CD4+ T cell-induced macrophage cytotoxicity against tumor cells

Doctoral thesis by

Fredrik Hellem Schjesvold

Center for Immune Regulation

Institute of Immunology

University of Oslo

Oslo University Hospital, Rikshospitalet

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List of papers

Paper I

Indirect CD4⁺ T cell-mediated elimination of MHC II^{NEG} tumor cells is spatially restricted and fails to prevent escape of antigen-negative cells. Eur. J. Immunol. 2014. 44: 2625–2637,
Fredrik Hellem Schjesvold*, Anders Aune Tveita*, Olav Sundnes, Ole Audun Werner Haabeth, Guttorm Haraldsen and Bjarne Bogen
* *These authors contributed equally to the paper*

Paper II

Tumors escape CD4⁺ T-cell-mediated immunosurveillance by impairing the ability of infiltrating macrophages to indirectly present tumor antigens. Cancer Res. 2015. 75(16): 3268-78
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Paper III

CSF-1R-inhibition delays growth of myeloma cells in a non-T-cell-dependent manner Submitted Manuscript (PLOS One).
Fredrik Hellem Schjesvold, Ole Audun Werner Haabeth, Bjarne Bogen and Anders Aune Tveita

Selected abbreviations

Arg1 – Arginase-1
ACT – Adoptive cell therapy
APC – Antigen presenting cell
BCR – B cell receptor
BCR/ABL – Breakpoint cluster region/Abelson murine leukemia viral oncogene homolog 1
CAR – Chimeric antigen receptor
CCL – Cysteine-cysteine chemokine ligand
CXCL – Cysteine-X-cysteine chemokine ligand
CD – Clusters of differentiation
CEA – Carcinoembryonic antigen
CLP – Common lymphoid progenitor
CMP – Common myeloid progenitor
CTL cell – Cytotoxic T lymphocyte
CTLA-4 – Cytotoxic T lymphocyte antigen 4B
CSF-1 – Macrophage colony stimulating factor (also known as M-CSF)
CSF-1R – CSF-1 receptor
DAMP – Damage-associated molecular pattern
DC – Dendritic cell
EGF – Epidermal growth factor
ER – Endoplasmatic reticulum
FDA – Food and drug administration
FoxP3 – Forkhead box P3
GM-CSF – Granulocyte-monocyte colony stimulating factor
HIF – Hypoxia inducible factor
HDAC – Histone deacetylase
HSC – Hematopoietic stem cells
Id – Idiotype
IFNγ – Interferon gamma
IgA – Immunoglobulin A
IκB – Inhibitors of NFκB
IL – Interleukin
iNOS – Inducible nitric oxide synthase
iTregs – Induced T regulatory cell
IRF5 – Interferon response factor 5
KLF – Krüppel like factor
M-CSF – Macrophage colony stimulating factor (also known as CSF-1)
MAGE-A3 – Melanoma associated antigen 3
MDSC – Myeloid derived suppressor cell
MGUS – Monoclonal gammopathy of undetermined significance
MHC – Major histocompatibility
NK cell – Natural killer cell
nT_{reg} – Natural T regulatory cell
JAK2 – Janus kinase 2
LPS – Lipopolysaccharide
MCP1 – Monocyte chemotactic protein
MOPC – Mineral induced plasmacytoma
NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells
NO – Nitric oxide
PAMP – Pathogen-associated molecular pattern
PD1 – Programmed cell death protein 1
PDL1 – Programmed cell death ligand 1
PDGF – Platelet derived growth factor
PPAR-γ – Peroxisome proliferator-activated receptor gamma
PSA – Prostata specific antigen
RAG2 – Recombination activating gene 2
RNS – Reactive nitrogen species
SCID – Severe combined immunodeficiency
TAA – Tumor associated antigen
TCR – T cell receptor
Tg – Transgenic
TGF – Transforming growth factor
Th cell – T helper cell
TLR – Toll like receptor
Treg cell – T regulatory cell
TSA – Tumor specific antigen
TIL – Tumor infiltrating lymphocyte
TNFα – Tumor necrosis factor alfa
Tyrp-1 – Tyrosinase-related protein 1
V(D)J – Variable (Diversity) Joining
VEGF – Vascular endothelial growth factor
WT – Wild-type
XBP – X-box binding protein

1. Introduction

With increasing average lifespan, the incidence of cancer is expanding in almost every corner of the world. In the Western world, by 2020 almost one in two persons will be diagnosed with cancer during their lifetime (Maddams, Utley et al. 2012). Detection and treatment of cancer is improving, leading to increased overall survival, but still, with currently available treatment modalities, the majority will still succumb to the disease. For most cancers, the only potentially curative treatment option is surgery. Adjunctive chemotherapy or radiation treatment has improved the cure rate from surgery, but are rarely curative as monotherapy. The last decade has also seen the advent of more targeted therapies, made possible by increased knowledge of the molecular characteristics of particular types of malignancies. For some cancers, this has transformed treatment and life expectancy, but for the majority, surgery still remains the only curative option.

Immunotherapy has been contemplated since the 18th century, when the Duke of Kent injected malignant cell into himself as a cancer prophylactic measure. Just over a century later, in 1891, Coley injected streptococcus pyogenes in miscellaneous cancer patients, observing in some patients tumor regression in response to the systemic inflammation induced by the bacterial inoculum (Coley 1891). In 1909, Paul Ehrlich postulated that the immune system orchestrates a continuous surveillance and eradication of newly formed cancer cells, a theory further developed by Burnet and Thomas in the fifties, commonly referred to as the tumor immunosurveillance hypothesis (Burnet 1957).

Today, the immune system's ability to kill cancer cells is widely accepted, a property that is being exploited in treatment for a number of cancer types. Cancer immunotherapy was awarded the status of "breakthrough of the year 2013" in Science magazine (Couzin-Frankel 2013), and an increasing number of immunotherapeutic strategies are approaching the clinic.

The immune system consists of the innate and the adaptive immune system. Broadly, the adaptive system comprises a multitude of cells with different and specific receptors, able to recognize virtually any molecular structure, either directly (B cells) or in the form of processed peptides presented on major histocompatibility (MHC) molecules (T cells). Since cancer cells often produce proteins not found in normal cells, or express proteins in larger quantities or in different contexts than normal cells, they can be targeted by adaptive immune responses, in cooperation with the innate immune system. Still, for the many patients being diagnosed with cancer, this inherent surveillance is obviously not sufficiently effective, and thus it is important to understand more of what controls tumor inhibition and tumor escape.

The current thesis is focused on the complex interactions and interdependence of the adaptive and innate immune system in the context of immunosurveillance. We explore limitations in the immune response, spatial and phenotypical, leading to tumor escape, and we investigate the tumors dependence on stromal interaction. In the following passages, I will briefly introduce important players in the adaptive and innate immune system, the theory of cancer immunoediting, and the disease model used in our experiments.

1.1 T cells

The immune system is divided in two major parts, with complex interaction; the innate and the adaptive immune system. The adaptive immune system consists of T and B-lymphocytes, which harbor an impressive target recognition receptor diversity. An immune reaction against identified

targets – when successful – will in addition to eliminating the disease, create memory cells that will make the response much stronger upon a second encounter. This is what we know as immunity. When the innate immune system responds to a suspected threat, be it microbes or tissue damage, it responds with inflammation facilitating activation of the adaptive immune system.

