mediators or vice versa on eliciting antitumor responses via adoptive cell transfers and vaccines or the blocking of immune checkpoints (Thallinger, Fureder et al. 2018). Tumor stroma evolution strongly affects cytotoxic effector cell trafficking into the tumor and could impede effectiveness of immunotherapies. Blood vessel leakiness, hypoxia-driven metabolic alterations, ECM stiffness and capsule formation can affect immune effectors homing and function into tumors. The understanding of the processes involved in stromal remodelling could help improve current immune based therapy with new combinatorial strategies targeting tumor stromal barriers to enhance antitumor immune responses (Vignali and Kallikourdis 2017).

1.4.1 Immune targeted therapies focused on Myeloid Cells

Among myeloid cells, tumor associated macrophages exert several protumor activities that could be targeted with different therapeutic strategies (Mantovani, Marchesi et al. 2017). In preclinical and initial clinical studies, CSF-1R, the major receptor involved in monocyte myeloid cell activation and differentiation at the tumor site, has been targeted through soluble inhibitors or specific humanised antibodies. The inhibition of CSF-1/CSF-1R axis impairs macrophage accumulation, improving cytotoxic T cell density in tumors. While these molecules improve survival in preclinical models, they display minor clinical relevance in cancer patients. Yet they can potentially be combined with other therapies to improve their efficacy, by blocking intratumoral
immunosuppressive TAM accumulation (Butowski, Colman et al. 2016). Other drugs that display cytotoxic effects on TAM are currently administered to oncologic patients, e.g. trabectedin (anti neoplastic agent) and bisphosphonates (inhibitor of cholesterol synthesis). In patients undergoing these therapies, the reduction of TAM density at tumor site correlates with improved free survival duration (Colotta, Peri et al. 1984; Diel, Solomayer et al. 1998).

TAM targeting can be achieved also by blocking their recruitment to the tumor site. Chemokines are the main molecules involved in monocyte and macrophage tumor chemotaxis. Their targeting trough specific antibodies, such as anti-CCL2 or anti-CCR-2, anti-CCL5 or anti-CCR5, seems to partially ameliorate or stabilise disease progression in some cancer patients, in combination with chemotherapy (Halama, Zoernig et al. 2016) (Nywening, Wang-Gillam et al. 2016). Finally, preclinical studies have shown how specific targeting of proangiogenic macrophages, such as Tie2 expressing macrophages, via inhibition of Tie2 or angiopoietin signalling, results in reduction of tumor growth. Indeed the blocking of pro-angiogenic macrophages results in impaired angiogenesis and switch to M1-like differentiation of TAM (Mazzieri, Pucci et al. 2011; Schmittnaegel, Rigamonti et al. 2017).

1.4.2 Immune targeted therapies focused on Lymphoid Cells

Based on the concept that cytotoxic lymphocytes are the main effectors of specific and prolonged antitumor responses, several strategies have been studied to elicit their functions in cancer therapies.

Among immunotherapies, adoptive T cell therapies directly transfer tumor specific cytotoxic T cell in cancer patients. Cancer eradication could be achieved by ex vivo-expanded autologous T cells or genetically modified T cells, which are made to express chimeric antigen receptors (CARs) or tumor antigen specific TCR. Despite their high specificity and efficacy in cancer cell killing, these strategies can be hampered by immune escape mechanisms occurring at the tumor site and by the lack of tumor homing due to unfavourable cancer stromal architecture (Vignali and Kallikourdis 2017).
Enhancing of T cell responses can also be achieved indirectly with vaccine strategies. Vaccine immunisation consists of administration of short tumor surface antigenic peptides or of re-infusion of patient derived peripheral DCs loaded with tumor peptides *ex vivo*. Upon administration, tumor peptides must be loaded on MHC of APCs to elicit T cell responses. Therefore the main limits of this strategy could be the clearance of the peptides before their uptake by antigen presenting cell and the capability of the patient to develop an efficient immune response against the selected tumor antigens (Guo, Manjili et al. 2013). Differently, dendritic cell transfer has the advantage of utilising professional antigen presenting cells able to elicit strong cytotoxic T cell responses, thus it reported improved survival in clinical studies (Sabado and Bhardwaj 2013).

Finally, antitumor T cells can be impaired by immunosuppressive mechanisms present at tumor site. Immune checkpoint inhibitors, such as anti PD-1, anti-PDL-1 or anti CTL-4, have been used to recover the full activation of cytotoxic T cells. Blocking of checkpoint receptors enhances effective and durable antitumor immune responses in patients with solid and hematologic tumors, even if some clinical studies have shown limits in efficacy and a risk to develop autoimmune reactions (Thallinger, Fureder et al. 2018).

1.4.3 Targeting Stromal cells to improve Anti-tumor Immune Responses

During cancer progression, stromal changes and the building up of physical barriers could impede antitumor immune cell trafficking into tumors. The absence of a mature regular vascular network formation could alter leukocyte infiltration within tumors. On the contrary, normalisation of blood vessels has been demonstrated to improve cytotoxic T cell homing into tumors (Qin, Schwartzkopff et al. 2003; Carretero, Saktioglu et al. 2015). In fact, excessive tumor angiogenesis results in poorly perfused vessels. Normalisation of tumor vasculature through anti-angiogenic treatments, such as blocking of VEGF, angiopoietin or endhotelin, could directly facilitate tumor specific T cell homing and restore the expression of integrins (e.g. ICAM-1) and downregulate immunosuppressive molecules (CD95L) on endothelial cells (Shrimali, Yu et al. 2010) (Motz, Santoro et al. 2014; Schmittnaegel, Rigamonti et al. 2017); . Similarly, in advanced cancers the formation of dense fibrotic capsules can impede cytotoxic effector cell trafficking. Thus anti-fibrotic therapies based on direct cancer
associated fibroblast targeting (vaccination against FAP) or indirect inhibition of pro-fibrotic molecules (anti-TGFβ or anti-focal adhesion kinase) may decrease collagen deposition, improving T cell homing and consequent immune cell mediated killing of cancer cells (Principe, DeCant et al. 2016) (Jiang, Hegde et al. 2016). Interestingly, in a murine model of mammary carcinoma, anti-TGFβ therapy in combination with anti-neoplastic doxorubicin has shown that CAF elimination can enhance anti-tumor cytotoxic T cell response by reducing ECM density, thus increasing tumor vasculature perfusion. Vasculature perfusion can improve drug delivery, with overall positive effects on antitumor responses, both in primary and in secondary metastatic sites. In vivo targeting of CAF via DNA vaccines resulted in improved efficacy of doxorubicin but also higher IL-2 and IL-7 levels, and reduced myeloid derived suppressor cells and M2-like TAM infiltration (Polydorou, Mpekris et al. 2017) (Liao, Luo et al. 2009). The above highlight the cooperation of cancer fibrosis and vessel perfusion in forming obstacles to antitumor responses. Despite these encouraging results, depletion of CAF in a model of pancreatic adenocarcinoma, both at early or at late stages of the disease, worsened tumor progression, with increase tumor invasion and enhanced immunosuppression despite the suppression of tumor angiogenesis (Ozdemir, Pentcheva-Hoang et al. 2014). In summary, the functional contribution of CAFs in cancer progression and fibrogenesis requires further investigation to enable more efficacious CAF targeting in therapy.

In conclusion, the evidence from immunotherapies and therapies targeting the tumor stroma suggests that an optimal therapeutic protocol may require the combination of therapies enhancing both myeloid and lymphoid antitumor immunity, whilst also acting on tumor stroma. This could impede cancer cell invasion, whilst improve the recruitment of immune cells mediating the antitumor immune response.

SPECIFIC BACKGROUND OF THE PROJECT

1.5 Type 1 versus type 2 responses in tumors
As explained in detail in the previous section, possibly driven by the chronicity of the tumor-associated inflammatory responses, a prevalence of type 2 cytokines (IL-4, IL5, IL9 and IL13) occurs at tumor sites. The main effect mediated by these cytokines on tumour-associated macrophages (TAMs) is polarisation toward M2-like phenotype (Mantovani, Marchesi et al. 2017). Direct consequences of this M2-like polarization are i) the inhibition of innate type 1 antitumor immune responses, ii) tumor growth support through release of soluble mitogens and iii) tissue architecture remodelling via release of proangiogenic factors and enzymes supporting angiogenesis and reorganisation of extracellular matrix (Schmid and Varner, Journal of Oncology 2010). Indeed predominance of type 2 cytokine profiles and Th2 responses have been demonstrated to correlate with tumour progression in human pancreatic cancer (De Monte, Wormann et al. 2016). Similarly in mammary adenocarcinoma, enrichment of CD4+ T helper 2 effector cells and improved M2-like profiles of myeloid cells in secondary organs augment the risk of metastasis (DeNardo, Barreto et al. 2009).

Up to now the effects of type 2 cytokines have been studied in tumors mainly by considering their polarizing effect on macrophages, using animal lacking single cytokines or through inhibition of single or double cytokine. However these cytokines have pleiotropic effects on multiple cell types. The overall effects of type 2 responses on tumor progression are therefore still poorly understood.

1.5.1 Type 2 responses and fibrogenesis

Fibrogenesis is the accumulation of extracellular matrix components in a tissue. During fibrogenesis, basement membrane and interstitial matrix may vary their collagen content, localisation and orientation, depending on influence from immune cells, vascularisation and stromal cell activation (Baiocchini, Montaldo et al. 2016). Several studies characterised the role of type 2 cytokines in pathologic tissue fibrogenesis, such as insulin sensitivity, (Odegaard, Ricardo-Gonzalez et al. 2007); and in tissue repair (Costa, Ruiz-de-Souza et al. 2011). Furthermore, in these studies, scar formation in studied organs was traced back to fibroblast hyperactivation and chronic type 2
response, mainly due to the presence of CD4+ T cells. Indeed transient wound healing (characterised by angiogenesis, collagen production and release of mitogenic growth factors to sustain immune responses and restoration of tissue homeostasis) can become chronic if the injury is maintained in time. Consistently, perpetuation of chronic inflammation and fibrogenesis progression in fibrosis, have been observed for example in persistent pathogen infections (Harris, De Haro et al. 2007), severe asthma and allergies (Ying, Humbert et al. 1997) (Nath, Leung et al. 2007), liver fibrosis (Chiaramonte, Donaldson et al. 1999), pulmonary fibrosis (Murray, Argentieri et al. 2008) or fibroproliferative cardiac diseases (Weirather, Hofmann et al. 2014). In all these conditions, myofibroblast activation is strongly dependent on production of immune-derived mediators by monocytes/macrophages, such as growth factors (FGF, HGF, EGF, VEGF, PDGF and IGF), cytokines (such as TGFβ, IL-4 and IL-13) and chemokines (CCL2, CCL8, CCL7 and SDF-1), but also reactive oxygen and nitrogen species and stress signals. (Ramachandran, Iredale et al. 2015) (Karlmark, Weiskirchen et al. 2009). In pulmonary fibrosis it has been further shown that Th2 immune responses, involving interleukin-4, IL-5 and IL-13, favour fibrosis by inducing the expression of key proteins and enzymes involved in collagen deposition and ECM remodelling, including pro-collagens, LOXs, MMPs by myofibroblasts as well as pro-fibrotic mediators by macrophages (Sandler, Mentink-Kane et al. 2003). Among Th2 cytokines, IL-13 is the main cytokine involved in stimulating production of TGFβ and in activating fibroblasts (Lee, Homer et al. 2001) (Liu, Meyer et al. 2011). Indeed fibroblasts express IL-4Rα and IL-13 Rα1 signalling receptors, that bind and transduce IL-4 and IL-13 signalling, and IL-13Rα2 decoy receptors (Liang, Zhang et al. 2017) (Jakubzick, Choi et al. 2004). Signal transduction of IL-4 and IL-13 stimulate collagen production in fibroblasts (Chiaramonte, Donaldson et al. 1999), while IL-5 indirectly recruits eosinophils, which are an important source of IL-13 and TGF-β. TGF-β amplifies pro-fibrotic functions inducing myofibroblast collagen deposition, expression of Arginase-1 in macrophages (thus promoting switching to M2 phenotype) (Yamaguchi, Hayashi et al. 1988): (Rosenberg, Dyer et al. 2013). The depletion of macrophages in bleomycin model of lung fibrosis resulted in reduction of fibrogenesis related to decrease in “alternative macrophage activation” (Gibbons, MacKinnon et al. 2011).
1.5.2 Innate and Adaptive responses affecting cancer fibrosis

Chronic wound healing responses, and thus fibrosis, sustained by an ongoing tissue injury are hallmark of cancer progression (Rybinski, Franco-Barraza et al. 2014). Indeed continuous production of pro-fibrotic factors released by the same cancer cells or by the infiltrating immune cells, both from the innate and the adaptive compartments, govern the recruitment and activation of fibroblasts into tumours. Macrophages can receive signals from stromal cells, innate and adaptive immune cells, and integrate them to promote tumor fibrosis. M2-like macrophages and fibroblasts are associated with cancer progression, persistent inflammation, ECM matrix remodelling and poor prognosis in several types of cancers (Comito, Giannoni et al. 2014) (Herrera, Herrera et al. 2013) (Hashimoto, Yoshida et al. 2016). Recent findings from our group show that the formation of peri-tumoral fibrotic capsules is partially dependent on T cell presence. This is important as peri-tumoral dense collagen deposition constitutes an obstacle to antitumor T cell homing, and thus to tumor mass eradication (Garetto et al. 2016;). Moreover De Monte and colleagues found a correlation between tumor infiltrating Th2 frequencies, enhanced cancer-associated fibroblast activation and pancreatic cancer progression (De Monte, Reni et al. 2011). Taking into account that fibrogenesis is strongly linked with the development of type 2 immune responses, specifically Th2, and that immune activated CAFs are associated with cancer progression, the conjunction of the effects of type 2 cytokines on tumour growth and cancer fibrosis are thus a crucial understudied aspect of tumor immunology.

1.6 The effect of type 2 responses on the progression of cancer

Several studies identified T cell presence as being essential for ECM matrix remodelling in both physiological and pathological contexts characterised by intense fibrogenesis (Gawronska-Kozak 2011). In damaged tissues, Th2 lymphocytes, in concomitance with group 2 innate lymphoid cells (ILC2s), and type 2 mediators (basophils, eosinophils and mast cells) orchestrate IL-4 and IL-13 responses, which
induce an M2-like phenotype switch of resident macrophages. All together, these cells influence tumor formation and growth, promoting tumor stroma remodelling. From early stages of progression, until advanced carcinoma with persisting type 2 mediated inflammation, the type 2 immune responses mediate several pro-tumoral effects, indirectly influencing stroma remodelling. This establishes a favourable milieu for cancer cell invasion, and favors survival and growth of cancer cells.