T cells develop and are educated in the thymus (hence the designation T cells). They recognize antigen epitopes presented on other cells by their major histocompatibility complex (MHC) molecules. By a coordinated selection process in the thymus, cells that are potently auto-reactive or have insufficient target affinity are deleted, while the rest enter the bloodstream as naïve T cells. Every T cell has T cell receptors (TCRs) reacting to antigens containing molecular motifs with affinity towards the relevant TCR. Generally, TCRs affinity for the combination of MHC molecules and antigen is of moderate strength, and cross reaction is common. When naïve, these cells will circulate between the blood stream and peripheral lymphoid organs until they meet their antigen, presented by MHC-molecules on other host cells. The two major types of T cells, T helper cells and T cytotoxic cells, are defined by the expression of either the co-receptor CD4 (T helper cells; Th) or CD8 (cytotoxic T cells; CTL), binding to respectively MHC class II and MHC class I molecules. MHC class I is an antigenic display framework molecule expressed on virtually all cells, and the major function of $CD8^+$ T cells is to kill cells which display intracellular foreign peptides bound to MHC class I molecules. $CD4^+$ cells on the other hand, are the orchestrators of the adaptive immune response, with multiple important functions. They recognize antigens in the context of an MHC class II molecule. MHC II expression is normally limited to a subset of cells of the innate immune system collectively referred to as professional antigen presenting cells (APCs). The prototypical APCs, residing in lymph nodes where they encounter naïve T cells, are dendritic cells, with macrophages and B cells constituting other important APC subsets.

1.1.1 Initial interaction with antigen presenting cells – priming

When a T cell is appropriately activated (primed) by interaction with MHC molecules, the cell proliferates, differentiates into effector cells, and gives rise to long-lived memory cells providing immunological memory and immunity. This result, however, depends on co-stimulatory signals from the APC to the T cell. This secondary signals (signal 2), are signals that arise upon detection of prototypical tissue-damage or pathogen-associated molecular structures by innate immune cells, signifying the presence of compromised tissue homeostasis (i.e. cellular stress or injury). Without this secondary signal, the interaction with the APC will lead to functional inactivation or clonal deletion of the T cell. Hence, additional signals by APCs offer contextual information that limits adaptive immune cell responses to situations of tissue damage.

Naïve T cells home to the lymph nodes where they have brief encounters with a multitude of APCs, screening them for presentation of target antigens. Many integrins and other adhesion molecules participate in the interaction, stabilizing the association when recognition occurs. The co-receptors CD4 and CD8 strengthen the association with MHC II and MHC I molecules, respectively, underscoring the importance of these molecules, but their binding is not enough for priming to occur. The most extensively characterized signal 2 co-stimulatory molecules are the B7 family molecules (CD80/86). These molecules are expressed when dendritic cells migrate from inflammatory areas to regional lymph nodes, and are only present on cells that stimulate T-cell proliferation. The corresponding cell surface receptor on the T cell is CD28, and their interaction together with the MHC:TCR binding lead to clonal expansion of the naïve T cell. $CD8^+$ T cells need

more co-stimulatory activity to induce the differentiation process. This can be accomplished by the presence of more mature APCs, but often is provided by help from CD4⁺ T cells that either induces more B7 molecules on the dendritic cell through CD40L-CD40 interaction, or provides interleukin 2 (IL-2), which promotes CD8⁺ T cell differentiation and expansion. Activated T cells leave the lymph nodes and re-enter the bloodstream, and are guided to sites of infection by chemokines and newly expressed adhesion molecules. Thus, activated T cells are generated in the lymph nodes and accumulate within sites of injury, where they exert their effector functions in a spatially restricted manner.

The differentiation of T cells, in particular CD4⁺ T cells, is extensively shaped by the microenvironmental factors during priming in the lymph node. Such factors include cytokines, giving rise to different T cell phenotypes in different situations (figure 1). These phenotype-defining influences are sometimes referred to as signal 3. Classically, there was a dichotomy of CD4⁺ T cell phenotype, defined as the Th1 and Th2 subsets. Th1 cells are typically formed in immune responses against bacteria, protozoa and viruses. They are involved in the priming CD8⁺ T cells, activating macrophages, and providing help to B cells for antibody production. Th2 cells are thought to be of importance in defense against extracellular parasites such as helminthes and protozoa, stimulate antibody class switching to IgE in B cells, and are implicated in the pathogenesis of asthma and allergic diseases. With the discovery of additional CD4⁺ T cell subsets the CD4⁺ family has been expanded to include T_{regs} and Th17 cells. The T_{regs} have important functions in suppressing or terminating ongoing adaptive immune responses to avoid unnecessary tissue damage and autoimmunity, and Th17 cells have effector functions against extracellular bacteria and fungi, and have been implicated in several forms of autoimmune diseases.

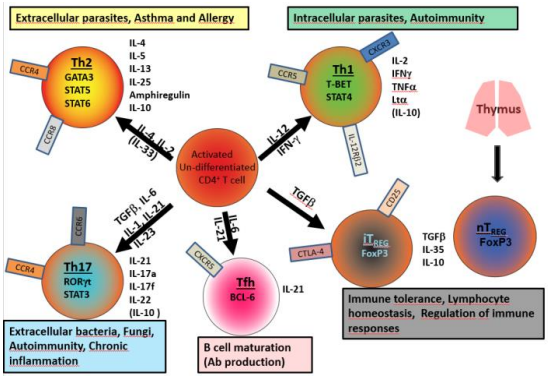

Figure 1. Conventional CD4+ T cells differentiate into phenotypically distinct T helper (Th) subsets depending on cytokine signals

1.2 CD4⁺ T cells in cancer

Since tumors largely contain the same proteins found in normal cells, there was previously controversy regarding the ability of tumor-derived antigens to activate CD4⁺ T cells, as this challenges the concept of immunological self-tolerance. However, the basis of malignant transformation includes extensive mutational and epigenetic changes, resulting in the production of structurally altered proteins containing neoepitopes not expressed in healthy cells. Also, quantitative changes in antigen expression and ectopic expression of antigens is commonly seen during malignant transformation. Several tumor-associated antigens have been identified, with considerable clinical impact in diagnostics (prostate-specific antigen; PSA and carcinoembryonic antigen; CEA), prognosis (p53, JAK2) and treatment (the BCR/ABL fusion protein and BRAF). It is now accepted that tumors can display antigens with the potential of facilitating adaptive immune responses. The term tumor-specific antigen (TSA) signifies antigens that are uniquely expressed by tumor cells, BCR/ABL being

one example. Tumor-associated antigens (TAAs) are normal proteins expressed aberrantly, either quantitatively or in terms of cell type, with PSA and CEA as prominent examples. This latter category includes differentiation antigens, which are normally found only in small subsets of cells during differentiation or are normally expressed only during embryogenesis. We know from animal experiments that the adaptive immune system is capable of keeping transformed cells in a dormant phase for an unknown amount of time by a process referred to as immunosurveillance (Koebel, Vermi et al. 2007). Anecdotal reports suggest that this may also happens in humans (MacKie, Reid et al. 2003)the conclusion being that the adaptive immune system can have anti-tumor effects, at least during malignant transformation.

Strategies for T cell immunotherapy have mostly focused on $CD8^+$ cells, for logical reasons. $CD8^+$ cells have an established role in anticancer immunity, their infiltration in tumors is correlated with better prognosis (Fridman, Pages et al. 2012), and their direct cytolytic effect make them an obvious choice. Several approaches of inducing cytotoxic T cell (CTL) responses have been tested, some of them with promising initial responses(Besser, Shapira-Frommer et al. 2010, Wu, Forget et al. 2012), but long-term outcomes of such studies have been largely disappointing (Klein, Schmidt et al. 2011). By means of immunoevasion, tumors commonly escape $CD8^+$ T cell responses (see later), leaving the CTLs either unable to identify tumor cells or unable to mediate cytotoxicity because of impaired effector functions (Zippelius, Batard et al. 2004). Such adaptation is facilitated by the need for efficient presentation of tumor antigens on MHC class I in the tumor cell itself, which constitutes an "Achilles heel" of such immune responses. Thus, despite initial anti-tumor effects, the T cells are in most cases unable to maintain protective, long-lasting immunity (Appay, Jandus et al. 2006). To induce longer-term anticancer response, $CD4^+$ T cell help to the $CD8^+$ CTLs might be necessary (Ossendorp, Mengede et al. 1998). Some results also indicate that $CT4^+$ T cells alone are more efficient effector cells than $CD8^+$ T cells, albeit the mechanism might be indirect(Perez-Diez, Joncker et al. 2007).