The definition of tumors as “wounds that do not heal”, may be strictly related to enhanced and chronic type 2 immune responses, which continuously sustain tumor progression. Even if fibrosis has been extensively characterised in different types of solid tumors, a potential role of type 2 responses in tumour-associated fibrotic processes has never been investigated formally (Dvorak 2015). Our central hypothesis is that, considering the role of IL-4, IL-5, IL-13 cytokines in wound healing and pathological matrix remodelling, type 2 responses could exert a fundamental role in tumor progression and in cancer-associated fibrosis formation. Indeed, several cell subsets of the innate immune response have been described as mediators of stroma remodelling (see chapter 1.3.2). Our study, using different tumor models in an in vivo system lacking all four type 2 cytokines, set out to assess for the first time how these different immune cell types may actively remodel tumor stroma architecture and directly affect tumor progression. Innate and adaptive immune cells with a type 2 polarization may cross-talk with stromal cells, leading to changes in collagen deposition and peritumoral capsule formation, which are critical for T cell recruitment. Thus blocking type 2 cytokines in different tumors may also help in defining their putative roles in tumor stroma remodelling and its consequences on immunosurveillance (Salmon, Franciszkiewicz et al. 2012) (Levental, Yu et al. 2009).
2. MATERIALS AND METHODS

2.1 MATERIALS

Animal strains
BALB/c IL-4/5/9/13 \textsuperscript{-/-} mice (kindly provided by Andrew N.J. McKenzie, Laboratory of Molecular Biology, Medical Research Council Cambridge, UK From Charles River Laboratory
BALB/c mice

Cell lines
4T1 cells (kindly provided by Raffaella Bonecchi, Humanitas Clinical and Research Center, Milano, Italy)

In vivo antibodies
From BioXCell
\textit{InVivo plus} armenian hamster anti-mouse CD3e (clone 145-2C11)
\textit{InVivo plus} armenian hamster IgG isotype control (clone BP0091)

In vivo procedures
From Sigma
3-methylcholanthrene (cat. N 213942)
From Thermo Scientific

From Merial Laboratorios S.A.
Ketamine hydrochloride Imalgene 1000 (10g/100ml)
From Bayer
Xylazine sterile injectable (20mg/ml)

Cell culture
From Lonza
Dulbecco’s modified eagle medium (DMEM) high glucose
Roswell park memorial institute (RPMI) 1640 medium without L-glutamine
Penicillin/streptomycin (10000 U Penicillin/ml; 10000 U Streptomycin /ml)
Ultraglutamine (Ultraglutamine 200nM in 0,85 NaCl)
Hepes (1M)
Phosphate buffer saline without calcium/magnesium (PBS -/-) 1X

From Sigma-Aldrich
Trypsin EDTA solution 1X (0,25% - 0,53mM)
Fetal bovine serum (FBS)

Tissue culture
From Sigma-Aldrich
collagenase IV

From Primo Euroclone
12 well plates
6 well plates
flask 25cm²
flask 75cm²

From BD
50ml polypropylene tubes
70µm pore cell strainers
10ml syringes with plungers

Molecular Biology
From Biorad
PureZol RNA isolation reagent
From Qiagen
RNeasy mini Kit
From Applied Biosystems
High Capacity cDNA Reverse Transcription kit
TaqMan Universal Master Mix
TaqMan Probes

*Rn18s* Mm03928990_g1 (internal control)

*Cd3e* Mm005996484_g1

*Tnfa* Mm00443260_g1

*Ifng* Mm01168134_m1

*Tgfb1* Mm01227699_m1

*Il10* Mm00439614_m1

*Il6* Mm00446190_m1

*Il1b* Mm00434228_m1

*Il12b* Mm01288909_m1

*Ccl2* Mm00441242_m1

*Cxcl9* Mm00434946_m1

*Cxcl10* Mm00445235_m1

*Cxcl11* Mm00444662_m1

*Lox* Mm00495386_m1

*Colla1* Mm00801666_g1

*Col3a1* Mm00802300_m1

*Acta2* Mm00725412_s1 (aSMA)

Molecular biology equipment

From Applied Biosystems

AB7900HT cycler

ViiA7 instrument

From Miltenyi Biotec

GentleMACS

Immunohistochemistry

From Thermofisher

Monoclonal rabbit anti-CD3e (clone SP7; Cat. N. MA5-14524)

Superfrost plus adhesion microscope slides
From Biocare Medical
Streptavidin-peroxidase
Rodent block M (Cat. N. RBM961 G)
Mach 1 universal HRP-polymer rodent (Cat. N. MRH538L10)
3,3’ diaminobenzidine (DAB) Chromogen
DAB substrate buffer

From VWR
Bouin’s solution
FeCl 37%
Phosphotungstic acid

From Merk Millipore
Green light
Fucsin acid
Ponceau S
Phosphomolybdic acid

From Sigma-Aldrich
Tween 20
EDTA solution (0.5 M)
PAP pen

From Fluka
Eukitt

Immunohistochemistry equipment
From Olympus
Olympus VS120 dotSlide

From Media Cybernetics
Image-Pro Premier software version 9.1

Second-harmonic generation imaging equipment
From LaVision
TrimScope II multiphoton microscope
From Olympus
40x/1.0 LUMPLFL-WI water immersion objective

Open source
Image J (http://imagej.net/Contributors)

Flow cytometry
From BD
Rat anti-mouse CD16/CD32 (clone 2.4G2)
Rat anti-mouse CD45 BV605 (clone 30-F11)
Armenian hamster anti- CD3e Pecy7 (clone 145-2c11; Cat. N. 552774)
Rat anti-mouse CD4 alexa fluor 700 (clone RMA-5)
Rat anti-mouse CD19 PE (clone 1D3)
Rat anti-mouse Ly6G Pecy7 (clone 1A8)
Rat anti-mouse Ly6G PE-CF594 (clone 1A8)
Rat anti-mouse Ly6C BV421 (clone AL-21)
Rat anti-mouse Ly6C FITC (clone AL-21)
Rat anti-mouse CD11b PerCP cy5.5 (clone M1/70)
Hamster anti-mouse CD11c alexa fluor 700 (clone HL3)
Hamster anti-mouse CD11c FITC (clone HL3)
Hamster anti-mouse CD11c PE (clone HL3)
Rat anti-mouse MHC II FITC (2G9)
Rat anti-mouse Siglec-F PE (clone E50-2440)

From Ebioscience
Armenian hamster anti-mouse CD8 eFluor 450 (clone 145-2C11)
Rat anti-mouse CD4 APC-eFluor780 (clone RM4-5)
Rat anti-mouse FOXP3 FITC (clone FJK-16s)
Rat anti-mouse CD25 APC (clone PC61.5)
Rat anti-mouse Ly6C PE (clone HK1.4)
Rat anti-mouse MHC II AP CeFluor780 (clone M5/114.15.2)
Rat anti-mouse MHC II APCCH7 (clone M5/114.15.2)
Armenian hamster anti-mouse CD103 PerC PeFluor710 (clone 2E7)
Rat anti-mouse CD8 PE (clone 53-6.7)
Rat anti-mouse CD19 eFluor450 (clone eBio1D3(1D3))
Armenian hamster anti-mouse CD27 APC-eFluor780 (clone LG.7F9)
Rat anti-mouse CD44 FITC (clone IM7.8.1)
Rat anti-mouse FOXP3 APC (clone FJK-16s)

From Thermo Fisher
LIVE/DEAD fixable aqua kit

From Biolegend
Rat anti-mouse CD45 PerCP (clone 30-F11)
Rat anti-mouse CD8 Alexa Fluor 488 (clone 53-6.7)
Rat anti-mouse DX5 Pecy7 (clone DX5)
Rat anti-mouse CD62L BV570 (clone MEL-14)
Rat anti-mouse CD206 APC (clone C068C2)
Rat anti-mouse F4/80 Pecy7 (clone BM/8)
Rat anti-mouse F4/80 APC (clone BM/8)
Rat anti-mouse CD11b BV785 (clone M1/70)
Rat anti-mouse CD11b Pacific Blue (clone M1/70)
Rat anti-mouse ICAM APC (clone YN1/1.7.4)

Flow cytometry equipment
From BD
FACSCanto II
LSR Fortessa
FACSymphony
From Tree Star
FlowJo Version 9.9.4
2.2 METHODS

Tumor cell lines

4T1 cells derived from murine breast carcinoma (Pulaski and Ostrand-Rosenberg 2001) were cultured in DMEM high glucose supplemented with 10% fetal bovine serum, 1% ultraglutamine, 1% penicillin/streptomycin, 1% Hepes.

Fibrosarcoma cells were isolated from primary tumors induced with 3-MCA in wild type BALB/c mice. Briefly tumor samples were harvested in PBS without calcium/magnesium, cut in 2ml 0,1mg/ml collagenase (dissolved in PBS without calcium/magnesium) and incubated 1 hour at 37°C. Aggregates were broken up with 10ml syringe without needle and cell suspension was filtered using 70μm strainer. Cells were counted in Turk’s solution and 5x10⁶ cells were plated in a T25 flask. Isolated 3-MCA fibrosarcoma cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% ultraglutamine.

All cells were cultured at 37°C in humidified 5% CO₂ incubator. PBS without calcium and magnesium and trypsin EDTA were using for passaging. Cell lines were tested for mycoplasma. Only mycoplasma free cells were used for the experiments.

Mice

Male and female BALB/c mice were sourced from Charles River Laboratories. Male and female BALB/c IL-4/5/9/13 \(-/-\) quadruple KO mice, which feature a complete deficiency in type 2 cytokines, were kindly provided by Andrew N.J. McKenzie (Laboratory of Molecular Biology, Medical Research Council Cambridge, UK) (Nath, Leung et al. 2007).

Induction of 3-MCA-induced tumors

BALB/c male or BALB/c IL-4/5/9/13 \(-/-\) quadruple KO male mice were shaved and injected subcutaneously in the right side (into the depilated area) with a single dose of 200 μg of 3-methylcholatrene (3-MCA) (Sigma-Aldrich) dissolved in corn oil. Mice used for experiments were 8/13-week-old. Tumors were measured twice a week over the course of 5 months, using a calibrated caliper. Two perpendicular caliper measurements were recorded. The tumor volume was calculated using the formula volume = (width (2) x length) / 2. When tumors reached the diameter 15-20mm, mice...
were sacrificed and the tumors were processed. In short term experiments, mice were sacrificed on day 21 and tissue at site of injection was harvested and processed. Procedures were performed according to protocols approved by Humanitas Clinical and Research Centre animal welfare committee and the Ministry of Health, in compliance with national and EU legislation and policies.

**Tumor transplantable model**

For 4T1 model, BALB/c female or BALB/c IL-4/5/9/13 −/− quadruple KO female mice were injected with 5*10^4 4T1 at the site of the mammary gland.

For injectable 3-MCA fibrosarcoma model, BALB/c male or BALB/c IL-4/5/9/13 −/− quadruple KO male mice were injected subcutaneously into the right side with 5*10^4 3-MCA fibrosarcoma cells.

Mice used for the experiment were 8/13-week-old. Tumours were measured every two days with caliper. On day 21 tumors were harvested and processed. At the end of the experiment, tumors harvested from BALB/c or BALB/c IL-4/5/9/13 −/− mice had significant different dimensions. Thus, the analysis performed are representative of tumors at maximum volumes but substantial differences in tumor dimensions, such as formation of necrotic or hypoxic areas, between the two groups may influence immune infiltration and stroma components.

**In vivo T cell depletion**

In order to analyse the contribution of T cells in tumor growth and tumor fibrosis, four days before tumor injections, BALB/c or BALB/c IL-4/5/9/13 −/− quadruple KO mice received T cell depleting armenian hamster anti-mouse αCD3 (clone 145-2C11) 250μg/mouse or isotype/vehicle control via intra-peritoneal injection. T cell depletion in the blood was monitored via FACS for CD4 and CD8 one day before tumor injection and at days 3, 16 and 21 after tumor injection.

**Quantitative gene expression analysis**

Tumor samples were collected, snap frozen in liquid nitrogen, and stored at -80°C. Tissues were resuspended in 1ml PureZol, moved in GentleMACS tubes and homogenised with GentleMACS. Aqueous phase of RNA was isolated with chloroform. Thus, RNA was extracted following the instructions of RNeasy mini kit.
from Qiagen. Upon RNA quantification, the same amount of RNA was retrotranscribed with High Capacity cDNA Reverse Transcription kit. Quantitative expression of selected genes was performed using TaqMan real-time qPCR reactions on ViiA7 instrument.

**Immunohistochemistry for T cell detection**

Tumors were harvested, fixed in 4% formalin overnight and processed for paraffin embedding. Sample sections of 3μm were collected on polarised slides. For T cell staining samples on slides were deparaffinised and rehydrated through descending scale of alcohols. Antigen retrieval was performed in EDTA (25mM) for 20 minutes at 98° in the water bath. Sections were cooled, washed in PBS without calcium/magnesium with 0.05% Tween 20. Endogenous peroxidase blocking was performed by incubation in 2% H$_2$O$_2$ for 20 minutes at room temperature. Upon washing, non-specific binding of the antibody to tissues was blocked with rodent block incubation for 20 minutes at room temperature. Sample sections were incubated with rabbit anti-CD3e (clone SP7) diluted 1:100 (in PBS without calcium/magnesium with 0.05% Tween 20) for 2 hours at room temperature. Negative immunoistochemical controls were incubated replacing primary antibody with PBS/-/ with 0.05% Tween. Washed slides were incubated with Mach 1 universal HRP polymer for 30 minutes at room temperature. Upon washing, antigen-antibody-HRP complex were incubated with DAB substrate for the dark brown staining development. Sections were counterstaining with hematoxylin, dehydrated via ascending scale of alcohols and xylene, and coverslip were attached with mounting medium Eukitt.

Images of whole tumor slides (or 3MCA-injection site tissue slides) were acquired with 20x magnification using an Olympus VS120 dotSlide slide scanner. Image pro premier was used for the analysis of CD3$^+$ cells. Within tumor tissue 2 to 8 regions of interest (ROIs) were randomly drawn with fixed area for each sample. In 3-MCA injected pre-tumor tissues T cells were counted in one single ROI drawn at the site of injection. DAB staining was selected with smart segmentation, noise was excluded trough watershed algorithm, and CD3$^+$ cells were counted automatically in the range size 10μm-infinity. For each ROI tissue areas were measured. Within each ROI, T cell density was calculated using the formula: T cell density = number of T cells / μm$^2$. 
ROIs were analysed independently. T cell densities for each ROI of the same sample were averaged to yield the mean T cell density at sampling site [intratumoral for 4T1 model, injection site for 3MCA].

**Histology for collagen content assessment in tumors**

For the detection of collagen fibers 3 μm sample sections were collected and stained following Masson’s trichrome staining protocol. Slides were deparaffinised and rehydrate through descending scale of alcohols. A secondary fixative, Bouin’s solution, was applied, for 1 hour at 60°C. Sections were cooled, washed in running water at room temperature, to remove yellow color of Bouin’s solution. Nuclei were stained with iron hematoxylin for 10 minutes at room temperature. Upon washing under running water for 10 minutes, cytoplasms were stained with ponceau of xylidine for 1 minutes and quickly washed for three times. To prepare sections to uptake collagen staining, samples were incubated in phosphomolybdic/phosphotungstic acid for 15 minutes. Sections were drained without dry and incubated in green light for 3 minutes, in order to stain collagen. Upon quick wash in water, collagen staining was differentiated in acetic water for 3 minutes. Sections were dehydrated quickly through ascending scale of alcohols, cleared in xylene and coverslip were mounted in medium Eukitt.

Images of all tumor slides (or 3MCA-injection site tissue slides) were acquired with 20x magnification using an Olympus VS120 dotSlide slide scanner. Image pro premier software was used for the analysis. Smart segmentation was used to define image segmentation of nuclei, cytoplasm and collagen, drawing a reference area on each of them and adjusting with background correction. The same segmentation was applied to all the samples. At least 3 ROIs were randomly drawn with a fixed area for each sample. In tumors, peritumoral collagen density and intratumor collagen density were analysed in different times. Region of interest in peritumoral capsule collagen were drawn including only capsule region, randomly around tumor, avoiding skin area. Region of interest to quantify intratumor collagen deposition were randomly drawn within tumor mass. Collagen area and total tissue area were measured in each ROI. Percentage of collagen density was calculated with the formula: collagen density = collagen area (μm²) / tissue area (μm²))*100. Collagen densities for each ROI of the same sample
were averaged to yield the mean of percentage density at sampling site [intratumoral for 4T1 model, injection site for 3MCA]

Before the staining the slides were analysed using second harmonic generation 2-photon microscopy to confirm collagen quantification, as described below.

**Second harmonic generation (SHG) imaging**

Tumors were harvested, fixed in 4% formalin overnight and processed for paraffin embedding. Sample sections of 3μm were collected on polarised slides. Images were captured with 2 multiphoton microscope using 20x/1.0 objective. For second harmonic generation (SHG), excitation was provided by laser tuned at 840nm for collagen signal or at 920nm for green auto-fluorescent 3MCA (polycyclic aromate structure) (Qin, Kim et al. 2002). For each slide the operator selected from 3 to 5 regions at 3MCA-injection site, in the capsule regions or in the intratumor regions. Images were saved as TIFFs and analysed with ImageJ/Fiji. Following exclusion of background and artefacts, collagen density analysis was conducted with fixed threshold levels in collagen signal channel, avoiding skin area. White collagen pixels on white/black total tissue pixels were measured and density of collagen was calculated as % of collagen area (μm$^2$)/total tissue area (μm$^2$). Automatic particle counting was used to count 3MCA particles. Threshold levels were fixed in green channel and images were converted to binary. Background noise was excluded with de-speckle filter and automatic count particle was used to analyse 3MCA particles, setting area from minimum 5 μm to infinity maximum. Particle density was calculated using the formula density = particle counts/total tissue area (μm$^2$).

**Flow cytometry**

Peripheral blood for flow cytometry was collected in EDTA treated tubes and treated with lysis buffer (NH4Cl 0.15M, KHCO3 10nM, NaEDTA 0.1nM pH7.2-7.4) for erythrocyte lysis. For the staining 100μl of blood were lysed. Spleens were collected in PBS without calcium and magnesium, single cell suspension were obtained using 70μm strainer in 50ml tubes. Cell suspensions were washed and counted in Turk’s solution. For the staining 1.6x10$^6$ cells were used.
Tumor samples were collected in PBS without calcium and magnesium, transferred in 6 multiwell and cut in 2ml of 0,1mg/ml collagenase (dissolved in PBS without calcium and magnesium). Samples were incubated 1 hour at 37°C. Aggregates were broken up with 10ml syringes without needle and cell suspension was filtered using 70μm strainer. Upon washing in RPMI medium 10% FBS, 1% P/s, 1% Ultraglutamine cells were counted in Turk’s solution. For the staining 1.6x10^6 cells were used. For 3MCA-cancer cell culture 5 x10^6 cells were plated in a T25 flask overnight, to allow cell adhesion. Medium was changed the next day.

For the staining, single cell suspensions were washed three times in PBS and were stained with aqua live and dead, following kit instructions, to exclude dead cells. To reduce Fc binding of antibodies of interest, cells were incubated with Fc blocking (CD16/CD32) for 5 minutes at 4°C. Single cell suspension was stained with antibodies listed in materials. Antibody panels were set up taking into account the features of tissue to analyse and are described in the table below.

Facs samples were analysing on FACSCanto II, LSR Fortessa and FACSymphony. Facs data were analysed with FlowJo software (gate strategies are described below *table 1*).

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism software version 5.0a. Data sets were tested for normal distribution using normality tests to apply parametric or non-parametric tests. Outliers have been identified using Grubb’s test. For normal distribution, unpaired t-test, 1-way ANOVA with Tukey post-test or 2-way ANOVA with Bonferroni were used to test statistical significance. For data without normal distribution, statistical analyses were performed using Mann Whitney non-parametric test and 1-way ANOVA tests.
## Table 2.2.1 Facs panel for 4T1 breast carcinoma

<table>
<thead>
<tr>
<th>Spleen</th>
<th>Blood</th>
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</thead>
<tbody>
<tr>
<td><strong>Innate Immune Panel</strong></td>
<td><strong>Innate Immune Panel</strong></td>
</tr>
<tr>
<td>antigen</td>
<td>fluorochrome</td>
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Table 2.2.2 Facs panel for 3-MCA induced tumors and 3-MCA transplantable tumors.

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Figure 2.2.1 Gating strategy for FACS analysis of tumor infiltrating leukocytes in 4T1 breast carcinoma.

Innate Immune Panel

Adaptive Immune Panel
Figure 2.2.2 Gating strategy for FACS analysis of splenocytes isolated from 4T1 tumor bearing mice.

Innate Immune Panel

Adaptive Immune Panel
Figure 2.2.3. Gating strategy for FACS analysis of blood leukocytes in 4T1 tumor bearing mice.

Innate Immune Panel
Figure 2.2.4 Gating strategy for FACS analysis of tumor infiltrating leukocytes in 3MCA induced sarcoma.

Innate Immune Panel
Adaptive Immune Panel

Gated on CD3+ cells

Gated on CD11b-CD45+ cells

Gated on NOT T cells

WT

T2KO
The results described in the next chapters are solely the product of my own work, with the exceptions of figure 1, which was obtained in collaboration with Dr Kapka Miteva and Msc Marco Cremonesi, who performed the experiments and the analysis of molecular biology and Dr Lorenzo Drufuca, who generated heat map representation.

Dr Matteo Massara performed 4T1 cell injections in breast cancer transplantable model. Dr Andrea Ponzetta and Dr Kapka Miteva performed 3-MCA injections in carcinogen induced tumor model. Dr Diego Morone performed image acquisition through 2-photon microscopy.

The analysis of type 2 response associations with tumor incidence in asthmatic patients in Norwegian and in Italian cohorts have been performed respectively by Dr Maria Markaki, Dr Dimitri Roe and Dr Enrico Brunetta.
3. THE ROLE OF TYPE 2 RESPONSES IN A MURINE TRANSPLANTABLE BREAST CANCER MODEL

3.1 INTRODUCTION: Type 2 cytokines in 4T1 Breast Carcinoma

Malignant progression has been thought to correlate with type 2 polarisation of immune responses in solid cancers, such as breast and pancreatic cancers (De Monte, Reni et al. 2011) (DeNardo, Brennan et al. 2011). Indeed immune cell plasticity allows innate and adaptive component to differentially express functional cytokines depending on the perceived local damaged tissue signals. There is some evidence that in chronic inflammation associated with carcinoma the prevalence of type 2 cytokines contribute to set a pro-tumorigenic environment (Steenbrugge, Breyne et al. 2018). Among type 2 cytokines, IL-9 could favour immune-evasion via tolerance induction, in different types of tumors. Moreover in cancer patients, the enhancement of IL-9 serum levels preceding metastasis formation could suggest their involvement in metastatic predisposition (Carlsson, Wingren et al. 2011). IL-4 seems to exert a direct positive influence on survival and proliferation of cancer cells (Ponzetta, Carriero et al. 2019); it indirectly favours the formation of cancer metastasis via TAM activation (DeNardo, Barreto et al. 2009). Similarly to IL-4, IL13 can stimulate cancer cell proliferation and survival. These cytokines have similar peptide structures and can activate signal transduction in epithelial tumor cells, upon binding to IL4-Rα, resulting in dimerization with IL13Rα1 into type II IL4-R, or after binding to IL13-Rα2 (LaPorte, Juo et al. 2008) (Hallett, Venmar et al. 2012) (Koller, Hwang et al. 2010). Since these cytokines can induce cancer proliferation in vitro and both ligands and receptors are expressed in different types of tumors (such as breast, lung, pancreas and ovarian carcinomas) they have been postulated to be involved in tumor progression. Moreover, these cytokines can influence pro-tumorigenic and immune-suppressor functions of myeloid immune cells as well as of stromal cells, since both cell types express their receptors (DeNardo, Barreto et al. 2009). Finally, IL-5 has been described to be involved in lung metastatic processes, again by modulating the immune milieu at the site of cancer metastasis. (Quail, Olson et al. 2017) (Zaynagetdinov, Sherrill et al. 2015). IL5R, which is activated upon IL5 binding and dimerization with the common β-chain receptors shared by the receptors for IL3 and CM-CSF, is mainly expressed on two immune cell
populations: eosinophils and B cells. The activation of IL-5/IL-5R axis is crucial for the expansion and recruitment of eosinophils in type 2 mediated pathologies, such as asthma or allergy and helminthic infections, (Nakajima, Iwamoto et al. 1992) (Yamaguchi, Hayashi et al. 1988) and for the terminal differentiation of B cells into IgG1 antibody secreting cells (Horikawa and Takatsu 2006). The effects of individual type 2 cytokines in tumor growth and in the progression of the disease have been largely investigated in these studies trough preclinical models with genetic or pharmacological deletion of single cytokines or partial of type 2 responses (Hoelzinger, Dominguez et al. 2014); (Gaggianesi, Turdo et al. 2017) (DeNardo, Barreto et al. 2009).

However several studies have described a redundancy of type 2 cytokines in different pathological conditions, such as worm clearance, as well as their coordinate expression and function (McKenzie, Fallon et al. 1999; LaPorte, Juo et al. 2008) In type 2 mediated inflammation, the transcription factors GATA3 and STAT6 promote Th2-locus transcription and the coordinate expression of IL-4, IL-5 and IL-13 cytokines (Lee, Spilianakis et al. 2005). Moreover these cytokines share common receptor subunits, thus activating common intracellular pathways (LaPorte, Juo et al. 2008) (Zurawski, Vega et al. 1993).

3.2 Experimental design

Considering the overexpression of all type 2 cytokines in solid tumors and their overlapping effects on immune cells, stroma cells and cancer cells, due to the complex interactions with shared and ubiquitous type 2 cytokine receptors, we decided to investigate the overall influence of type 2 responses on tumor progression taking advantage of a BALB/c IL-4/5/9/13 −/− quadruple knock-out model. The KO of type 2 cytokines (T2KO) has been developed in A.N.J McKenzie Lab and has been previously widely characterised to investigate complementary and redundant roles of type 2 cytokines in different type 2 mediated diseases: chronic allergic airway inflammation, (Nath, Leung et al. 2007) and in parasite mediated granulomas, both gastrointestinal and pulmonary helminthic infections (Fallon, Jolin et al. 2002). Importantly the lack of type 2 cytokines did not affect the immune cell compartment in lymphoid organs under basal physiological conditions (Fallon, Jolin et al. 2002)). For our investigation,
we adopted a primary mammary carcinoma transplantable model: 4T1 breast cancer cells injected orthotopically into fat pad of female mice. Three main reasons directed our model choice:

I) This cell line reflects the immunological features of breast carcinoma. Indeed immune cell tumor infiltration evolves with tumor progression. As previously described the tumor immune compartment presents both innate and adaptive cells, with a predominance of myeloid cells, described as CD11b+ population that constitutes the 86% of CD45+ infiltrating leukocytes. On the other side, lymphoid cells (CD3+ T cells and CD19+ B cells) together constitute around 5% of CD45+ infiltrating leukocytes (DuPre, Redelman et al. 2007). Importantly among the innate immune compartment, it is possible to identify M1-like and M2-like activated tumor-associated macrophages, which both progressively increase during 4T1 tumor progression (Movahedi, Laoui et al. 2010).

II) Among injectable solid tumor models, the 4T1 breast carcinoma is highly immunogenic, with slow growth rate, rare infiltrating T cells and high expression of immunosuppressive molecules, that could be reverted in response to immunotherapy (Lechner, Karimi et al. 2013). 4T1 cells show adequate levels of MHC class I, which can be increased through immunotherapy regimens, making this breast model suitable to test T cell-based immunotherapy protocols as well as to define markers of immunogenicity and predictive immune profiles clinically relevant (Pulaski and Ostrand-Rosenberg 2001); (Lechner, Karimi et al. 2013). Thus the presence of the two distinct type 1 and type 2 polarised innate immune responses and the capability to elicit T cell mediated tumor responses make 4T1 model suitable for our study.

III) Finally breast cancer progression is characterised by a major impairment of extracellular matrix remodelling. With age, breast tissue undergoes several changes through which mammary gland develops, expands or involves, depending on age, pregnancy and lactation. Morphogenesis of mammary gland branching depends on activation of mammary stroma cells, and in particular fibroblasts, adipocytes, pre-adipocytes and endothelial cells. The homeostatic remodelling of mammary stroma allows the formation and invasion of ducts into the fat pad and epithelium expansion. Immune mediators, such as TGFβ, and epithelial derived type 2 cytokines IL-4 and IL13 finely regulate these processes (Siegel and Massague 2003) (Khaled, Read et al.
In breast cancer progression, the disruption of physiologic stromal homeostasis is characterised by collagen remodelling, crosslinking enhancement and stiffener of ECM, following processes similar to those occurring in fibrotic pathologic conditions. Indeed in advanced stages solid tumor collagen cross-linking is mainly mediated by lysyl oxidase (LOX) activity (Levental, Yu et al. 2009); while overexpression of different types of matrix metalloproteinases have been shown to favour matrix degradation and cancer cell invasion (Hasebe, Sasaki et al. 2002); (Chakraborti, Mandal et al. 2003) (Butcher, Alliston et al. 2009). In orthotopic 4T1 mammary carcinoma, previous studies highlighted the presence and the heterogeneity of cancer associated fibroblasts (Sugimoto, Mundel et al. 2006). Moreover in this tumor model it has been previously shown that stroma remodelling and collagen deposition are enhanced (Han, Burke et al. 2008).

In summary 4T1 breast carcinoma has been described has a high immunogenic tumor, recruiting T cells and a fibros-prone tumor. Since our aim is understating the roles of type 2 cytokines in tumor growth and the possible involvement of Th2 cells in tumor associated remodelling in vivo (considering the role of type 2 cytokines in fibrotic processes of several diseases), 4T1 breast carcinoma model perfectly match a fibrotic prone carcinoma and T cell infiltrating tumor.

3.3 The Lack of Type2 Responses Reduces Tumor Growth

In order to investigate the roles of type 2 responses on primary epithelial tumour growth we injected 4T1 breast cancer cells into the fat pat in BALB/c wild type mice and IL-4/5/9/13/-/- “quadruple” KO mice, deficient for the type 2 cytokines. The lack of type 2 responses in IL-4/5/9/13/-/- KO inhibited tumour growth starting from 13 days post tumour injection (p<0.01, 2-way ANOVA) (Figure 1A).
**Figure 1A. The lack of type 2 responses inhibits tumor growth in transplantable breast carcinoma.** 4T1 breast cancer growth was monitored in the presence (BALB/c recipients) or absence of type 2 cytokines (in IL-4/5/9/13−/− recipients, T2KO). Each dot represents mean volume ± SEM; two independent experiments are shown with 9-10 mice per group (BALB/c recipients n=19; IL-4/5/9/13−/− recipients n=21). Statistical analysis: 2 way analysis of variance (ANOVA) with Bonferroni post-test: *** P value<0.001, **** P value<0.0001.

Since the 4T1 breast carcinoma cell line derives from a spontaneously arising BALB/c mammary tumour and is not deficient for IL-4/5/9/13 cytokines (Pulaski and Ostrand-Rosenberg 2001), any effects on tumor growth are due to the host immune system.
Figure 1B. The inhibition of type 2 responses favours immunosurveillance in transplantable breast cancers. Gene expression profile of mediators of inflammation and stroma remodelling within tumors in WT versus T2KO recipients (collected at day 21 post tumor injection) by TaqMan real-time qPCR. In red the genes expressed at significant levels. Heat map reflects protective immune responses in T2KO; color-coded based on expression levels relative to average (blue=down-regulated, orange = upregulated).

To gain deeper insights into tumor protective immune responses occurring in T2KO mice, we assessed the presence of soluble immune mediators by quantitative PCR.
(Figure 1B and C). We found that genes associated with general immune activation were up-regulated albeit not significantly in established tumors grown in T2KO (Figure 1B). The significantly increased expression of CD3 and IL12b genes suggested an enhancement of type 1 immune responses with anti-tumoral effects occurring in T2KO (Figure 1C).