Whereas the direct, cytolytic effect of $CD8^+$ CTLs is fairly easy to understand and quantify, the actions and effects of $CD4^+$ Th1 cells are more complex. As previously described, they secrete interferon-γ (IFNγ), causing macrophage activation and promoting phagocytic activity and tumoricidal effects. $CD4^+$ Th1 cells also secrete IL-2, which is the most important inducer of activation and proliferation of $CD8^+$ T cells. IL-2 promotes the acquisition of a cytolytic phenotype in $CD8^+$ cells (Kim, Imbert et al. 2006). It also promotes $CD8^+$ memory cell development(Williams, Tyznik et al. 2006), facilitating long-lasting responses. $CD4^+$ Th1 cell help is therefore of great importance during the priming phase. To be able to attain such synergistic effects, the $CD4^+$ and the $CD8^+$ T cells have to recognize antigen at the same time on the same dendritic cell (DC) (Bennett, Carbone et al. 1997). $CD4^+$ T cells promote up-regulation of co-stimulatory molecules on the DC surface, release of cytokines (e.g.Interleukin-12; IL-12) from the DC, and themselves release IL-2, which affects nearby $CD8^+$ T cells. Transfecting tumors with MHC class II genes leads to increased anti-tumor immune responses, indicative of an augmenting effect of $CD4^+$ T cells(Ostrand-Rosenberg, Thakur et al. 1990, Ostrand-Rosenberg, Roby et al. 1991). In sum, these events support the differentiation, survival and memory of $CD8^+$ T cells, leading to improved efficiency of tumor-reactive $CD8^+$ T cells(Gao, Khammanivong et al. 2002, Janssen, Lemmens et al. 2003). Alone, $CD8^+$ T cells probably have limited anti-tumor effects (Lee, Wang et al. 1999, Dudley, Wunderlich et al. 2002, Dudley, Wunderlich et al. 2002, Boon, Coulie et al. 2006).

Data on the importance of $CD4^+$ T cells have accumulated (Bogen, Munthe et al. 1995, Hung, Hayashi et al. 1998, Mumberg, Monach et al. 1999, Qin and Blankenstein 2000, Lundin, Hofgaard et al. 2003, Corthay, Skovseth et al. 2005, Perez-Diez, Joncker et al. 2007, Muranski, Boni et al. 2008, Corthay, Lundin et al. 2009, Quezada, Simpson et al. 2010, Xie, Akpinarli et al. 2010, Haabeth, Lorvik et al. 2011), proving the mechanisms behind $CD4^+$ T cell cancer protection to be multifaceted. The T cell phenotype matters. Th1 cells are considered to have an anti-tumor effect, based on results from animal models and clinical studies. In mice, depletion of $CD4^+$ T cells led to increased tumor size (Benigni, Zimmermann et al. 2005), and class II restricted epitope vaccinations has been shown to prevent tumor development and metastasis (Caserta, Alessi et al. 2008). Adoptive transfer with $CD4^+$ T cells from donors immunized with tumor, to mice depleted of other lymphocyte subsets, has been shown to induce tumor-specific immunity (Fujiwara, Fukuzawa et al. 1984, Greenberg, Kern et al. 1985). Importantly, studies of tumor biopsies suggests a correlation between Th1 tumor infiltration and better survival (Fridman, Pages et al. 2012). The results of such correlation analyses are less clear for other $CD4^+$ subsets, but point towards an association with poorer prognosis. Intuitively, this might be expected, given the ability of these subsets to skew the macrophage towards a growth-promoting phenotype (see later). Alternatively, the prevention of an effective Th1 response might in itself explain some of their negative impact.

To establish the mechanisms behind $CD4^+$ T cell tumor protection, several groups have worked with TCR transgenic models. Our group has established murine T cell lines in which the TCRs recognize epitopes within the somatically hypermutated immunoglobulin light chain variable region of a malignantly transformed plasma cell (Lauritzsen, Weiss et al. 1994, Bogen, Munthe et al. 1995). Since the B cell receptor is unique to a particular clone of B cells, these structures constitute natural tumor-specific antigens. Others have used tumors expressing non-compatible minor histocompatibility antigens, xenogeneic antigens or viral antigens (Marzo, Lake et al. 1999, Nishimura, Avichezer et al. 1999, Klein, Trautman et al. 2003, Chamoto, Wakita et al. 2006, Zhou, Drake et al. 2006, Perez-Diez, Joncker et al. 2007, Marabelle, Kohrt et al. 2013). Muranski et al. have utilized the non-mutated differentiation antigen tyrosinase-related protein 1 (Tyrp-1), also expressed in normal melanocytes (Muranski, Boni et al. 2008). Since expression of a self-antigen in healthy cells precludes the generation of reactive T cells, TCR clones were generated by immunization of syngeneic Tyrp-1-deficient mice, where the antigen was considered foreign.

$CD4^+$ T cell recognition of antigens is dependent on display in the context of MHC class II molecules. When interpreting results from experiments addressing $CD4^+$ T cell responses, it is therefore important to consider the MHC class II status of the tumor cell line utilized. B cell lymphomas commonly express high levels of MHC class II (Lauritzsen, Weiss et al. 1994, Nishimura, Avichezer et al. 1999, Lundin, Hofgaard et al. 2003, Lundin, Screpanti et al. 2004), while other tumors have no MHC class II expression (Greenberg, Kern et al. 1985, Lauritzsen and Bogen 1993, Mumberg, Monach et al. 1999, Qin and Blankenstein 2000, Perez-Diez, Joncker et al. 2007). In some tumor cell types, the MHC class II can be induced by exposure to IFNγ (Perez-Diez, Joncker et al. 2007, Muranski, Boni et al. 2008, Quezada, Simpson et al. 2010, Xie, Akpinarli et al. 2010). MHC class II status determines the possibility of direct interactions between $CD4^+$ T cells and the tumor cells; for MHC class II negative tumor cells, the T cell is not capable of recognizing the tumor cell directly (Lauritzsen and Bogen 1993) but is dependent on display on MHC-compatible APCs (see figure 2). Traditionally, $CD4^+$ T cells have been portrayed as accessory cells, helping macrophages, $CD8^+$ T cells and B cells to differentiate and proliferate. *In vitro* observations have long suggested the presence of direct, cytotoxic effects of $CD4^+$

T cells (Fleischer 1984, Tite and Janeway 1984, Bogen, Malissen et al. 1986, Lauritzsen, Weiss et al. 1993, Quezada, Simpson et al. 2010). Later, $CD4^+$ T cells have also been showed to confer efficient elimination of MHC class II-positive tumors in vivo (Lauritzsen, Weiss et al. 1994, Lundin, Hofgaard et al. 2003, Horna, Cuenca et al. 2006, Perez-Diez, Joncker et al. 2007, Muranski, Boni et al. 2008, Quezada, Simpson et al. 2010, Xie, Akpinarli et al. 2010) More recently, such cytotoxic $CD4^+$ T cells have been showed to exist in low numbers in most individuals (Appay, Zaunders et al. 2002). These cells have a differentiated memory phenotype, with preference for peripheral tissue migration, and little potential for proliferation. Presence of such cells containing granules with granzyme and perforin seem to correlate with chronic or strong activation. In addition to its cytotoxic effector functions, mediated by granzyme/perforin granules, these cells are also capable of inducing cytolysis through Fas-dependent mechanisms (Lundin, Screpanti et al. 2004, Brown, Kamperschroer et al. 2009). In the Tyrp1-specific TCR-transgenic model, rejection of melanoma was abrogated in granzyme B or perforin-deficient mice, indicating their importance in direct $CD4^+$ T cell-mediated killing(Quezada, Simpson et al. 2010).