**Figure 1C. The inhibition of type 2 responses favours immunosurveillance in transplantable breast cancers.** aSMA, CD3e and IL12b genes were expressed at significant levels in tumors grown in T2KO mice compared with those grown in WT mice; Mann Whitney test: *P value<0.05.

Chemokine genes and ECM remodelling genes (such as Coll, CollIII and Lox) had higher expression levels, but not significant (Figure 1B). The significant differential expression of αSMA, marker of cancer associated fibroblast (Luhr, Friedl et al. 2012), in tumors collected from T2KO mice suggested enhanced presence of fibroblasts. Overall, the gene expression results may suggest an enhanced T-cell mediated inflammation and an enhanced tumor fibrosis formation in T2KO recipients. Since we previously demonstrated in subcutaneously injected pancreatic tumor (Garetto, Sardi et al. 2016) that recruited T cells are potentially involved in peritumoral collagen formation, these data may indicate that 4T1 tumors, in the absence of type 2 cytokines, grow less, associated with T cell-mediated inflammation and ECM changes.
To further characterise cancer associated anti-tumoral immune responses, we next analysed by flow cytometry the immune cellular compartment infiltrating 4T1 tumors collected at their maximum volume (300mm² in WT mice; 170mm² in T2KO mice) (Figure 2A; representative flow cytometry gating strategies are shown in methods). Consistently with our above findings, we observed a significant increase of CD3⁺ T lymphocytes. CD8⁺ cytotoxic T cells showed a significant increase in tumors grown in T2KO mice. Among tumor infiltrating myeloid cells, we observed that neutrophils and eosinophils were significantly less abundant within tumors in T2KO mice compared to WT mice. We did not observe statistically significant differences in CD11b⁺ myeloid cell frequencies. Nonetheless tumor infiltrating macrophages showed significant higher expression of MHC II, marker of maturation and polarisation to M1-like phenotype, in T2KO mice. Since there are no differences in dendritic cell frequencies (Zitvogel and Kroemer 2014) and neutrophil frequencies are lower in T2KO tumors (Ponzetta, Carriero et al. 2019), it is likely that among tumor infiltrating cells, M1-like macrophages can source IL12 (Tsung, Dolan et al. 2002); other populations (not investigated here) may participate, such as innate lymphoid cells (Sgadari, Angiolillo et al. 1996; Eisenring, vom Berg et al. 2010)).

Figure 2A. Type 2 cytokine deficiency promotes protective inflammatory responses in transplantable breast carcinoma. (A) Immune infiltrate of 4T1 tumors

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from BALB/c recipient and T2KO recipient assessed by flow cytometry. Two independent experiments are shown as mean volume ± SEM Statistical analysis: 2 way analysis of variance with Bonferroni post-test: ** P value<0.01, *** P value<0.001. (B) Expression of MHC class II on CD45+CD11b+LY6c-MHC II+ tumor infiltrating macrophages determined by flow cytometry. Unpaired t test with Welch’s correction: * P value <0.05. (A, B) Each dot represents a single mouse.
Figure 2C and D. Type 2 cytokine deficiency influences systemic immune responses in transplantable breast carcinoma. (C) Splenocytes of 4T1 tumor bearing mice, BALB/c versus T2KO mice, by flow cytometry at the end of the experiments. (D) Peripheral leukocytes of 4T1 tumor bearing mice, BALB/c versus T2KO mice, by flow cytometry at the end of the experiments. (A, B and D) Two independent experiments are shown as mean volume ± SEM Statistical analysis: 2 way analysis of variance with Bonferroni post-test: ** $P$ value<0.01, *** $P$ value<0.001.

We next assessed leukocyte cellular components in the periphery, peripheral blood and spleen, by flow cytometry (Figure 2C and D).

In accordance with cancer-associated inflammation, we observed a significant increase of T lymphocytes, both CD8$^+$ T lymphocytes and CD4$^+$ T lymphocytes, in the spleen of T2KO tumor bearing mice (Figure 2C).

Likewise, we observed a significant reduction of eosinophil frequency among splenocytes of T2KO recipient. Additionally we observed a significant expansion of dendritic cells (CD45$^+$LY6G$^-$$^-$CD11b$^-$$^-$CD11c$^+$MHCII$^+$ cells); while the frequencies of CD19$^+$ B lymphocytes were significantly reduced in the spleen of T2KO mice (Figure 2D). These data suggested that splenic immune cell populations contributed to immunosurveillance in T2KO mice. Finally we assessed the frequencies of innate cellular components within circulating leukocytes. We observed a significant reduction of circulating neutrophils and the expansion of monocytes in the blood of tumor bearing
T2KO mice, which reflects tumor responses ongoing into tumors. Taking together all these data suggested that host immune responses have a fundamental role in tumor surveillance. Specifically the significant enhanced recruitment of CD8$^+$ cytotoxic T cells, CD4$^+$T cells may slow tumor growth, as was previously demonstrated in other in vivo studies (Shankaran, Ikeda et al. 2001) (Aires, Yoshida et al. 2019); (Malik, Byrne et al. 2017) (Milner, Toma et al. 2017).

3.4 T CELL PRESENCE IS ESSENTIAL TO INHIBIT TUMOR GROWTH IN T2KO

Since T cells do mediated tumor immunosurveillance (Anichini, Tassi et al. 2018) and we previously demonstrated that tumor growth inhibition depends on the ability of T cells to infiltrate into tumor mass (Garetto, Sardi et al. 2016), we next performed a staining for CD3e via immunohistochemistry (Figure 3A).
Figure 3. The lack of type 2 cytokine favours T cell-mediated immunosurveillance in transplantable breast cancers. (A) Representative images for CD3 staining in 4T1 tumors in BALB/c recipient (right) and in IL-4/5/9/13−/− recipients (left) at study endpoints. (B) Density of tumor infiltrating T cells quantified as T cell counts per squared μm in 4T1 transplanted tumors. Unpaired T test: *** P value<0.001. Each dot represents one animal BALB/c (n=9) versus IL-4/5/9/13−/− KO (n=8). (C, D) T cell infiltration correlates with tumor growth in 4T1 tumor in T2KO and WT mice (C), and in 4T1 tumor in T2KO or in WT mice (D). Spearman r= -0.8064; *** P value (two-tailed) <0.001 (WT group Spearman r = 0.6802, * P value; T2KO group Spearman r = 0.4279). (E) preliminary analysis of CD3+ T cell density distribution across T2KO (n=1) and WT (n=1) tumors. Y axis values show T cell density in concentric regions from outer capsule border moving towards the center of the tumor. X axis values represent the distance of the region from the capsule border to the tumor center.

Notably in the lack of type 2 responses we observed a significant increase in CD3+ T cell density within tumors (Figure 3B), which is consistent with our gene expression analysis and flow cytometry analysis. Interestingly, the density of tumor infiltrating T lymphocytes inversely correlated with tumor growth (Figure 3C and D). Further analysis of T cell distribution across the tumor would potentially reveal whether T cells are likely to be found deeper within T2KO tumors compared to WT tumors. Understanding how T cells can infiltrate and move to the center of the tumors in T2KO mice can possibly explain how tumor inflammation can shape stroma remodelling and favour tumor immunosurveillance.
3.5 T CELL DEPLETION ABOLISHES TUMOR IMMUNOSURVEILLANCE AND AFFECTS PERITUMORAL FIBROSIS FORMATION IN MURINE ADVANCED BREAST CANCER

In order to investigate if the dramatic effects on tumor growth were T cell dependent, we assessed 4T1 breast cancer cell growth in WT and in T2KO immunocompetent mice after T lymphocyte depletion with anti-mouse CD3e antibody (Figure 4A).

Figure 4A. Host T cells inhibit tumor growth in transplantable breast carcinoma. (A) Schematic representation of experimental design: WT and T2KO mice were injected with anti-mouse aCD3 250μg/mouse, and 4 days later with 5*10^5 4T1 breast cancer cells. CD8^+ and CD4^+ T cell frequencies were monitored before tumor injection (day -1) and for the following 21 days in the peripheral blood. (B) 4T1 primary tumor were implanted in WT (n=10) and T2KO (n=7) recipients after T cell depletion with anti-mouse aCD3 250μg/mouse (BALB/c treated mice n=3; IL-4/5/9/13 KO treated mice n=6). Data are displayed as mean tumor volume +/- SEM. Statistically significant differences: 2-way ANOVA (applied to the last timepoint): *** P value<0.001, **** P value<0.0001.
Wild type mice where T cells were depleted showed significantly worse tumor progression compared to non-depleted wild type mice, as assessed by primary tumor burden measurements (Figure 4B). Likewise, T cell depletion in IL-4/5/9/13-/- KO mice resulted in significant increased tumor size starting from 15 days after tumor injection (Figure 4B). These data indicated that T lymphocytes are necessary for tumor immunosurveillance both in the presence and in the absence of type 2 immune responses. Nevertheless, the difference in tumor size between WT ant T2KO mice was not abolished, suggesting that other cell populations can partially contribute to tumor immunosurveillance.

**Figure 4B.** Host T cells promote peritumoral capsule formation in transplantable breast carcinoma. Representative images of 4T1 peritumor tissue sections from BALB/c recipient with or without T cell depletion (left) or from T2KO recipient with or without T cell depletion (right) were acquired by 2-photon microscopy. Second harmonic generation signals derived from collagen-rich tissue are shown in white. Scale bars represent 100 μm.

Considering our previous finding that T cell depletion significantly reduced peritumoral collagen deposition in a murine transplantable model of pancreatic cancer, we next investigated i) if the lack of type 2 cytokines impacts collagen deposition and ii) if even in the absence of type 2 responses, T lymphocyte depletion could change tumor fibrotic architecture in advanced breast carcinoma. Thus we analyzed collagen density in
random images acquired in peritumoral area in second-harmonic generation (SHG) microscopy in combination with two photon-excited fluorescence (2-P). Indeed SHG microscopy consists in a nonlinear optical technique with high sensitivity and specificity for collagen detection, in combination with 2-P it’s possible to assess unstained collagen deposition and localisation in harvested tumors (Figure 4B).

**PERITUMORAL COLLAGEN**

**Figure 4C.** Host T cells inhibit tumor growth and promote peritumoral capsule formation. (C) Density analysis of peritumoral collagen deposition within 4T1 tumors in T cell depleted WT and T2KO recipient mice, after 21 days from tumor implantation. Collagen density as mean of percentage of collagen area per total tissue squared μm (mean collagen frequencies have been calculated from 2-5 acquired fields). Each dot represents one animal. Statistical analysis: unpaired T test: * P value<0.05.

Surprisingly, despite the involvement of type 2 cytokines have been described in several fibrotic processes (summarised in (Gieseck, Wilson et al. 2018)), we did not observe any difference in peritumoral collagen density comparing WT versus T2KO tumor bearing mice. However consistently with our previous data, T cell depletion strongly impaired collagen capsule formation in WT tumors (Figure 4C). Similarly, the
depletion of T cells appeared to have marginal effects on peritumoral collagen formation in T2KO ($P$ value = 0.056).

Taking together all these findings suggests that T cells are fundamental for controlling tumor growth and for promoting peritumoral capsule formation in advanced breast carcinoma. As we could expect, T cell depletion abolished tumor immunosurveillance even in mice deficient of type 2 responses. The most striking result was the presence of peritumoral collagen deposition in tumor bearing mice that lacks type 2 responses. Indeed, despite the involvement of type 2 cytokines in fibrotic processes, we did not observe any difference in peritumoral collagen density comparing WT versus T2KO tumor bearing mice.

3.6 THE LACK OF TYPE 2 RESPONSES DOES NOT AFFECT PERITUMORAL COLLAGEN DEPOSITION WHILE SIGNIFICANTLY ENHANCES INTRATUMORAL FIBROSIS DEPOSITION

Recent second-harmonic generation microscopy studies have revealed enhanced collagen deposition in breast carcinoma (Levental, Yu et al. 2009); (Tilbury and Campagnola 2015), we therefore decided to assess if type 2 cytokine absence could affect intratumor-associated fibrosis in 4T1 breast carcinoma. To test this, we analyzed collagen density in images of the intratumoral area in SHG microscopy.
Figure 5. Type 2 response lack significantly improves intratumoral fibrosis deposition. (A) Representative images from BALB/c recipient (left) or from T2KO recipient (right) of 4T1 intra-tumor tissue sections acquired by 2-photon microscopy. Second harmonic generation signals derived from collagen-rich tissue are shown in white. Scale bar = 100 μm. (B) Density analysis of collagen deposition within 4T1 tumors harvested from WT and T2KO mice at 21 days. Collagen density as mean of percentage of collagen area per total tissue squared μm (mean collagen frequencies have been calculated from 2-4 acquired fields). Each dot represents one animal BALB/c (n=9) versus IL-4/5/9/13 -/- KO (n=8). Statistical analysis: unpaired T test: *** P value<0.0001.

In contrast to the described role of type 2 mediators in the development of pathological fibrosis (Gieseck, Wilson et al. 2018), we observed a significant increase of intratumoral collagen density in T2KO mice compared to WT mice (Figure 5A and B). Surprisingly the lack of type 2 cytokines seemed to promote the deposition of collagen within tumors.

We next performed Masson trichrome staining to confirm the SHG collagen analysis. In trichrome staining, green light acidic dye binds specifically to acidophilic collagen.
Figure 6. Host T cell and type 2 responses enhanced intratumoral fibrosis formation. (A) Representative images of histological Masson’s trichrome stain of 4T1 breast carcinoma tissues harvested from BALB/c (left) or IL-4/5/9/13−/− mice (right), at day 21 after tumor transplantation. Collagen stain detected in green; cytoplasm, muscle fibers and red blood cells stained in pink; nuclei stained in dark brown to black. Peritumoral collagen density (B) and intratumoral collagen density (C) in the presence of type 2 cytokines (BALB/c recipients), in T cell depleted BALB/c, in the absence of type 2 cytokines (in T2KO recipients) and in T cell depleted recipient mice. Each dot represents one animal; BALB/c (n=10), BALB/c with T cell depletion (n=3), T2KO (n=8) and T2KO with T cell depletion (n=6). Mean collagen frequencies have been calculated from 3-4 ROIs). Unpaired t test: *P value<0.05; **P value<0.01.
We observed a significant impairment of peritumoral fibrosis formation both in WT and in T2KO mice depleted of T cells, which confirmed the fundamental role of T lymphocytes in the process of tumor fibrotic capsule formation (Figure 6). Moreover we observed similar effects of T cell depletion on intratumoral fibrosis. T cell depletion significantly impaired intratumoral collagen deposition both in WT and in T2KO mammary tumor bearing mice. In agreement with SHG analysis, we observed enhanced collagen density in tumors grown in T2KO mice versus WT mice.

Taken together, all these findings indicated that the lack of type 2 responses inhibited 4T1 breast carcinoma growth, likely promoting antitumor T lymphocyte recruitment. The inhibition of tumor progression was accompanied by collagen deposition and fiber formation, suggesting that stroma remodeling and tumor fibrosis are compatible with antitumor immune cell infiltration.

3.7 DISCUSSION

During tumor progression both the innate and the adaptive branches of the immune system are involved in the antitumor responses. It is now well accepted that efficient antitumor responses mainly depend on tumor infiltrating T cells with cytotoxic phenotype. It is likely that CD4+ T helper cells influence CD8+ T cell responses against cancer cells through the production of pro-inflammatory cytokines, such as IFNγ, IL-2 and IL-12 (Benigni, Zimmermann et al. 2005) (Antony, Piccirillo et al. 2005). These cytokines preferentially activate cell mediated type 1 immune responses (Nishimura, Iwakabe et al. 1999). On the contrary Th2 cells could polarise immune responses by the production of IL-4, IL-5, IL-9 and IL-13. Th2 mediated immune responses are followed by tumor promoting cytokines and growth factor production and the inhibition of Th1 antitumor responses ((Hung, Hayashi et al. 1998); (Jovanovic, Radosavljevic et al. 2011)).