In MHC II^{NEG} tumors, $CD4^+$ T cells cannot recognize the tumor cell directly, but may still be able to induce killing of tumor cells. Several mechanisms could be at play, notably activation of $CD8^+$ T cells as described, and activation of antigen-presenting cells such as macrophages and dendritic cells. In addition, NK-cells activated by $CD4^+$ T cells can under some circumstances be important(Perez-Diez, Joncker et al. 2007), even though their effector mechanism and general importance has yet to be elucidated. Th1-polarized $CD4^+$ T cells have also been suggested to inhibit tumor growth through modulatory effects of secreted IFNγ on neoangiogenesis within and near the tumor stroma (Qin and Blankenstein 2000).

In our model system, the tumor cells are MHC class II negative. Nonetheless, mice harboring $CD4^+$ T cells specific for this tumor are protected against tumor challenge (Lauritzsen, Weiss et al. 1994), independent of the presence of $CD8^+$ T cells and B cells (Bogen, Munthe et al. 1995). In vivo and in vitro data suggest that macrophages that have taken up secreted tumor antigen, and are activated by cognate interaction with antigen-specific $CD4^+$ T cells, are major contributors to the inhibition of tumor growth in this model(Lauritzsen and Bogen 1993, Dembic, Schenck et al. 2000, Dembic, Rottingen et al. 2001, Corthay, Skovseth et al. 2005, Corthay, Lundin et al. 2009, Haabeth, Lorvik et al. 2011).

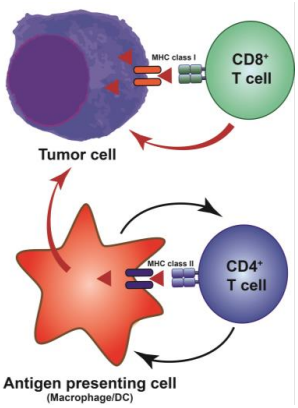

Figure 2. Indirect and direct recognizing of tumor antigen, with respectively indirect and direct killing of tumor cell (A.Tveita)

Quezada et al. have published data regarding co-injection of tumors with and without recognized antigen, with no apparent bystander effect (Quezada, Simpson et al. 2010). However, in their model, $CD4^+$ T-cells demonstrated a directly cytolytic phenotype, and were able to kill cancer cells by secretion of granzyme and perforin. This contrasts the indirect mechanism seen in our system. In the model used by Schietinger et al., bystander killing was seen when tumor cells were simultaneously expressing two antigens recognized by both $CD4^+$ T-cells and $CD8^+$ T cells, but not when production of the two antigens occurred in different cells (Schietinger, Philip et al. 2010). Despite this documentation of other mechanisms such as direct cytotoxicity or effects on

angiogenesis (Ohminami, Yasukawa et al. 1999, Qin and Blankenstein 2000, Quezada, Simpson et al. 2010), most clinical trials utilizing $CD4^+$ cells have focused on their helper cell functions (Ostrand-Rosenberg 2005). Delineating the specific contribution of cytotoxic $CD4^+$ subsets in clinical trials is difficult. Are they a byproduct with negligible impact, or an important player in the adaptive anti-tumor immune response? The physiological roles of cytotoxic $CD4^+$ T cells and the functional plasticity of $CD4^+$ T cells warrants further scrutiny in future studies.

1.2.1 $CD4^+$ T cells in cancer immunotherapy

Three principal strategies currently exist to utilize $CD4^+$ T cells in cancer immunotherapy. The first is to vaccinate with peptides, or dendritic cells pulsed with peptides to induce or boost endogenous T cell response. Such treatment is commonly complemented by the use of adjuvants, cytokines or growth factors that support T cell expansion and polarization towards a Th1 phenotype. The second possibility is to infuse *ex vivo* expanded autologous or allogeneic T cells, pursuing the establishment of a long-lasting immune response. Such T cell populations can be either antigen-specific or based on heterogeneous populations of T cells. The latter may also involve the use of artificial T cell receptors with specificity against relevant tumor antigens(Hong, Stastny et al. 2014). A third strategy is to try to enhance the activation, differentiation and proliferation of $CD4^+$ T cells that are already present by the use of cytokines or checkpoint inhibition.

1.2.1.1 Vaccines

For decades there have been clinical trials trying to prove the effect of different kinds of tumor vaccine preparations. Results have at large been disappointing. In many cases, measurable antigen-specific immune responses have been seen, but without translating to clinical responses. In 2010, Sipuleucel-T, the first, and so far only, therapeutic cancer vaccine was approved by the FDA. This dendritic cell vaccine increases median survival in patients with metastatic prostate cancer by an average of 4 months (Kantoff, Higano et al. 2010). The vaccine represents a highly personalized treatment, where a laboratory prepares autologous cells for infusion for individual patients. The approval of Sipuleucel-T has led to increased enthusiasm in the cancer vaccine field, with 150 therapeutic cancer vaccines currently undergoing evaluation in phase I-III trials(Kudrin 2014). Most of these (60%) are not personalized, and hence not cell-based, with the advantage of being commercially easier to distribute. Nonetheless, vaccines based on pure antigen +/- adjuvant still have not shown effect in clinical trials. In September 2013, results from a phase III study of one of the most promising vaccines (anti-MAGE-A3 for metastatic melanoma) were announced in a press release, showing no clinical benefit. Some of the difficulties in achieving significant responses with vaccines alone might be due to the potent, inherent ability of tumor cells to suppress the immune system and evade immune responses (Kerkar and Restifo 2012). As an adjunct to other treatment modalities especially in tumors with high mutational load, vaccination could likely still confer clinical benefits.

1.2.1.2 Adoptive transfer

The most widely tested strategy of adoptive cell therapy (ACT) using T cell is the use of expanded tumor-infiltrating lymphocytes (TILs) isolated from excised tumor tissue, with $CD8^+$ cells regarded as the most important constituent(Barth, Mule et al. 1991, Wilmott, Long et al. 2012). The addition of $CD4^+$ T cells or exclusive transfer with $CD4^+$ cells, have been able to induce long-term responses (Hunder, Wallen et al. 2008). Such cells may have a better likelihood to inducing endogenous responses to non-targeted antigens (epitope spreading) (Hunder, Wallen et al. 2008). This might be

an important adjunct to the direct cytolytic effects of the infused T cells themselves. Because of these issues, the attention to $CD4^+$ T cells in the adoptive transfer setting is increasing(Muranski and Restifo 2009).
One key challenge is that T cell functions tend to get exhausted in the face of prolonged stimulation, preventing long-lived effects of immunotherapy. In the absence of $CD4^+$ T cell help, $CD8^+$ T cells tend to get exhausted(Matloubian, Concepcion et al. 1994), implying an important facilitating function of $CD4^+$ T cells. $CD4^+$ cells are also able to suppress tumor growth by themselves, as early papers in the field showed in a murine model of leukemia(Greenberg, Cheever et al. 1981, Greenberg, Kern et al. 1985).

The phenotype, number and specificity of $CD4^+$ T cells are issues of importance; optimization of protocols is essential for the success of treatment using such cells. In one study, TILs with specificity for antigens from autologous or HLA-matched cell lines were expanded *ex vivo*, and re-infused after lymphodepletion with remarkable results (Rosenberg, Yang et al. 2011). This study demonstrated both the presence of antigen specific T cells within the tumor, and the possibility of expanding them to attain tumoricidal effects. To limit the number of transferred immunosuppressive T cells might be necessary (Paulos, Suhoski et al. 2008). It is also worth noticing that increased survival have been documented by using naïve vs. effector $CD4^+$ T cells (Aubert, Kamphorst et al. 2011).

The most recent addition to the field of ACT, and the most promising to date, is the use of T cells with engineered chimeric antigen receptors (CARs). CARs are trans-membrane protein chimeras, expressing an extracellular single-chain antibody fragment specific for native cell surface tumor antigens. This antibody fragment is fused to the ζ chain of the CD3 protein, and variably fused to the signaling domains of co-stimulatory molecules(Turtle and Riddell 2011). The result is a receptor that exploits the antigen recognition ability of antibodies, but responds by inducing intracellular signaling similar to that of T-cell receptor engagement. The advantages of this approach are several. Firstly, there is no MHC restriction and there is no pairing with endogenous TCR chains. There is also no requirement for antigen processing and presentation for activation. CAR therapy has shown impressive results in small clinical trials, particularly in B-lymphoid neoplasms, where CD19-specific CARs have been utilized (Porter, Levine et al. 2011). Limitations to the efficacy of CAR therapy include the identification of robust and specific target molecules, and achieving persisting responses. For instance, CD19 is abundantly present on most healthy B cells, and a prominent consequence of CD19 CAR treatment is profound B cell depletion(Davila, Kloss et al. 2013). Another issue is the long-term safety concerns of introducing virally transfected cells, and strategies are under development to allow the option of selective elimination of transfected cells at some point following tumor regression(Budde, Berger et al. 2013).

1.3.1.3 Checkpoint inhibition

As previously discussed, many cancer cells contain antigens with the potential to act as targets of productive immune responses. Still, clinical evidence of active immune control of cancers has been sparse, causing great skepticism within the scientific community. Lately this has changed. The problem in cancer immunology has been that the tumor and/or the tumors microenvironment adapt the capability of suppressing the immune system, even taking advantage of the growth promoting capacities of the innate immune system. Avoidance of immune destruction is now proposed as one of the hallmarks of cancer in the conceptual framework of tumorigenesis set forth by Hanahan and Weinberg(Hanahan and Weinberg 2011). Immunoevasion strategies are thought to exploit immune

checkpoint modifiers; cell-to-cell-interactions that have evolved as negative regulators of immune responses, and which contribute to limit tissue destruction and autoimmunity. Whilst highly desirable for the maintenance of normal tissue homeostasis, such mechanism have unfortunate effects in the context of cancer, as it counter-acts anti-tumor immune responses. Hence, strategies to inhibit such immunomodulatory influences is an attractive strategy. The checkpoints are predominantly affecting T cells, with inhibition of the proliferation and survival of CD4+ subsets as the central mechanism. Ipilimumab – an antagonistic anti-CTLA4 antibody – and nivolumab – a blocking anti-PD1 antibody – have reached the commercial market so far, but several others are on its way. Most promising is probably the anti-PD1/anti-PDL1 inhibitors. Activated $CD4^+$ T cells express PD1 (Porichis, Kwon et al. 2011), and PDL1 expression is found on many tumor cell types as well as on certain subsets of APCs. Interaction between these molecules prevents T cell effector functions(Keir, Butte et al. 2008). Suppression by the PD1/PDL1 pathway can be rescued by antibody blockade (Butler, Moebius et al. 2012), which has yielded successful preclinical (Goding, Wilson et al. 2013) and clinical results (Wolchok, Kluger et al. 2013). Whether the best approach will be combination of different checkpoint inhibitors, or combination of checkpoint inhibitors and adoptive transfer approaches, remains unknown. Nonetheless, by using these drugs, anti-tumor responses extending beyond what has been seen with any current treatment regimens have been observed in some types of cancer, in some cases possibly even cure(Wolchok, Kluger et al. 2013).

1.3 Macrophage differentiation and polarization

Macrophages have multiple roles in health and disease. They are key players in the innate immune system, and intimately cooperate with the adaptive immune system. As the name implies, they are "big eaters", and the main task is phagocytosis (eating) and clearing of cellular debris and pathogens. Most tissues contain fixed, specialized macrophage subsets; osteoclasts in bone, Kupffer cells in liver and microglia in neural tissue, to name a few. Macrophages generally comprise up to 10-15% of tissue mass, and are particularly abundant in the liver, lung and testis. The phenotypes of the tissue-infiltrating macrophages differ tremendously, reflecting their involvement in diverse tissue homeostatic mechanisms.

Macrophages are thought to originate from either hematopoietic stem cells (HSCs) in the bone marrow or self renewing tissue resident macrophages seeded through embryogenesis. These stem cells, which show unlimited self-regenerative potential, give rise to committed progenitors of either lymphopoiesis (Common lymphoid progenitors – CLPs) or myelopoiesis (CMPs). The CMPs eventually are the precursors of monoblasts, destined to become circulating monocytes.

The monocytes circulate in the blood, typically for a couple of days, before entering tissues in response to chemotactic and adherence signals which are expressed in conditions of stress or tissue damage. Upon entering the tissues, the cells take on the characteristics of macrophages or dendritic cells; two broad subsets of monocyte-derived cells with both shared and unique functions(Randolph, Jakubzick et al. 2008). While the dendritic cells specialize in antigen presentation of endocytosed material, the macrophages have multiple tasks. Their primary function appears to be as scavenging phagocytes, digesting dying neutrophils (pus), pathogens and cellular debris. They present remaining peptides from their phagolysosomes to the adaptive immune system, mainly to T helper cells in a MHC II-restricted manner. In this way, they screen the phagocytosed material for non-self molecules.

A T cell that recognizes peptides presented on MHC II molecules on a macrophage, will interact with the macrophage in a manner defined by its phenotype and by the co-stimulatory signals it receives from the macrophage and other parts of the stroma. A small subset of macrophages will carry MHC II loaded with peptide, also in a non-inflammatory situation (Pozzi, Maciaszek et al. 2005). If the interaction results in classical activation of the macrophage – as will be discussed later – the macrophage will enhance its phagocytic capacity, its amount of MHCII on its surface, and its production of cytokines and other secreted products.

1.3.1 APC-function

Macrophages and dendritic cells (DCs) are two cell types with important roles in antigen presentation. There are differences and similarities, with the DCs being the most potent antigen-presenting cells (APCs), vital to the priming of adaptive immune responses(Steinman and Hemmi 2006). Their main function is to process material from the external environment, degrade it and present the resulting fragmented peptides to T-cells in lymph nodes. This interaction induces proliferation and differentiation, as described earlier, with naïve T cells developing to effector T cells. The effector T cells then re-enter the bloodstream, adhere to blood vessels at sites of inflammation, and enter the inflammatory site through diapedesis. At the inflammatory site they may either interact directly with infected cells (typically in the case of $CD8^+$ T cells) or tissue-infiltrating APCs (in the case of $CD4^+$ T cells), the most abundant of which are macrophages. I will here focus on the APC function of the classical tissue macrophage with phagocytic, effector and stimulatory abilities. The macrophages have a large capacity for engulfing and digesting cellular debris, foreign substances, microbes and cancer cells, but compared to DCs, the capacity for antigen retainment and delayed presentation is lower (Delamarre, Pack et al. 2005). This means they will present peptides in the vicinity of where antigens are taken up, providing them with the ability to interact with local T cells in e.g. a tumor. Macrophages are drawn to sites of inflammation, and have a key role in amplifying and orchestrating the inflammatory response, scavenge tissue debris and mediate its resolution by inducing wound healing responses from fibroblasts and endothelial cells. Their infiltration is usually only preceded by neutrophils(Clark, Hingorani et al. 2007).

The APCs play key roles in adaptive immune responses, serving as a link between the adaptive and the innate immune system. Unlike B cells, T cells do not bind to antigen directly, but recognizes fragments resulting from internalization and cleavage of whole proteins by antigen presenting cells(Ziegler and Unanue 1981). After partial enzymatic digestion, peptide fragments are saved from complete degradation by binding to MHC molecules in endosomal vesicles(Donermeyer and Allen 1989), and MHC:peptide complexes are transported to the cell membrane. A high binding affinity to MHC is crucial for the peptide to avoid degradation (Carrasco-Marin, Petzold et al. 1999). The meaning of antigen presentation is to sample both the environment and the interior of cells, showing epitopes to T cells that continuously screen for epitopes from non-self proteins. The macrophage was the first known antigen-presenting cell, and was pivotal to the understanding of T cell activation. Later, the dendritic cell have been recognized as the APC *par excellence*, but at inflammatory sites, including tumors, macrophages play the dominant role, sometimes making up half the mass of a tumor. Non-activated macrophages have not upregulated their antigen presenting apparatus, but still present peptides to a certain degree. Upon activation, the macrophages increase the expression of MHC II on their surface, and hence their capacity for antigen display to T cells (Pozzi, Maciaszek et al. 2005). On the other hand, activated Th1 cells produce IFNγ (Schroder, Hertzog et al. 2004) to

induce macrophage activation. It is this interaction between T cells and macrophages that, given the right tumor microenvironment, can elicit an anti-tumor response.