In this chapter we investigated the role of type 2 responses in the progression of a murine transplantable model of advanced breast carcinoma. For the first time we examined the growth of 4T1 breast carcinoma in the complete lack of type 2 cytokines. Tumor growth of 4T1 cancer cells injected in mice with genetic deletion of IL-4, IL-5, IL-9 and IL13 was significantly slower compared with WT mice. In our study, type 2
response inhibition enhanced antitumor immune inflammation, via expression of type-1 pro-inflammatory cytokine IL-12. Furthermore, after tumor transplantation we observed an increased splenic lymphopoiesis and enhanced T cell recruitment at tumor site. In line with our studies, Jovanovic et al. have demonstrated that genetic deletion of th2-activator axis IL-33/ST2, could impair the type 2 immune responses while enhancing a potent type 1 antitumor response in 4T1 tumor model, which slow down tumor growth (Jovanovic, Radosavljevic et al. 2011). Similarly it has been previously demonstrated that model genetic depletion of IL-9 promote 4T1 tumor rejection, accompanied by an enhanced T cell mediated antitumor response. (Hoelzinger, Dominguez et al. 2014). It should be noted that these models may from redundant effect of other cytokines (in the case of IL-9KO), or Treg mediated effects (in IL-33KO) (Ameri, Moradi Tuchayi et al. 2019).

The lack of type 2 responses promotes the recruitment of pro-inflammatory antitumor responses, characterised by general inflammation with increased frequency of circulating monocytes, splenic dendritic cells and enhanced T cell recruitment and activation. We observed an increase of cytotoxic CD8+ T cells frequency within the tumors, compared to WT mice, assessed by flow cytometry and immunohistochemical analysis. While among innate immune cell, we observed a reduction in frequencies of tumor infiltrating neutrophils, which has been shown to favour tumor progression and immunosuppression in breast carcinoma, both in the 4T1 transplantable model and in the PyMT transgenic model (DuPre, Redelman et al. 2007); (Park, Wysocki et al. 2016); (Spiegel, Brooks et al. 2016); (Casbon, Reynaud et al. 2015) In support of our findings, Bunt et. al., observed a progressive enhancement of innate immune cells and a decreasing infiltration of T cells during tumor progression in 4T1 transplantable model (Bunt, Yang et al. 2007).

We also observed a reduction of the eosinophil fraction in tumors transplanted in T2KO recipients, compared to WT recipients. Even if apparently eosinophils could directly or indirectly promote tumor surveillance by sustaining T cell recruitment and activation, and tumor blood vessel normalisation, in our hands less eosinophilia correlated with tumor immunosurveillance. A possible explanation for our results would be that although less abundant eosinophils infiltrating in a type 1 polarised milieu could
contribute to antitumor effector functions of cytotoxic T cells, similarly to those observed by Carettero et. Al. in a co-transplant of activated eosinophils and T cells, inhibit tumor growth via improved T cell antitumor activity (Carretero, Sektiglu et al. 2015). Nevertheless the role of eosinophils, as well as that of neutrophils, in tumor progression is still largely unclear. (Reichman, Karo-Atar et al. 2016); (Brandau, Dumitru et al. 2013). In summary, therefore, these results provide further support to the thesis that T cells participate to tumor surveillance together with general pro-inflammatory responses (Shankaran, Ikeda et al. 2001) and a decrease in polymorphonucleated cells, which foster antitumor immune responses. This is highly relevant to solid cancers. Our data suggest that in growing solid tumor the blockade of type 2 cytokines would probably improve antitumor immune responses. The significant increased infiltration of CD3$^+$ T cells, and in particular of CD8$^+$ cytotoxic T cells, in the tumor confirmed the pivotal role of T cells in antitumoral immune responses in 4T1 breast carcinoma. Previous studies have shown the ability of autologous T cells to recognise 4T1 cancer cells and maintain antitumor activity after adoptive transfer. ((Filatenkov, Baker et al. 2014) (Cha, Graham et al. 2010)). Corroborating these findings, we performed blockade of T cells, using specific CD3e depleting antibody. We observed significantly increased tumor growth both in WT and in T2KO recipients, confirming tumoricidal role of T lymphocytes. Similarly other studies demonstrated a strong antitumoral role of tumor-antigen specific T lymphocytes, both on primary and on secondary tumors, via specific CD4$^+$ or CD8$^+$ T cell depletion in combination with other therapeutic treatments in breast cancer models, both 4T1 model and MMTV-PyMT tumor models (Huang, Le Marchand et al. 2018) (DeNardo, Brennan et al. 2011) (Kokulus, Capitano et al. 2013). The detectable tumor burden differences in WT T-cell depleted mice compared with T2KO depleted mice could indicate that other cells could actively contribute to antitumor immune responses. Targeting of macrophages by blocking CD115, also known as macrophage colony-stimulating factor (M-CSF) receptor, worsened 4T1 tumor growth in WT mice, but did not abolished the significant difference in tumor size between WT and T2KO depleted mice (data not shown). Suggesting that tumor surveillance in the absence of type 2 responses can partially depend on T cells and partially on innate immune cells. It is therefore likely that M1-like macrophages could support antitumor activities, indeed
we can hypothesise that in T2KO recipients both M1-like macrophages and Th1 cells play a tumoricidal role, while in T cells depleted T2KO we could observe only antitumor contribution of M1-like macrophages. In line with this hypothesis, Hoves et al. have recently demonstrated in different types of murine transplantable cancer (id est in orthotopic E0771 breast carcinoma, in subcutaneous T241 sarcoma and MC38 adenocarcinoma) that treatment with CSF-1R inhibitor in combination with CD40 agonists could induce TAM reprogramming into a pro-inflammatory phenotype. Thus favouring enhancement of IL-1β and IL-12 cytokine expression, as well as CCL2 and CCL5 chemokines, followed by the establishment of a strong T cell-mediated antitumor response. Indeed CD8+ T cell depletion could abolish the antitumoral effects in tumor bearing mice treated with αCSF-1R receptor and αCD40 (Hoves, Ooi et al. 2018). Also other immune subsets have been demonstrated to display major antitumor activities, such as NK cells. Notably it has been demonstrated that in TS2 genetic depleted mice, the depletion of NK cells abrogates the protective effects of faint type 2 immune responses in 4T1 tumors (Jovanovic, Radosavljevic et al. 2011).

Additionally we assessed for the first time the role of type 2 responses in advanced cancer associated fibrogenesis. We previously demonstrated the association of T cell presence with peritumoral cancer fibrosis (Garetto, Sardi et al. 2016). However the role of Th2 cell in fibrogenesis has yet to be formally demonstrated in tumor progression. Contrary to expectation, in tumors harvested from mice lacking type 2 cytokines versus WT mice we observed similar peritumoral fibrosis; while we found a significant increase of intratumoral collagen deposition compared to WT mice. In contrast, in physiologic tissue remodelling as well as in wound healing and in other fibrotic diseases, IL-33 / ST2L receptor axis trigger the activation of type 2 immune responses, in particular IL-13 and IL-5 production, thus activating stroma remodelling and repairing activities. Tumors have been historically considered as wound that do not heal characterised by persistence of chronic inflammation mainly type 2 polarised (de Kleer, Kool et al. 2016) (Rankin, Mumm et al. 2010) (Kotsiou, Gourgoulianis et al. 2018); (Dvorak 1986) (De Monte, Reni et al. 2011). In our model, the enhancement of tumor associated-fibrosis in the lack of type 2 cytokines could be driven by the expression of TGFβ. Indeed, (in experiments performed by M Sc Cremonesi after the end of my thesis, in our Lab) among genes of cytokine inflammation-related, we observed a up-
regulation of Tgfβ signalling at transcription levels in T2KO tumor bearing mice (Figure 1A), compared to WT. In this regard, it has been shown that activated fibroblasts could produce and activate TGFβ via pro-TGFβ interactions with αvβ6 and other integrins. TGF-β and αvβ6 integrin act in a common pathway to suppress pancreatic cancer progression (Hezel, Deshpande et al. 2012); (Munger, Huang et al. 1999). Moreover integrin-mediated TGFβ activation is critical in several fibrotic diseases, such as in renal, pulmonary and hepatic diseases (Wipff and Hinz 2008); (Reed, Jo et al. 2015); (Henderson, Arnold et al. 2013). However it has never been described before if fibrosis formation induced by integrin-mediated TGFβ activation would be pathogenic or protective (Gieseck, Wilson et al. 2018). Apart from a study of Kojima et al., that demonstrated a tumor-promoting role of myofibroblasts, upon TGFbeta and SDF-1 autocrine signaling activation in a mammary tumor xenograft model, thus in a mouse lacking the immune system (Kojima, Acar et al. 2010).

Even more surprising, increased fibrosis in the lack of type 2 responses is associated with less tumor growth and enhanced T cell tumor infiltration. In contrast to our findings, several reports have shown that increased fibrosis in advanced solid carcinomas correlates with stroma stiffening, immunosuppressive milieu and tumor progression (Levental, Yu et al. 2009); (Goetz, Minguet et al. 2011) (Costa, Kieffer et al. 2018)). Moreover we previously reported that formation of tumor capsule could impede tumor targeting in adoptive T cell transfer strategies (Garetto, Sardi et al. 2016). Likewise, cancer-associated fibroblast targeting strategies seem to have a positive antitumor effects, specially in combination with adoptive T cell transfer therapy ((Rhim, Oberstein et al. 2014); (Provenzano, Inman et al. 2008); (Ozdemir, Pentcheva-Hoang et al. 2014); (Jiang, Hegde et al. 2016)). Nevertheless in our model we observed a significant increase in collagen deposition associated with reduced 4T1 tumor grown in T2KO mice. Thus it can be suggested that in 4T1 breast carcinoma in the absence of type 2 cytokines the establishment of a “good” tumor-associated fibrosis could possibly support immune antitumor responses and favour a reduction of tumor growth. While a compact collagen deposition into the stroma surrounding the tumor can directly impair the ability of T lymphocytes to reach and attack tumor cells. Relaxed collagen network enriched in thin fibers seems to favour T cell infiltration within tumors. T cells can
preferentially follow linear fibers, paralleled to blood vessels. \((Salmon, Franciszkiewicz et al. 2012) (Peranzoni, Rivas-Caicedo et al. 2013)\). Thus we can hypothesize that even if we do not observe a difference in the % of area of collagen deposited around the tumors on the area of tissues examined in our analysis, the network of fibers in peritumoral capsule in T2KO mice could potentially be more permeable to T cell trafficking, compared to WT mice. Collagen fiber orientation could be affected by cancer cell proliferation, which could induce a deformed configuration of fiber networks \((Ristori, Notermans et al. 2018)\). Similarly the presence of intratumoral collagen fibers could direct T cell path and localisation in proximity of cancer cells, favouring their interactions in the immune synapses. Adhesive phenotype is fundamental for activated T cell to perform their tumoricidal activities \((Krivacic and Levine 2003)\). Indeed several studies have described the role of collagen in T cell immune synapse formation, through collagen and T lymphocyte integrin interactions, as well as the capability of T cells to induce collagen formation and deposition \((de Fougerolles, Sprague et al. 2000); (Gunzer, Weishaupt et al. 2004); (Meharra, Schon et al. 2000); (Dustin and de Fougerolles 2001); (Shiina, Kobayashi et al. 2002); (Schnittert, Bansal et al. 2018) (Garetto, Sardi et al. 2016)\). Collagen fibers could store several growth factors and cytokines, such as TGFβ, EGF or FGF, which could released via matrix remodeling processes, favouring inflammatory processes associated with tumor surveillance \((Bonnans, Chou et al. 2014)\). Finally we observed a significant reduction in collagen deposition after T cell depletion, confirming our previous data in a murine model of pancreatic cancer. \((Gawronska-Kozak, Bogacki et al. 2006)\). Thus all our data confirmed that in advanced carcinoma, T cells are partially responsible for collagen deposition, and interestingly we demonstrated for the first time that a tumor-associated fibrosis concomitant with effective antitumor immune response, which could be generated only in the absence of type 2 cytokines. These findings raise intriguing questions regarding clinical immunotherapies in patients with advanced carcinoma and the possibility to improve Th1 mediated immune responses. Further studies will need to be undertaken in order to assess clinical value of “good” fibrosis in an oncological context. A description of collagen fiber orientation, anisotropy \((Alfano, Nebuloni et al. 2016)\), would possibly suggest if T cell infiltration within tumor is influenced by fiber networks.
4. THE ROLE OF TYPE 2 RESPONSES IN MURINE CARCINOGEN-INDUCED FIBROSARCOMA MODEL

4.1 INTRODUCTION: Type 2 responses in 3-MCA-induced tumor model

Over the past decades, the involvement of inflammation as a co-factor in tumorigenesis has been widely described (Balkwill and Mantovani 2001). Chronic inflammation has been associated with chemical carcinogens, such as asbestos-derived inflammation which can induce lung carcinoma, and with persistent pathogen infections, such as chronic gastritis due to Helicobacter pylori that can predispose to cancer (Yoshida, Kato et al. 2014); (Hiraku, Kawanishi et al. 2010). We decided to assess the role of type 2 cytokines in carcinogen-induced tumorigenesis and tumor growth. We chose 3-methylcholanthrene (3-MCA)-induced sarcoma, that has been widely characterised in the three phases of tumor evolution and immunoediting: elimination, equilibrium and escape (Schreiber and Podack 2009). Among polycyclic aromatic hydrocarbons, 3-methylcholanthrene is a mesomethylbenz[a]anthracene derivative, capable of undergoing methylation through a dimethylene bridge that can react with nucleic acids and generate passenger mutations, thus promoting DNA damage and cell transformation, leading to carcinogenesis (Lehner A. F. 5 June 2017). A single subcutaneous injection of 3-MCA dissolved in oil in immunocompetent mice can cause an early tissue reaction at the site of injection, followed by tumor development within 3-5 months (Qin, Kim et al. 2002) (Krelin, Voronov et al. 2007). Early reaction includes local inflammation, immune cell recruitment and granuloma formation, which is mainly composed by innate immune cells. Furthermore, 3MCA-induced tissue damage is characterised by intense stromal responses, mediated by activated fibroblasts, and enhanced angiogenesis (Swann, Vesely et al. 2008) (Qin, Kim et al. 2002); (Bonavita, Gentile et al. 2015); (Krelin, Voronov et al. 2007). Subsequently, innate and adaptive branches of the immune response contribute to establishing chronic inflammation (Prehn and Main 1957). Of interest for our study, during tumor progression tumor-specific T cell responses are fundamental in the tumor equilibrium phases and in host-derived immunoserveillance (Shankaran, Ikeda et al. 2001); (Smyth, Crowe et al. 2002) (Swann, Hayakawa et al. 2007) (Swann, Vesely et al. 2008); (Teng, Swann et al. 2010);
T cell depletion, by anti-CD4 and anti-CD8 antibodies, and the blocking of IFNγ may promote rapid tumor growth after 3-MCA injections (Koebel, Vermi et al. 2007). Several other inflammatory molecules, such as cytokines (IL1b, IL12, IFNs, perforin etc) and cytotoxic immune cells (T cells, NK, NKT and γδT cells) have been demonstrated to be essential for 3-MCA fibrosarcoma protection and control (Swann, Hayakawa et al. 2007), (Swann, Vesely et al. 2008). Thus 3-MCA carcinogen-driven tumor model allowed us to study tumor protective immune-mediated dynamics.