1.3.2 The M1/M2 paradigm and the plasticity of macrophage differentiation

Cells in the monocyte-macrophage lineage are profoundly dynamic. The macrophage itself can change between a number of very different functional states, and is likely the hematopoietic cell type with the most pronounced plasticity (Mantovani, Sozzani et al. 2002, Mantovani, Sica et al. 2004, Mosser and Edwards 2008, Martinez, Helming et al. 2009, Pollard 2009, Deban, Russo et al. 2010, Gordon and Martinez 2010, Biswas and Mantovani 2012, Sica and Mantovani 2012). Earlier, there was an understanding of macrophage activation as a stereotypic transition from a resting state to a microbicidal or tumoricidal phenotype (Adams and Hamilton 1984). Today, a continuum of activational states with varying effector properties are recognized. (Sica and Mantovani 2012) At one extreme we find so-called "classical activation"; typically induced in response to Toll-like receptor (TLR) signaling, lipopolysaccharide (LPS), granulocyte-monocyte colony stimulating factor (GM-CSF) and IFNγ. At the opposing end of the spectrum is "alternative activation", typified by the response to IL-4 and IL-13(Stein, Keshav et al. 1992) signaling. The classically activated macrophage is designated M1, and the alternative M2, mirroring the Th1/Th2 T helper cell nomenclature of $CD4^+$ T cell polarization(Mantovani, Sozzani et al. 2002). Accordingly, M1 or M2 polarization is thought to dominate in the course of Th1- or Th2-type adaptive immune responses, respectively.
Early evidence suggests that the activation phenotype is determined by alteration in gene expression occurring at the epigenetic level(Chen, Barozzi et al. 2012), with the Signal Transduction and Activator of Transcription (STAT) pathway signaling patterns forming an instructive influence (STAT1 for M1 and STAT3/5/6 for M2(Sica and Bronte 2007, Kuroda, Ho et al. 2009)). Specifically, it has been proposed that the balance between STAT1 and STAT3 activation regulates macrophage polarization(Sica and Mantovani 2012). In the M1 macrophage, the transcription factor interferon response factor 5 (IRF5) is important in inducing production of typical M1 cytokines (IL-12, IL-23, tumor necrosis factor; TNFα)(Krausgruber, Blazek et al. 2011). In the M2 macrophage, a large array of transcription factors appear to be involved in controlling the phenotype, notably PPAR-γ(Szanto, Balint et al. 2010) and -δ(Odegaard, Ricardo-Gonzalez et al. 2008), and c-Myc(Pello, De Pizzol et al. 2012).

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) proteins regulate response to cellular stress through regulation of transcription in response to pro-inflammatory signals. The name derives from its binding to "kappa-light-chain-enhancer"(Sen and Baltimore 2006), but it also targets many other genes related to inflammation. The protein family consists of five members sharing the Rel homology domain: RELA (p65), RELB, c-REL, p105/50 (NF-κB1) and p100/p52 (NF-κB2). The shared domain permits dimerization which leads to translocation to the nucleus(Hayden and Ghosh 2012). The RELA:p50 heterodimer is the primary mediator of inflammatory signals from TLRs and other inflammatory cytokine signaling pathways. NF-κB dimers are in non-stimulated cells bound to inhibitors of κB (IκB), retaining them in the cytosol. Upon phosphorylation of IκB induced by a variety of signals, IκB gets degraded and the nuclear localization signal is exposed. Nuclear translocation then leads to activation of a number of target genes (Monaco, Andreakos et al. 2004). This is called the canonical pathway. In the non-canonical pathway, there is induced proteosomal processing of p100 to p52, also leading to nuclear translocation and subsequent transcription(Chen and Chen 2013) (see figure 3)(Mowla, Perkins et al. 2013). The NFκB signaling pathway is active in both types of macrophage phenotypes. Following TLR signaling, NF-κB activation leads to production of inflammatory mediators (Bonizzi, Bebien et al. 2004). At the same time, a transcriptional program favoring the resolution of inflammation is also induced(Lawrence and Gilroy 2007), serving as a negative feedback mechanism. This process is mediated by the formation of NF-κB p50 homodimers, which competitively inhibits productive signaling through NF-κB. It has been demonstrated that the formation of p50 homodimers is required for the induction of M2 polarization (Porta, Rimoldi et al. 2009). Broadly, the M1 phenotype is thought to be the result of a pro-inflammatory transcriptional program, whilst the M2 phenotype mediates a wound healing process.

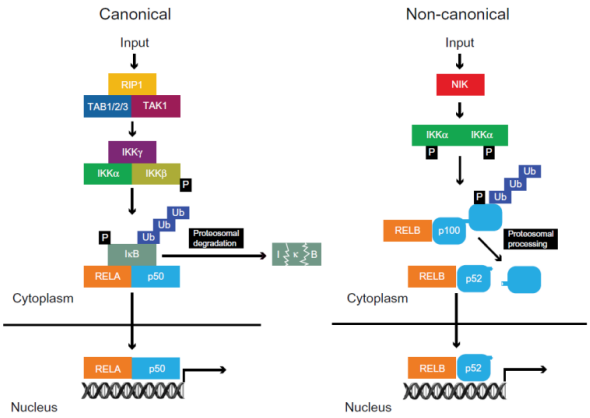

Figure 3 Courtesy of Prof Jat

Repeated exposure to LPS induces so-called LPS tolerance in macrophages and dendritic cells, rendering them hyporesponsive to subsequent challenges (at least within the timeframe of 2-3 days). This reorientation towards an immunosuppressive state is thought to have evolved to ensure limitation of inflammatory damage(Medzhitov, Schneider et al. 2012). Transcriptome analysis of such tolerant cells largely mirrors that of alternatively activated (M2) macrophages (Biswas and Lopez-Collazo 2009), including high expression of IL-10, arginase 1, CCL17 and CCL22. Resolution of viral infections can also lead to long-lasting macrophage desensitization (Didierlaurent, Goulding et al. 2008). Both examples illustrate the phenotypic alterations occurring in macrophages after the initial inflammatory effector phase. Even though IL-4 and IL-13 are the main alternative activators, other cytokines are also associated with M2 polarization. IL-33 is one example, amplifying IL-13-induced polarization by increasing YM1, arginase 1, CCL24 and CCL17 (Hazlett, McClellan et al. 2010). IL-21 is another example (Pesce, Kaviratne et al. 2006), as is M2 skewing by the chemokines CCL2 and CXCL4 (Gleissner, Shaked et al. 2010).

M1 macrophages are important effector cells in Th1 responses, through their production of potent cytotoxic effector molecules (e.g. reactive oxygen derivatives and nitrogen intermediates) and cytokines with pro-inflammatory properties (IL-1β, TNFα, IL-6, IL-12, IL-23). By this capacity, they can mediate cytotoxicity against microbes and tumors. The M2 macrophages, on the other hand, highly express anti-inflammatory cytokines such as IL-10 and Transforming growth factor beta (TGF-β), and contribute to tissue remodeling(Mantovani, Biswas et al. 2013) and angiogenesis. This includes potent suppression of both innate and adaptive immune responses(Biswas and Mantovani 2010). M2 macrophages typically express high levels of scavenger receptors (such as mannose receptor), but show low levels of IL-1 and caspase 1 signaling(Dinarello 2005).