Moreover it has been shown that established 3MCA-induced fibrosarcomas are heterogeneously collagen enriched, featured by the presence of type I, type III and type V collagens (Tryniszewska and Bankowski 1991); (Sobolewski and Bankowski 1984); (Asokan, Puvanakrishnan et al. 1993). So this model would provide us new insights about type 2 cytokine involvement in tumor collagen deposition.

4.2 THE LACK OF TYPE 2 RESPONSES REDUCES TUMOR PROTECTING FIBROSIS

To assess the role of type 2 cytokines in carcinogenesis, wild type male BALB/c mice and T2KO mice were subcutaneously injected with 200μg of the chemical carcinogen 3-MCA in corn oil. Three weeks later, the skin tissues that included site of 3-MCA/oil injections were harvested and fixed sections were analysed by 2-photon microscopy, enabling the visualisation of 3-methylcolanthrene crystal autofluorescent signals and collagen SHG signals. As fertile female mice are in part protected from 3MCA induced carcinogenesis, possibly because of hormones ((Hilf, Goldenberg et al. 1971; Trutin-Ostovic, Golubic et al. 1986) and estrogen-induced T cell-mediated immunosuppression, we performed injections in male mice (Pollow, Uhlorn et al. 2019).Since previous studies demonstrated that early foreign body reactions could protect from cancer development through carcinogen encapsulation, thus we analysed carcinogen entrapment into newly formed collagen networks (Qin, Kim et al. 2002).
Figure 7. Host Th2-immune responses mediate prompted 3-MCA encapsulation.

(A) Representative images of 3-MCA induced pre-tumor tissue damage in BALB/c (left) and IL-4/5/9/13 $^{−/−}$ mice (right), day 21 after carcinogen injection. Tissue slides were acquired by 2-photon microscopy. SHG signals from collagen-rich tissue is shown in white, autofluorescent signal from polycyclic aromatic 3-MCA particles is shown in green. (B) Increased dispersion of 3-MCA particles in IL-4/5/9/13 $^{−/−}$ (T2KO recipients), compared to BALB/c recipients, at 21 days post 3-carcinogen injections. (C) Significant density reduction of the collagen surrounding 3MCA particles in the absence of type 2 cytokines versus BALB/c recipients. B, C: each dot represents one analysed image (2 images of each tissue slides have been acquired for each animal; in BALB/c recipients (n=10 slides collected from five mice) versus (n=12 collected from samples T2KO recipients). Statistical analysis: Mann Whitney = *$P$ value $< 0.05$; ***$P$ value $< 0.0005$.

We observed a significant increase of particle retention in WT mice compared to T2KO recipients, thus the absence of type 2 cytokines favours particle spreading (Figure 7A.
and B). Interestingly, we found that collagen deposition at the site of 3-MCA injection was significantly higher in WT mice, compared to T2KO mice. These findings suggested an involvement of type 2 cytokines in protective foreign body reactions against the injected carcinogens.

4.3 T CELL PRESENCE CORRELATES WITH CARCINOGEN ENCAPSULATION

Considering that 3-MCA usually induces leukocyte recruitment at injection site since the early time points (7 days), (Bonavita, Gentile et al. 2015), and that T cells have been thought to be essential in equilibrium phase (Koebel, Vermi et al. 2007), skin section that contain injected MCA/oil were stained with CD3e antibody and recruited T cells were analysed (Figure 8A).

Figure 8. 3-MCA enhanced host T cell recruitment. (A) Representative immunohistochemistry images for CD3e staining of BALB/c (left) and IL-4/5/9/13 −/− mice (right), at site of methylcolanthrene and oil injection site *, at 21 days after 3-MCA injections. (B) CD3+ T cells quantification as cell counts per squared micrometre in pre-tumor tissues at 21 days after carcinogen injections; Mann Whitney test, BALB/c recipients (n=5) versus IL-4/5/9/13 −/− recipients (n=6) *P value< 0.05.
We observed significant enhancement of T cell recruitment in WT mice injected with 3-MCA, in line with our previous data showing T cell presence correlated with collagen deposition (Figure 8B).

4.4 THE LACK OF TYPE 2 RESPONSES FAVOURS FIBROSARCOMA CARCINOGENSES

To ask whether the difference in foreign body responses and carcinogen encapsulation would affect tumor incidence and tumor progression, we next assessed tumor growth in 3-MCA injected WT and T2KO male mice for 145 days. We observed significantly higher incidence and tumor size in the absence of type 2 responses (Figure 9A and B). For instance, 23% of T2KO mice (3/13) versus only 5% wild type mice (1/19) developed fibrosarcoma at week 9 (Figure 9A). While at day 145, 92% (12/13) T2KO versus 78% (15/19) WT mice developed fibrosarcoma.

The decreased tumor development in wild type mice suggested that type 2 cytokine mediated immune responses are essential to improve foreign body responses that protect from carcinogenesis, as well as to impair fibrosarcoma progression.

![Figure 9](image.png)

**Figure 9. Type 2 cytokines protects against carcinogenesis.** 3-MCA induced sarcoma growth was monitored in the presence (BALB/c recipients) or in the absence of type 2 cytokines (IL-4/5/9/13/– recipients); two independent experiments are shown. (A) Tumor size is shown as tumor volume in BALB/c recipients (n=14) versus IL-
4/5/9/13 */- recipients (n=11). Each dot represents mean volume ± SEM; 2-way ANOVA: *P value< 0.05; **P value< 0.01. Only mice with tumors are shown (B). Incidence of 3-MCA induced sarcoma in BALB/c recipients and in IL-4/5/9/13 */- recipients. Statistical analysis: BALB/c recipients (n=23) versus IL-4/5/9/13 */- recipients (n=17), 2-way ANOVA ***P value<0.001.

4.4.1 THE ROLE OF T CELLS IN TUMOR PROTECTION

We next aimed to define the recruitment of adaptive immune cells in 3MCA carcinogen-driven fibrosarcoma, by flow cytometry (Figure 10). In established tumors (collected at maximum volume allowed) the percentage of T cells (CD3+ cells) and in particular CD4+ T cells (CD4+CD3+ cells) out of CD45+ leukocytes were significantly higher in WT mice, compared to T2KO mice. Whereas the frequencies of NK (DX5+ cells), NKT (DX5+CD3+), and B cells (CD19+ cells) were comparable in the two groups. These data confirmed a possible role for T cells in immunosurveillance in 3-MCA induced fibrosarcoma (Koebel, Vermi et al. 2007). Moreover they highlighted a possible detrimental role of chronic type 1 polarized immune responses, which could worsening chemical-induced carcinogenesis; while type 2 immune responses seemed fundamental for immune-mediated tumor surveillance in this context. Finally, considering the innate branch of immune cells, we did not observe significant differences in CD11b+ subsets, even if we could observe an increased in mast cell and macrophages frequencies and reduced eosinophil frequency in T2KO tumors compared to WT tumors (not statistically different from each other). Among them the mast cells can be a source of several citokines, such as TNFα, IL-1β, IL-2, -3, -5, -6 and IL-9 potentially involved in tumor inflammation (Mukai, Tsai et al. 2018).
CD11b+ cells

- Neutrophils
- Mast Cells
- Eosinophils
- DC
- Macrophages
- Monocytes
Figure 10. Tumor adaptive immunity generated in the presence of Type 2 cytokines protects against carcinogenesis. Analysis of leukocyte infiltrating 3-MCA induced sarcoma by flow cytometry at study endpoints (2 cm tumor size). A) Frequencies of tumor infiltrating T cells (CD3\(^+\)) and T cell subsets (CD4\(^+\) T cells and CD8\(^+\) T cells), B cells (CD19\(^+\)), NK cells (NK1.1\(^+\)), NKT cells (NK1.1\(^+\)CD3\(^+\)) and myeloid subsets out of CD45\(^+\) live leukocytes in BALB/c recipients (n=4) or in IL-4/5/9/13 \(^-\) recipients (n=7). Data are plotted as mean ± SEM; Statistical analysis: two-tailed unpaired t test. B) Frequencies of innate CD11b\(^+\) myeloid subsets: neutrophils (LY6G\(^-\)CD11b\(^+\)), mast cells (LY6G\(^-\)CD11b\(^+\)FceR1\(^+\)), eosinophils (LY6G\(^-\)FceR1\(^-\)CD11b\(^-\)SigleF\(^+\)), dendritic cells (LY6G\(^-\)FceR1\(^-\)SigleF\(^-\)CD11c\(^-\)F4/80\(^+\)), monocytes (LY6G\(^-\)FceR1\(^-\)SigleF\(^-\)CD11c\(^-\)CD11b\(^-\)LY6C\(^+\)) and macrophages (LY6G\(^-\)FceR1\(^-\)SigleF\(^-\)CD11c\(^-\)CD11b\(^+\)LY6C\(^-\)F4/80\(^+\)).

4.4.2 THE ABSENCE OF TYPE 2 RESPONSES IMPAIRS INTRATUMORAL FIBROSIS FORMATION

Considering the differences in fibrosis formation at the early time point and that fibrosarcoma is characterised by both enhanced fibroblast recruitment and strong collagen production (Qin, Kim et al. 2002); (Zhang, Chen et al. 2013), we next assessed peritumoral capsule formation (Figure 11A) and intratumoral collagen deposition in established tumors, by 2-photon microscopy (Figure 11C).
Figure 11. Enhanced intratumoral fibrosis in the presence of Type 2 cytokines. (A) Representative 2-P microscopy images of peritumoral collagen deposition of harvested sarcomas grown in BALB/c (upper) versus T2KO (lower) mice, at study endpoints. (C) Representative 2-P microscopy of intratumoral collagen deposition of harvested sarcomas grown in BALB/c (upper) versus T2KO (lower) mice. (B) Peritumoral and (D) intra-tumoral collagen density analysis in WT (n=5) and in T2KO (n=7) mice. Study endpoints: 2cm or 145 days after 3-MCA injections. Two-tailed unpaired t test *P value< 0.05.

We did not find any difference in the collagen density of peritumoral capsule in WT versus T2KO fibrosarcoma. However, the lack of type 2 cytokines strongly impaired
the intra-tumoral collagen deposition. To further assess changes in collagen deposition we performed Masson trichrome staining on tissue slides (Figure 12A). We confirmed the reduced intratumoral collagen density in the lack of type 2 cytokines (Figure 12C), whilst we did not observe any difference in peritumoral collagen quantification (Figure 12B). Thus reduced intratumoral collagen formation persisted in T2KO mice, but not in WT, until advanced tumor stages.
Figure 12. Peritumoral and intratumoral tumor fibrosis in the presence of Type 2 cytokines. (A) Masson trichrome staining of harvested sarcoma sections (study endpoints: 2cm or 145 days after 3-MCA injections). Representative images are from BALB/c recipient mice (upper) versus T2KO recipient mice (lower). Collagen stains in green. (B) Peritumoral and (C) intra-tumoral collagen density analysis in WT (n=5) and in T2KO (n=6) mice. Two-tailed unpaired t test **P value< 0.01

4.4.3 GROWTH OF 3-MCA FIBROSARCOMA DERIVED TUMOR CELL LINES IN THE ABSENCE OF TYPE 2 RESPONSES

In order to assess if the decreased tumors in MCA carcinogen tumor growth was associated with altered tumorigenicity and immunogenicity due to immune selection, we obtained 4 different tumor clones from WT 3-MCA–induced fibrosarcomas grown in immunocompetent mice, and we subcutaneously injected them in WT or T2KO recipients. Isolated clone tumor transplantation allowed us to assess the effects of antitumor immunity on pre-existing tumor cells, bypassing the phases of carcinogenesis. The WT 3-MCA isolated clones grew less in T2KO recipients compared to WT recipients (Figure 13A). This was a remarkable outcome, corroborating the results of 4T1 tumor experiments. The absence of type 2 cytokines had the opposite effect on tumor growth in carcinogen-driven fibrosarcoma compared
to transplantable tumor model with WT isolated MCA clones, meaning that the type 2-mediated protection affects carcinogenesis but not tumor growth and persistence.

Figure 13A. The lack of type 2 cytokines slows tumor growth of 3-MCA sarcoma cell lines derived from WT mice. Four different clones of sarcoma cell lines were derived from WT mice and subcutaneously injected in the presence (BALB/c n=21) or absence of type 2 cytokines (IL-4/5/9/13 $^-$ n=19) recipients. (A) Cancer growth was monitored (one experiment exemplary of two independent experiments). Each dot represents mean volume ± SEM; statistical analysis: 2 way analysis of variance (ANOVA) with Bonferroni post-test; ** $P$ value<0.01.

We assessed the presence of inflammatory molecules and immune cells involved in tumor immune responses. The gene expression of T cell recruiting chemokine CXCL9, and pro-inflammatory mediators TNF$\alpha$ and IFN$\gamma$ were significantly higher in the tumors grown in T2KO hosts, compared to WT mice (Figure 13B).
Figure 13B The lack of type 2 cytokines promote protective inflammation reducing tumor growth of 3-MCA sarcoma cell lines derived from WT mice. Four different clones of sarcoma cell lines were derived from WT mice and subcutaneously injected in WT and T2KO mice. Three weeks later tumors were harvested and gene expression of immune mediators was assessed. Cxcl9, Tnfa and Ifnγ were significantly increased in tumors from T2KO recipients, compared to WT recipients. qPCR analysis of selected genes from whole cancer tissues of WT (n=6) and T2KO (n=5) recipients normalised to housekeeping gene; each dot represents one animal; Mann Whitney test: *P value<0.05.

Similarly, via flow cytometry analysis, we observed significantly higher recruitment of T cells (CD3+), among CD45+ tumor infiltrating leukocytes in T2KO hosts compared to WT (Figure 13C). The enhanced levels of cytotoxic CD8+ T cells was significant in T2KO mice compared to WT mice. We also found a significant reduction in eosinophil frequencies in T2KO tumors compared to WT.
T2KO mice showed type 1 polarized responses most likely leading to enhanced continuous activity in an analogous manner to the 4T1 results.

**Figure 13C. Tumor infiltrating leukocytes in 3-MCA transplanted tumors.** Immune infiltrate of 3MCA transplanted tumors from BALB/c donor into WT recipient and T2KO recipient assessed by flow cytometry. Graph frequency of neutrophils (CD11b+LY6G+), eosinophils (CD11b+LY6C-MHCCI- differentiated by their side scatter), dendritic cells (CD11c+MHCCI+), monocytes (CD11b+LY6C+), M1-like macrophages (CD11b+LY6ClowMHCCI+), M2-like macrophages (CD11b+LY6ClowMHCCI-), T cells (CD3+), T cell subsets (CD4+ T cells and CD8+ T cells), B cells (CD19+), NK cells (NK1.1+) and NKT cells (NK1.1+CD3+) out of CD45+ live leukocytes in BALB/c recipients (n=5) or in IL-4/5/9/13-/ recipients (n=6). Graphs are exemplary out of two independent experiments; data are plotted as mean ± SEM; Statistical analysis: two-tailed unpaired t test.
In 2002 it was postulated for the first time that carcinogens could induce a foreign body reaction that activates tissue repair processes ending in chemical carcinogen encapsulation. This would protect from carcinogenesis specifically induced by chemical carcinogens (e.g. 3-methylcholanthrene, 7,12-dimethylbenz[a]anthracene or diethylnitrosamine) though not by spontaneous tumors (Qin, Kim et al. 2002; Zhang, Chen et al. 2013). It is interesting to note that immune responses actively participate and influence this early carcinogen-induced reactions, as it has been demonstrated in mice lacking fundamental immune mediators, such as proinflammatory cytokines in IFNγ, IL-1α or IL-1β knock out mice (Qin, Kim et al. 2002; Krelin, Voronov et al. 2007). Indeed in the absence of pro-inflammatory IFNγ, the lack of carcinogen encapsulation drives to higher incidence of fibrosarcomas (Qin, Kim et al. 2002). Presumably the IFNγ is necessary for inducing the first steps of a foreign body response, which is known to be subsequently mediated by type 2 responses (Boros and Whitfield 1999). (Wensky, Marcondes et al. 2001); (Ramalingam, Gieseck et al. 2016). Capsule formation has been shown to be essential for tumor protection, in fact collagenase disruption of fibrotic encapsulation or selective ablation of fibroblasts at the site of carcinogen injection favours carcinogenesis of tumors with epithelial morphology (Zhang, Chen et al. 2013).