Inducible nitric oxide synthase (iNOS; M1) and arginase-1 (Arg1; M2) catalyze reactions with the same substrate, L-arginine, and are regulated by the hypoxia-inducible factors HIF-1α and HIF-2α, respectively (Takeda, O'Dea et al. 2010). Induction of iNOS expression in M1 macrophages leads to increased secretion of nitric oxide (NO), which forms reactive nitrogen species (RNS) that have cytotoxic and anti-proliferative effects on neighboring cells. M2 type macrophages have high levels of Arg1, allowing the generation of ornithine. This typically induces proliferation of surrounding cells, and is a trigger for various tissue repair mechanisms(Wu and Morris 1998). In an elegant fashion, the intermediates of each pathway suppress the opposing pathway, creating a positive feedback mechanism that further skews the balance in one direction (Morris 2009). TGF-β is a central M2-maintaining cytokine, due to its strong inhibitory effect on iNOS expression (Vodovotz, Bogdan et al. 1993). Because of this important role in both macrophage phenotypes, arginine levels in inflammatory sites normally drops to extremely low levels(Albina, Mills et al. 1990).

The chemokine and chemokine receptor profile are different in the two macrophage counterparts, reflecting their cooperation with distinct T cell phenotypes, at least within a simplified framework of understanding. The M1 macrophage expresses chemokines to attract Th1 cells, typically CXCL9 and CXCL10; while M2 type generally secrete the Th2 chemokines CCL17, CCL22 and CCL24 (Martinez, Gordon et al. 2006). Other functional differences include metabolism of arginine, iron, folate and glucose (Puig-Kroger, Sierra-Filardi et al. 2009, Recalcati, Locati et al. 2010, Rodriguez-Prados, Traves et al. 2010, Biswas and Mantovani 2012), even though the physiological significance of these factors are not fully established as of today.

As described above, the M1 and M2 macrophage are at the extremes of macrophage phenotypes. Both in health and disease, we find macrophage accumulations resembling one of these types, but we also observe mixed phenotypes and coexistence of macrophages with different activation status. This reflects the dynamics of macrophage function, and its interaction with its surroundings. This is reflected by the use of more ambiguous terms such as “M2-like macrophages", sharing receptors but not chemokine repertoire with canonical M2s(Biswas and Mantovani 2010). More importantly, the polarization is a dynamic feature and can largely be reverted or interconverted to other archetypical or intermediate states (Guiducci, Vicari et al. 2005). Still, there are wide gaps in our knowledge of what happens on the single-cell level. Is the plasticity bi-directional? Is plasticity a widespread phenomenon, or is recruitment of new cells responsible for changes in macrophage behavior? Are intermediate states frequent, or are the so-called intermediates actually cells in transition? By ongoing studies of macrophage phenotype, these issues will likely become clearer in coming years.

1.3.3 Macrophage cell surface markers

Macrophage colony stimulating factor-1 (CSF-1; also referred to as M-CSF) is the major chemoattractant and growth factor for the differentiation of macrophages, and is necessary for them to reach a fully differentiated phenotype. CSF-1 promotes a "default" pathway of macrophage activation, mostly resembling the M2 phenotype (Martinez, Gordon et al. 2006) (see later). CSF-1 is the ligand for CSF-1 receptor (CSF-1R), which is increasingly expressed at the cell surface as the monocyte develops into a macrophage. In some self-renewing tissue-resident macrophages, the survival signals come through CSF-1R by an alternate ligand, interleukin 34 (IL-34) (Wang, Szretter et al. 2012). Macrophages constitutively express a number of receptors that recognize molecular structural patterns that are commonly present in invading microorganisms and cellular structures that are only exposed upon tissue damage; referred to as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), respectively (Janeway 1989, Kono and Rock 2008). Thus, these receptors mediate a degree of specific sensing of conditions of tissue damage. Examples include the mannose receptor, scavenger receptors, complement receptors and the Toll-like receptor (TLR) family. One of the TLRs – TLR4 – activates the cell when bound to PAMPs such as lipopolysaccharide (LPS) (Chow, Young et al. 1999), which is located in the outer cell membrane of Gram-negative bacteria. TLR4, in conjunction with the small extracellular protein MD2, interacts with the CD14-LPS complex to activate intracellular signaling (Poltorak, He et al. 1998). CD14 is known as the LPS receptor. In addition, TLR4 can also recognize various host-derived lipid metabolites and contribute to development of insulin resistance and glucose intolerance (Olefsky and Glass 2010). The PAMP/DAMP receptors are involved in the process of phagocytosis as well as macrophage activation, leading to higher expression of MHC class II molecules and B7 (B7 is a co-stimulatory molecule exclusively expressed on cells that activate naïve T-cells). In the classically activated state, the ability to damage microbes and some tumor cells are enhanced.

Scavenger receptors have roles in clearance of inflammation, maintenance of homeostasis and anti-bacterial immunity. CD163, until recently most known for scavenging hemoglobin-haptoglobin complexes, is one example, now shown to act as a macrophage receptor for bacteria (Fabriek, van Bruggen et al. 2009). This particular scavenger receptor is up-regulated when exposed to glucocorticoids (Ehrchen, Steinmuller et al. 2007), as part of the anti-inflammatory response and the resolution of inflammation partly driven by the macrophage in this setting.

INFGR (interferon gamma receptor) is an important cell surface molecule necessary for classical activation of the macrophage. $CD8^+$ and $CD4^+$ T_H1 cells as well as activated natural killer (NK) cells are the dominant sources of secreted IFNγ. Upon activation, macrophage expression of CD40 and TNF receptors is increased. CD40 binds to CD40 ligand (CD40L) on T cells, and TNF receptors respond to TNF secreted by activated macrophages in an autocrine fashion. Upon CD40-CD40L interaction the expression of B7 proteins and MHC class II molecules increases, making the macrophage a more potent stimulator of CD4 T cells. This activation also occurs upon ingesting bacteria and recognizing their molecular patterns. B7 recognition by CD28 on the T-cell functions as a second signal to the T-cell, in addition to recognition of MHC-II/peptide complexes, potentiation T cell activation and promoting IL-2 signaling and cell survival. IFNγ also primes the macrophage ensuring increased responsiveness to LPS, and increases the expression of pattern recognition receptors (TLR4 and MD2) on its surface, enforcing its effector machinery (Meltzer, Occhionero et al. 1982, Bosisio, Polentarutti et al. 2002).

Alternative activation of macrophages, resulting in a M2 phenotype, is a result of the macrophage being stimulated by Interleukin-4 (IL-4) and/or IL-13. Two receptors on the macrophage bind to IL-4. The type I receptor binds only IL-4, whilst the type II receptor binds to both IL-4 and IL-13, even though the response can differ (LaPorte, Juo et al. 2008). The primary sources of IL-4 are basophils and T_{FH} cells in the lymph nodes, whereas Th2cells produce a spectrum of either IL-4 and/or IL-13(Liang, Reinhardt et al. 2012). Both interleukins are part of allergic disease and immune responses against helminthes. As discussed later, they are also a part of metabolic homeostasis and the provision of at tumor-friendly environment.

In contrast to dendritic cells, which have a high expression of CD11c, macrophages are typically CD11c negative. Instead, they express CD11b, which is part of an integrin complex called Mac-1 that consists of CD11b and CD18 (CD11b:CD18). Although CD11b is well established as a surface marker on macrophages, it is also prevalent on other cell lines, including monocytes, granulocytes and natural killer cells. To distinguish macrophages from these other lines, another surface marker; F4/80 is often used. F4/80 is a transmembrane protein that a G protein coupled receptor. Little is known about its functions, but more about its distribution. With the exception of eosinophils, F4/80 is a highly specific marker for monocytoid and dendritic cell lines. The distribution is, however, not uniform. It is dim on monocytes in steady state, but highly expressed on extravascular macrophages in all tissues, including microglia and Langerhans cells. This makes it a good antigen for tumor immunohistochemistry. Expression is low or absent on macrophages in T cell areas, such as lymph nodes and Peyer's patches.