In agreement with carcinogen encapsulation as tumor protective mechanism, we observed that in mice lacking type 2 responses the impairment of collagen formation led to a higher carcinogen dispersion. This led to a significantly higher tumor incidence and higher tumor growth in mice lacking type 2 responses compared to WT in late time points (20 weeks after carcinogen injection). At initial phases of carcinogenesis type 2 responses seem to be involved in fibrosis deposition and consequently in 3-MCA encapsulation, and thus in the protection from tumors in WT mice. Further investigation would assess if encapsulation effectively protects host tissue from cell transformation, and initiation and promotion of tumorigenesis.
Nevertheless, pioneering studies showed that tumor protection required encapsulation. IL-1α and IL-1β deficient mice display 3-MCA encapsulation within a thin layer of collagen and they progressively develop less tumors compared to wild type mice. Protection against carcinogenesis in mice lacking pro-inflammatory IL-1β and in those lacking both IL-1β/IL-1α correlates with a strong reduction in leukocyte recruitment and in granuloma formation surrounding injected 3-MCA. Surprisingly, in mice lacking their cognate receptor, IL-1Ra KO, enhanced tumor growth correlates with a strong recruitment of leukocytes, in particular of neutrophils. Similar to IL-1βKO, the knock out mice lacking MyD88, the activator of pro-inflammatory immune responses mediated by IL-1, TNFα and IL-23, are protected from carcinogen-driven tumors, such as DMBA/TPA induced melanoma and 3-MCA induced fibrosarcoma (Swann et al., PNAS 2008). This means that both immune infiltration and fibrosis formation are crucial in early phases of carcinogenesis. Taking together all these findings suggest that the lack of pro-inflammatory IL-1β or MyD88 mediated immune responses protects from tumor incidence. Similarly, in our hands lack of type 2 response impairs capsule formation and leads to greater carcinogenesis. Nevertheless, the genetic ablation of single pro-inflammatory cytokines, such as in IFNγ−/−, IL-18−/−, IL12p40−/−, is not protective in 3-MCA-induced carcinogenesis (Swann, Hayakawa et al. 2007).

Thus the existing accounts fail to fully resolve the contradiction between pro-inflammatory mediators involved in the establishment of tumor promoting milieu at early stages of carcinogenesis and pro-inflammatory immune mediators activating processes that protect from carcinogenesis. However, our data clearly suggest that type 2 responses are protective in carcinogenesis whilst type 1 responses are detrimental.

Qin et al. described for the first time the protective role of encapsulation and could only speculate that T cells could be the main cellular component involved in foreign body reactions, because they observed only few recruited T cells at day 20 after 3-MCA injection. In our study, we show that enhanced T cell frequency corresponded to a significantly higher collagen mediated encapsulation in WT hosts compared to T2KO.
As tumor growth progresses, in established fibrosarcoma (145 days after 3-MCA injection) the persistence of higher T cell frequencies in smaller wild type tumors compared to T2KO suggests a putative role of T lymphocyte in immunosurveillance. This finding is consistent with previous studies demonstrating that T lymphocytes frequencies, and in particular CTL, NK, NKT and γδ T cell frequencies are higher in 3-MCA induced sarcoma at the equilibrium state compared to progressing tumors (Koebel, Vermi et al. 2007); (Wu, Peng et al. 2013). In line with previous studies, we observed significantly enhanced recruitment of CD8+ T cells in WT tumors compared to T2KO (Wu, Peng et al. 2013). We can thus hypothesise that tumor infiltrating T cells at least partially participate in controlling tumor growth.

Whilst several studies from 90s investigated the role of type 1 pro-inflammatory immune mediators in tumor carcinogenesis, very little is known about the role of type 2 cytokines in this context (Schreiber, Old et al. 2011). In agreement with our observations, IL4-genetic ablation in chemically induced skin carcinogenesis favours melanoma formation and progression, confirming a role of IL-4 cytokine in protection against tumor formation (Crawford, Hayes et al. 2018). In contrast to our findings, both C57BL/6 male and female mice lacking IL4 present tumor protection against low dose (25μg) 3MCA-induced sarcomas. These differences could be partly explained by the type 1 bias of the C57BL/6 strain (Kammertoens, Qin et al. 2012). Likewise, IL-4 does not participate in the protection against carcinogen-driven tumorigenesis upon prophylactic regulatory T cell depletion (Teng, Swann et al. 2010).

It is encouraging to compare our results with those of Simson et al. (2007) that found a significant reduction in 3-MCA induced carcinogenesis in opposite conditions: a transgenic mouse overexpressing IL-5, thus in the presence of enhanced th2-mediated responses, characterised by high level of eosinophils. Consistent with our hypothesis, IL-5 transgenic mice show encapsulation in fibrotic connective tissue of carcinogen and subsequent impairment of tumor growth. Vice versa the absence of eosinophils and probably type 2 cytokines in IL5/CCL11 double knock mice (chemo-attractive cytokine for eosinophils mainly secreted by fibroblasts) correlates with higher tumor incidence after the injection of 3-MCA into the flank (Simson, Ellyard et al. 2007). Corroborating our findings, it has been demonstrated by Demehri et al., that mice lacking Notch signaling with consequent enhancement of th2-mediated responses, induced by thymic
stromal lymphopoietin (TSLP) overexpression, are more resistant to both chemical-induced and spontaneous skin carcinogenesis. Moreover genetic TSLP receptor depletion in Notch deficient mice revert the phenotype, even after irradiation and bone marrow reconstitution from mice lacking adaptive immunity, Rag2−/− mice. Similarly Notch/TSLP receptor knock out mice reconstituted with wild type bone marrow and treated with depleting anti-CD4 loose tumor protection. Thus TSLP signals induce anti-tumor naïve CD4+ T cell differentiation, crucial in the establishment of a type 2 cytokine dominant milieu protecting from tumorigenesis (Demehri, Turkoz et al. 2012). Likewise Di Piazza et al., demonstrated in a TSLP knock out mouse that TSLP mediates tumor protection through T cell anti-tumor activity, while antibody depletion of CD4+ and CD8+ T cells impairs tumor protection in cutaneous cancer associated with Notch genetic ablation. Thus, in their study, the loss of TSLP signaling via TSLP receptor ablation, impairs T cell-mediated protection, favouring the establishment of tumor infiltrating myeloid subsets, in particular CD11b+Gr+ cells (Di Piazza, Nowell et al. 2012). A recent paper by Dalessandri has shown that in the absence of IL-13 mediated type 2 immune responses exposed to carcinogen DMBA are less protected from carcinogenesis compared to wild type animals (Dalessandri, Crawford et al. 2016). Curiously, in 2013 a group from Haifa University described spontaneous protection from carcinogen driven tumorigenesis (3-MCA induced fibrosarcoma and DMBA/TPA induced skin carcinoma) in subterranean rat Spalax. Spalax rats treated with DMBA/TPA developed necrotic wounds that subsequently healed, instead of papillomas progressing into squamous cell carcinoma. 3-MCA treated Spalax rats did not develop fibrosarcoma over 3 years, except for two animals that developed benign fibrotic tumors after 1 year and one animal that developed a malignant sarcoma. In both tumor models they observed massive fibrosis. Normal lung and skin-derived Spalax fibroblasts show potent tumor suppression effects in vitro, compared to normal fibroblasts isolated from lab mice and wild mice (Manov, Hirsh et al. 2013). Inhibition of cancer cell growth in vitro seems to be partially dependent on fibroblast-IFNβ production, which has been shown to directly inhibit cell growth, and necrotic cell death induction (Gorbunova, Hine et al. 2012); (Vitale, de Herder et al. 2006). These effects of fibrosis and fibroblasts on tumor protection reported by Manov are consistent with the enhanced collagen deposition that we observed in the smaller 3-MCA induced
tumors, grown in WT mice. Likewise, historically the improved ability of collagen producing tumor cells to degrade neo-synthetized collagen has been inversely correlated with tumorigenesis and cancer cell anchorage independence (Asokan, Reddy et al. 1992); (Smith, Mahoney et al. 1983). In line with all these findings, our results confirm that less encapsulation and less collagen deposition favour 3-MCA carcinogenesis and tumor growth in the absence of type 2 responses. Since all these groups did not investigate the possible effects of type 2 immune responses in fibrosis and in 3-MCA carcinogenesis protection in Spalax mice, we can only speculate that this type of inflammation is involved in carcinogenesis. It has been demonstrated that these animals seems to host different type 2 associated parasites, such as helminths and larval nematodes (FAIR J.M. 1990); (Nevo and Beiles 1992).

Finally, we investigated the growth of four different tumor clones isolated from wild type 3-MCA fibrosarcoma, subsequently grown in vitro and injected in WT and T2KO mice. MCA transplanted tumors grew more in WT mice compared to T2KO mice. Since wild type 3-MCA cancer cells could express type 2 cytokines and their cognate receptors, we could exclude autocrine factors influence on cancer cell growth. Therefore we could confirm that the tumor progression effects depended on host immune responses. In particular, the significant increase in T cell frequencies in tumors transplanted in T2KO host mice would suggest a fundamental role of cytotoxic T cells in tumor immunosurveillance. Our results confirm previous studies on the role of T cells in transplantable 3-MCA tumor protection (Tsung, Dolan et al. 2002), as well as our data in 4T1 model. Additionally, we confirmed findings that in established transplanted fibrosarcoma the type 1 mediated tumor surveillance could be impaired by the presence of IL-13, inhibitory cytokine (Terabe, Matsui et al. 2003).

In conclusion, considering the results described in chapter 3.2, we can speculate that the absence of type 2 cytokines inhibits different types of tumors: advanced transplantable 4T1 breast carcinoma and subcutaneous MCA derived fibrosarcoma. Moreover since 4T1 tumors were orthotopically injected into mammary fat pad, while 3-MCA derived fibrosarcoma cells were injected in the subcutaneous space, we can conclude that transplantable tumor growth control is not dependent on the type of the tissues of injection.
Thus, type 2 responses are protecting against tumor formation, via fibrotic-encapsulation of chemical carcinogens, while worsening tumor progression at advanced stages of the disease.
5. TYPE 2 CYTOKINE ASSOCIATIONS WITH TUMOR PROTECTION AND CLINICAL OUTCOME

5.1 INTRODUCTION

The experimental genetic deletion of type 2 cytokines could be considered the opposite immune state compared to Th2-mediated diseases, such as allergy or asthma. Retrospective studies in the last decades have investigated a possible relation between skin allergy (atopic dermatitis), allergic rhinitis or asthma and tumor protection, reporting conflicting results. Several factors could influence inconclusive results, such as limited number of patients, self-reported history of allergy and association with potential bias (Huang, Le Marchand et al. 2018); (Gomez-Rubio, Zock et al. 2017); (Gandini, Lowenfels et al. 2005). While recent prospective studies reported inverse associations between allergy and cancer risk for several types of cancers, e.g. brain cancer, glioma, colorectal cancer, melanoma and breast cancer (Cahoon, Inskip et al. 2014); (Wulaningsih, Holmberg et al. 2016); (Jacobs, Gapstur et al. 2013). Epidemiological studies of atopic dermatitis, in particular, have shown that inflammation-related allergic skin seems to be protective from skin carcinoma ((Hwang, Chen et al. 2012); (Van Hemelrijck, Garmo et al. 2010) (Wang and Diepgen 2005)). Th2–associated antibodies as well as type 2 cellular mediators of allergy, such as Th2 cells, type 2 polarised macrophages and dendritic cells, eosinophils, basophils and mast cells have been associated with good prognosis in different type of solid cancers and have been described as tumor protective players in several cancer models (Crawford, Hayes et al. 2018); (Cipolat, Hoste et al. 2014); (Demehri, Turkoz et al. 2012); (Ellyard, Quah et al. 2010); (Platzer, Elpek et al. 2015) (Gatault, Delbeke et al. 2015); (Eisenring, vom Berg et al. 2010). A clinical study reported inverse associations of increased eosinophil frequencies in the peripheral blood, typical of allergic reactions, and colorectal cancer incidence (Prizment, Anderson et al. 2011). In in-vitro studies, eosinophils isolated from allergic patients showed more efficient tumor killing abilities compared to eosinophils purified from healthy donors (Gatault, Delbeke et al. 2015); (Legrand, Driss et al. 2009). In view of these sometimes conflicting results, and as we found a possible protective role of type 2 mediators in carcinogen-induced
tumorigenesis, we asked if in asthmatic patients, the resultant pathophysiologic enhancement of type 2 responses could create a protective milieu preventing carcinogen-induced lung cancer.

5.2 THE ROLE OF TYPE 2 RESPONSES IN LUNG CANCER PROTECTION

To assess whether prior asthma history (considered a type 2 mediated disease) or other type 2 immunity enhancing conditions correlate with reduced smoke-associated lung cancer risk (considered a carcinogen-driven carcinoma), we analysed two different sets of patients from two different data sets: the Norwegian HUNT2 prospective Study data (covering 80000 patients followed up from 1995) and Italian Humanitas digital database (18000 partecipants). Norwegian HUNT2 patients were selected for having pollen allergy, matched for gender, smoking pack-years and age, and examined for lung cancer development subsequently. After exclusions, our analysis consisted of 885 total individuals (male N=540, females N=345). Subjects were monitored for an average period of 15 years. Among total 180 males with pollen allergy, 26 subjects developed cancer (14.4%); while 82 patients reported lung cancer events out of 359 not-allergic males (22.8%). We detected a significant inverse correlation between allergy histories with lung cancer in Norwegian males ((P value = 0.02; Table 5.2.1). On the contrary 23 allergic female patients developed lung cancer among total 102 lung cancer female patients (22.5%); while 46 patients reported lung cancer events out of 243 not-allergic males (18.9%). Thus there was no significant inverse correlation between pollen allergy history with lung cancer in females (P value = 0.46). In order to investigate if female allergic patients were not protected from lung cancer risk due to possible hormone-driven effects dampening inflammation, we examined the frequency of menopausal-age woman in the tested cohort. Physiologic regular fluctuation of hormones and related immunosuppressive Treg cell frequencies are seen in females but not in postmenopausal females (Benedusi, Martini et al. 2015). This could explain variations in immune responses to pathological conditions during carcinogenesis (Hampras, Nesline et al. 2012). 60.99% of women without cancer and the 60.9% of women with lung cancer were in menopause. These frequencies could suggest that immune variability conditions to hormonal fluctuations in pre but not post menopausal women could impact on cancer risk factors of female subjects.
Table 5.2.1 History of pollen allergy prevents lung cancer development in male patients of Norwegian prospective cohorts.

<table>
<thead>
<tr>
<th>Cohort Database</th>
<th>Gender</th>
<th>Pollen Allergy</th>
<th>No Event</th>
<th>Lung Carcinoma Event</th>
<th>Univariate conditional logistic regression test</th>
<th>Menopause (age&gt;55 years) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUNT2 Norwegian Patients</td>
<td>Male</td>
<td>No</td>
<td>309</td>
<td>108</td>
<td>0.034</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>84</td>
<td>24</td>
<td></td>
<td></td>
<td>60.9%</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>No</td>
<td>184</td>
<td>92</td>
<td>0.347</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>37</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


To further validate our findings we performed a similar analysis on patient data recorded in our institute’s digital database. The Humanitas dataset analysis consisted of 5502 total individuals (male N=3191, females N=2311) that have performed IgE level analysis. Men with high IgE levels (threshold validated at 97.5 units) were more significantly likely not to develop lung cancer (P value = 0.036). On the contrary, women with high IgE levels were not protected from lung cancer development (P value = 1; Table 5.2.2).

Table 5.2.2 History of type 2 responses prevents lung cancer development in patients of Italian prospective cohorts.
<table>
<thead>
<tr>
<th>Cohort Database</th>
<th>Gender</th>
<th>IgE High (&gt;97.5 units)</th>
<th>Lung Carcinoma Event</th>
<th>Fisher’s test P value</th>
<th>Menopause (age&gt;55 years) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUMANITAS Italian Patients</td>
<td>Male</td>
<td>No</td>
<td>2155</td>
<td>10</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>1026</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>No</td>
<td>1768</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>537</td>
<td>1</td>
<td>ND</td>
</tr>
</tbody>
</table>

Allergy, a type 2 mediated disease, protects male patients from lung carcinoma formation in Humanitas patient data. Two-sided P value from Fisher’s exact test.

Taking together these epidemiological data suggest that type 2 responses in allergic male patients could potentially protect from carcinogen-induced lung carcinoma.
5.3 DISCUSSION

In the previous chapter we have shown an antitumoral role of type 2 responses in carcinogen-driven carcinogenesis. At early stages of tumor development, type 2 immune responses antagonize tumor growth, rather than favour tumor formation. Our data suggest that type 2 mediators participate in the initial foreign body reaction against carcinogens, potentially impeding tumor formation.

To validate our preclinical data in a clinical setting, we asked if the enhancement of type 2 responses in Th-2 associated immune pathologies could protect from carcinogenesis. Allergic reactions are characterised by strong type 2 immune responses occurring in specific tissues that function as barriers between our body and the outer environment.

Interestingly, it has been recently demonstrated that the disruption of epithelial barrier, the outermost multilayer barrier of human body, could provoke allergic reactions, such as sever asthma and atopic dermatitis. These reactions include: i) epidermal hyper-proliferation to compensate barrier defects, ii) release of type 2 polarised immune activator TSLP, iii) CD4+ T cell recruitment, and iv) increase of IgE levels in peripheral blood (Palmer, Irvine et al. 2006); (Ziegler and Artis 2010); (Cipolat, Hoste et al. 2014). Some of these reactions have been described as protective at early stages of cancer development. Among them, the initial changes in tissue stroma architecture can favour chemical carcinogens encapsulation (Qin, Kim et al. 2002). CD4+ T cells may sustain CD8+ T response against cancer growth (Caserta, Kleczkowska et al. 2010).

We might speculate that subjects with allergies are prone to develope strong type 2 polarised immune responses that may trigger faster and stronger foreign body responses, potentially protective against chemical carcinogens. We observed a type 2 cytokine-dependent fibrotic encapsulation of carcinogen particles. Intriguingly, type 2 activator TSLP and type 2 cytokine IL-13 have been shown to be involved in atopic dermatitis (Oh, Oh et al. 2011) and in the progression of atopic dermatitis to asthma, so called Atopic March, that seems to be driven by enhanced TSLP serum levels, ((Demehri, Morimoto et al. 2009); (Zhu, Oh et al. 2011). Of interest, TSLP is involved in both skin and the lung fibrosis (Shin, Kim et al. 2016); (Datta, Alexander et al. 2013); (Nath, Leung et al. 2007), in particular in patients with asthma or idiopathic fibrosis,
high levels of TSPL have been proposed as drivers of lung fibrotic pathology (Cazzaniga, Nebuloni et al. 2016). All these data would explain a possible link between an unbalanced allergic type 2 immune responses driving fibrosis that, in oncology context, may trigger carcinogen-foreign body responses to carcinogens, via fibrotic capsule formation, potentially protective from carcinogenesis. Indeed, different preclinical studies have recently demonstrated the inverse association between atopic dermatitis and skin cancers. Three different groups demonstrated that the disruption of epithelial barrier could trigger a type 2 mediated atopic dermatitis, which protect from skin cancer development, via T cell recruitment (Demehri, Turkoz et al. 2012); (Di Piazza, Nowell et al. 2012); (Cipolat, Hoste et al. 2014). All these findings support our preliminary clinical analysis, suggesting a protective role of allergic immune responses against carcinogenesis occurring in patients with asthma or skin allergy. This inverse association is in agreement with a very interesting study by the Cunningham group, addressing the possibility of TSLP induction as prevention therapy for cutaneous squamous cell carcinoma. The aim of the study was to test the feasibility of TSLP-enhanced immune reactivity to block actinic keratosis premalignant lesion progressing to cutaneous squamous cell carcinoma, taking advantage of an FDA-approved TSLP enhancer, calcipotriol, that is topically administered in patients with psoriasis (Sato-Deguchi, Imafuku et al. 2012). They found that concomitant administration of calcipotriol (applied as short pulse) and skin carcinogen DMBA-TPA protects mice from cancer development through transitional increased TSLP levels. The genetic ablation of TSLP receptor abolishes the protective effects of the drug. In a clinical immunotherapy treatment, the combinatorial administration of calcipotriol and chemotherapeutic agent 5-fluorouracil in patients with actinic keratosis, favour immune stimulation leading to a significant reduction of premalignant lesions (Cunningham, Tabacchi et al. 2017). The drug could be eliciting CD4+ T cell mediated immune responses at tumor site. Thus, in summary, our findings in a clinical setting, in agreement with several other studies, strongly suggest a role for type 2 responses in protection from carcinogenesis.
6. CONCLUSION AND OUTLOOK

6.1 SUMMARY AND CONCLUSIONS

Malignant progression has been widely associated with type 2 polarisation of tumor immune responses. In an attempt to directly demonstrate in in vivo contexts the protumoral role of type 2 cytokines, we took advantage of quadruple IL-4/-5/-9/-13 KO mice. In the complete absence of type 2 mediators IL-4/-5/-9/-13 we observed a reduction in tumor growth in two different transplantable tumor models, orthotopic breast carcinoma and subcutaneous fibrosarcoma. Our data suggest that in advanced carcinoma, type 2 responses have harmful effects on tumor growth that is not necessarily dependent on tumor type or tissue. The characterization of immune cells involved in effective anti-tumor immune response, revealed that, in the prevalence of type 1 responses, T cell-mediated immunosurveillance has a pivotal role in highly tumorigenic and invasive cancers. This leads to the conclusion that, in advanced carcinoma, type 1 immune responses are essential to favour T cell mediated immunosurveillance.

Further investigation on the influence of type 2 cytokines on tumor architecture and tumor fibrosis revealed that despite the absence of pro-fibrotic IL-4/IL-13 (Kaviratne, Hesse et al. 2004); (Hashimoto, Gon et al. 2001), transplantable tumors showed similar collagen density in wild type recipients and in IL-4/-5/-9/-13 KO recipients. Peritumoral fibrotic formation may impede tumor T cell homing, as previously shown (Garetto, Sardi et al. 2016); (Vignali and Kallikourdis 2017), (Salmon and Donnadieu 2012), whilst surprisingly T2KO recipients had significantly higher T cell infiltration. A possible explanation is that the absence of type 2 cytokines may favour the formation of a highly permeable capsule structure surrounding the tumor. Moreover, higher intratumoral T cells correlated with significantly enhanced collagen deposition within tumors, as we previously observed in other contexts (Garetto, Sardi et al. 2016), (Trutin-Ostovic, Golubic et al. 1986). Intriguingly the enhanced intra-tumor fibrosis association is associated with the ability of T cells to trigger immunosurveillance, highlighting a potential contribution of collagen structures to: (i) direct T cell trafficking, via lymphocyte continuous adhesion to collagen fibers; (ii) sustain immune
response and T cell activation through cytokine enrichment inside reticular fiber networks; (iii) allow long-lasting contacts via stable interactions between antigen presenting cells and T cells, as well as target cells and T cells, inside fiber networks (Dustin and de Fougerolles 2001). Sustaining this hypothesis, it is notable that fiber networks in secondary lymphoid tissues, such as the spleen and lymph nodes, have a major role in the transport of soluble mediators and in cellular trafficking, as well as in establishing antigen encounter in immunological synapses (Sobocinski, Toy et al. 2010).

Thus according to this theory, we may talk, for the first time, about the possibility to establish a “good fibrosis” in tumor contexts. In line with this theory a recent preclinical study by Wolf demonstrates that replacement or co-injection with extracellular matrix scaffolds derived from biological tissues may promote tissue repair and CD4+ T cell recruitment in skin and in breast tumors, inducing type 2 responses through IL-4 producing T cells and M2 like macrophage and eosinophil accumulation, and support both T cell- and NK cell-mediated antitumor immune responses, even in cancer therapy contexts (Wolf, Ganguly et al. 2019).

In a different context, chemically induced carcinogenesis, it has been previously reported that early stroma response and collagen-mediated encapsulation of carcinogens may protect from tumor formation. Considering the significant impact of fibrosis on the phenotype of tumor bearing mice, we thus assessed the involvement of type 2 cytokines at early stages of tumor formation. Surprisingly, in chemical-driven carcinogenesis the absence of type 2 cytokines worsens tumorigenesis, leading to the establishment of bigger tumors, characterised by a reduction of antitumor infiltrating T cells as well as less fibrosis.

Back to 2002, the Blankenstein group demonstrated a fundamental role of enhanced IFNγ responses in promoting carcinogen encapsulation, in a process close to foreign-body reaction (Qin, Kim et al. 2002). In our study, we confirmed that early immune and stromal responses to carcinogens have important implications for the development of cancer. Intriguingly, in our hands, type 2 cytokines promoted collagen encapsulation of chemical particles, which impeded the carcinogen-spread into host tissues, partially impairing tumor formation. Taken together, these findings may suggest that, as in
foreign body responses, collagen-encapsulation of carcinogens may be triggered by initial presence, even if at low levels, of IFNγ followed by enhanced type 2 immune responses (Ramalingam, Gieseck et al. 2016). This mechanism would partially explain enhanced collagen deposition in the transplantable and in the carcinogen-induced tumor models. Hence IFNγ concurrently with pro-fibrotic cytokines would enhance collagen deposition in transplantable model. Similarly, induction of type 2 cytokines, due to initial expression of IFNγ, can favour tumor fibrosis formation in the arcinogen-driven cancer.

Thus we asked if the enhanced levels of type 2 responses of allergic reactions might pre-condition and preserve host tissues from tumor development. To address this in a clinical setting, we asked if asthmatic subjects with enhanced allergy-associated type 2 responses are protected from carcinogen-driven tumors. Corroborating our preclinical data, the analyses of two cohorts revealed that history of allergy or type 2 responses potentially prevented lung cancer development in men, but not in women. The absence of protection in female population, even in the presence of allergy-associated immune responses, may be due to the lack of sufficient sampling of post-menopausal, whose immune responses differ compared to pre-menopausal women. Indeed transcriptome analysis has recently enlightened sex effects on immune cell types, mainly in the expression of genes mediating type 1 immune responses (Schmiedel, Singh et al. 2018). There is also evidence that estrogen influences the immune system in females, favouring anti-inflammatory responses (Sgadari, Angiolillo et al. 1996); (Iannello, Rolla et al. 2018). In conclusion, the data of the clinical cohorts support and validate our preclinical findings.

Taken together all our findings formally demonstrate for the first time the synergistic effects of type 2 mediated immune responses on stromal remodelling in the prevention of chemical-induced carcinogenesis. Consistent with our previous study, we demonstrate in four different tumor models that T cell recruitment is fundamental to block cancer progression through the establishment of effective antitumor immune responses. We confirmed also that tumor-specific T cell responses positively correlate with collagen deposition (as we previously shown in (Garetto, Sardi et al. 2016).
Additionally we show for the first time the profound implications of type 2 responses on extracellular matrix architecture in tumorigenesis and in T cell mediated-tumor surveillance. Our experiments provide evidence that pro-healing environment may inhibit tumor growth. Thus, these findings contribute in several ways to our understanding of the interactions between immune cells and stromal remodelling in carcinogenesis. Moreover our study lays the groundwork for the introduction of new immune based approaches that may differ according to the stage of oncologic disease. Early strengthened of type 2 responses may block tumorigenesis. Similarly fostering of “good” fibrosis and the building up of T cell permeable matrix architecture may improve immune-therapy combatting growing tumors.
6.2 OUTLOOK

Our experiments provide evidence of the dual role of type 2 mediated immune responses in tumor contexts, which may depend on the stage of progression. We confirmed a detrimental role of type 2 cytokines in aggressive transplanted carcinoma at advanced stages of the diseases. Surprisingly, we found that type 2 responses sustain collagen-mediated encapsulation of carcinogens during tumor formation, which slows carcinogenesis. Consistent with our preclinical data, we found that allergic individuals, with a prevalent type 2 inflammation, are protected against the development of lung carcinoma. Our hypothesis could be further validated in different type of chemical-induced tumors.

Since tobacco smokers present augmented risks to develop skin squamous cell carcinoma, because of constant exposure to polyaromatic benz[a]antracene (Leonardi-Bee, Ellison et al. 2012), we could assess if the systemic enhancement of type 2 immune responses would protect allergic smokers from carcinogen-driven melanoma.

On the basis of epidemiological data and our preclinical findings, it would be interesting to better understand the role of associated allergy-inflammation in cancer protection and to dissect anti-tumor functions of allergy associated immune mediators.

To further investigate the role of type 2 immune mediators and the molecular mechanisms occurring in tumor-protective allergy inflammation, as well as their influence on extracellular matrix remodelling, we could assess the incidence and the progression of 3-MCA carcinogen-driven tumors in mice with established allergic airway inflammation. Since ovalbumin-sensitisation followed by allergen exposure improve local levels of type-2 cytokines followed by enhanced airway wall remodelling (Nath, Leung et al. 2007), as well as their circulating levels (Son, Jung et al. 2017), we can ask if airway allergy would enhance systemic type 2 responses that protect also secondary tissues from tumor formation. Allergy mediators, such as mast cells, have been directly associated with stromal remodelling and fibrosis enhancement (Pincha, Hajam et al. 2018). Therefore we could assess if allergy-induced type 2 polarised
immune cells have a role in tumor matrix and stromal remodelling, in early phases of
tumorigenesis and in particular in the encapsulation of chemical carcinogens.
We can also set up also a skin allergy model of atopic dermatitis, to further assess if the
systemic enhancement of type 2 responses, triggered by allergy in different context, can
protect from carcinogen driven sarcoma. Indeed inflammatory responses induced by
asthma and atopic dermatitis are characterised by increased levels of type 2 cytokines
(Dearman, Warbrick et al. 2000); (Belgrave, Granell et al. 2014).

Additionally, our epidemiological data poses the intriguing question about possible
influence of hormones on type 2 mediated protective responses in women. To
specifically understand if anti-inflammatory effect of estrogen impact on allergy
inflammation and tumor protection in women, we could perform allergy induction
experiment on female mice or on female mice after ovariectomy, and assess 3-MCA-
induced carcinogenesis and tumor growth.
In summary, the findings described in this thesis can form the basis of extensive and
novel questions on tumor and the immune responses that promote it or combat it.


Kammertoens, T., Z. Qin, et al. (2012). "B-cells and IL-4 promote methylcholanthrene-induced carcinogenesis but there is no evidence for a role of T/NKT-cells and their effector molecules (Fas-ligand, TNF-alpha, perforin)." Int J Cancer 131(7): 1499-1508.