1.4 Macrophages in cancer

The roles of macrophages in cancer, like in inflammation, are diverse and dynamic. As mentioned previously, macrophages form the bulk of tumor-infiltrating leukocytes, and can make up half the mass of a tumor (Kelly, Davison et al. 1988). The majority of published articles describe correlations between macrophage infiltration and tumor growth and metastasis, reporting a negative association with patient survival (Steidl, Lee et al. 2010, Kurahara, Shinchi et al. 2011). The correlation to tumor growth is likely a reflection of the function of M2 macrophages as a promoter of wound-healing processes. The macrophages orchestrate remodeling, induce angiogenesis and suppress the immune system to terminate local inflammatory responses(Biswas and Mantovani 2010). In support of this perspective, intratumoral macrophages most often adhere to the M2 phenotype(Sica, Schioppa et al. 2006). Angiogenesis is required for tumors to expand beyond a size of about one million cells (Hanahan and Weinberg 2011). As cancer cells normally do not produce angiogenic factors, they depend on external influence from tumor-infiltrating cells including M2 macrophages. Growth factors secreted by M2 macrophages (e.g. ornithine, EGF, VEGF, collagen) in the aftermath of tissue damage and healing might be the reason tumors preferentially appear at sites of wound repair (Sieweke, Thompson et al. 1990), and the reason why they promote tumor growth (Qian and Pollard 2010). One also observes decreased tumor growth in macrophage-depleted hosts (Gazzaniga, Bravo et al. 2007). Some tumors do contain M1 phenotype macrophages, correlating with better patient survival (Ohri, Shikotra et al. 2009), but in most cases, the tumor cells actively skew the macrophages towards M2 by producing factors such as prostaglandin E2 and TGF-β (Alleva, Burger et al. 1994). They even stimulate the macrophage to break down matrix allowing further growth of the tumor. Whether this represents an aspect of malignant transformation or is part of an inherent homeostatic mechanism of tissue cells is presently unknown.

One interesting possibility is that tumorigenesis involves development of immunological tolerance. This might include not only evasion of adaptive immune responses, but also skewing the macrophages in the direction of an M2 phenotype. The concept of immunoediting during tumorigenesis is well established for adaptive immune responses, but the dynamics of innate responses have been less explored. It seems likely that different scenarios are at play, depending on the immunogenicity of the tumor and the tumorigenic process. In cancer forms that are regarded as immunogenic, with melanoma as the most prominent example (Bronkhorst, Ly et al. 2011), one might expect there to be an initial immune reaction that is sufficiently potent to eliminate transformed cells in some cases. It could also turn in to a state of equilibrium, where the innate and the adaptive immune system keeps the tumor in check for some time, but eventually something happens that makes the tumor escape (Dunn, Old et al. 2004), and the cancer to become clinically apparent. One underlying mechanism could be that the evolutionary pressure from the macrophages promotes the outgrowth of tumor cells that secrete factors promoting a shift towards an M2 phenotype. However, cancer can also present itself with M1 macrophages still present, representing ongoing immune responses, and correlating with better survival, as stated above. Yet another scenario is an initial M2 macrophage phenotype dominance, whereupon an increase or appearance of a tumor specific antigen, occurring in the timeframe of increased inflammatory activity, induces an adaptive immune response. We know that e.g. IFNγ may reverse the immunosuppressive macrophages and repolarize them to immunostimulatory M1 cells (Duluc, Corvaisier et al. 2009). One should therefore bear in mind that the role and phenotype of tumor-infiltrating macrophages may be very different depending on whether or not they are part of an ongoing adaptive immune response. If M1 polarization is a reflection of an adaptive immune attack on tumor cells, this might offer an explanation of the seemingly contradictive associations of macrophage infiltration of tumors and cancer progression.

To add to the complexity, even though M1 phenotype macrophages can control, fight and eliminate tumor cells, the damage they inflict on nearby cells, including oxidative stress, may have mutagenic properties (Nardin and Abastado 2008), further complicating their role in cancer. This is especially true in the context of chronic inflammatory processes with danger signals causing sustained M1 responses resulting in damage to surrounding cells. Smoking is one example of this.

Initially, tumor cells recruit monocytes to the tumor site by secreting chemokines and growth factors, CCL2/MCP1 being especially important (Bottazzi, Polentarutti et al. 1983, Negus, Stamp et al. 1995, Ueno, Toi et al. 2000, Nesbit, Schaider et al. 2001, Monti, Leone et al. 2003, Gazzaniga, Bravo et al. 2007, Zhang, Patel et al. 2010). CCL2/MCP1 also induces M2 polarization of macrophages and promote macrophage survival (Roca, Varsos et al. 2009). Other chemokines and growth factors have been shown to serve as additional attractants for macrophages, including CCL5/RANTES, CXCL1/Gro-α, CCL7/MCP-3, CCL8/MCP-2, VEGF, PDGF, TGF-β and M-CSF/CSF-1 (Balkwill 2004, Mantovani, Sica et al. 2004, Allavena, Sica et al. 2008, Balkwill 2012). Some are secreted by tumor cells, and some by stromal cells, but all can be present in neoplastic tissue. Recently, it was shown that marked macrophage amplification and accumulation within the spleen occurs antecedent to their relocation to the tumor site in a mouse model of lung adenocarcinoma (Cortez-Retamozo, Etzrodt et al. 2012). This process was dependent on CCL2-CCR2, emphasizing the importance of this mechanism of chemoattraction. Normoxic environments are more prone to have M1 macrophages, whereas hypoxic areas are more likely to have M2s (Movahedi, Laoui et al. 2010). Hypoxia is known to develop within tumors because of their continued expansion, and is known to influence gene

expression of macrophages, including down-regulation of MHCII and induction of arginase 1 and pro-angiogenic factors (Murdoch, Muthana et al. 2005, Mancino, Schioppa et al. 2008). Intratumoral macrophages in more hypoxic areas, are associated with worse prognosis than peritumoral macrophages(Erreni, Mantovani et al. 2011), probably caused by microenvironmental influences on their phenotype.

Chemokines and growth factor from tumors mainly stimulate the macrophage to proliferate along the default pathway, the M2 "alternative" activation. However, there are results nuancing the picture. Liver macrophages, Kupffer cells, kill circulating tumor cells, and their depletion increases metastasis (Heuff, Oldenburg et al. 1993). A rat model of colon cancer showed increased growth and poorer survival in macrophage-depleted animals (Oosterling, van der Bij et al. 2005), even though tumor histology seemed more malignant in the presence of macrophages. In non-small-cell lung cancer, the macrophages are mostly M1 polarized, and are associated with better survival (Ohri, Shikotra et al. 2009). Osteosarcoma patients showed a significant correlation between the number of macrophages in the tumor and better survival (Buddingh, Kuijjer et al. 2011), and in colorectal cancer, some studies show benefit (Forssell, Oberg et al. 2007). Even so, the most important promise for M1 activity in malignant tumors is that clinical studies show potential for re-educating macrophages from the default M2 to tumoricidal M1 macrophages. One example of this is the treatment of pancreatic tumors by agonistic CD40 antibodies, substituting for the signal normally presented by activated T cells carrying CD40 ligand (CD154) (Vonderheide, Bajor et al. 2013). Since macrophages often constitute over 50% of a tumor, they represent an abundant mediator of cytotoxicity if they are collectively rendered tumoricidal. The potential could be vast, as suggested by widespread tumor regression seen in selected human patients (Beatty, Chiorean et al. 2011). Not all patients respond, however, so further investigations are require to define the optimal strategy to take advantage of macrophages in as many patients as possible. Another possible way to utilize the macrophages is to increase the $CD4^+$ T cell response by immune checkpoint inhibition. Ipilimumab is a CTLA-4 inhibitor, inhibiting an immune checkpoint and by that promoting anti-tumor immunity orchestrated by $CD4^+$ T cells (Quezada, Peggs et al. 2006). It was recently suggested that large numbers of intratumoral macrophages correlates to effect of treatment with ipilimumab(Tsaknakis, Schaefer et al. 2014). Whether this reflects an active role of macrophages as mediators of anti-tumor responses or serve as a marker of sustained immune responses is unknown, but these findings challenge the notion that $CD8^+$ cytotoxic T cells are the main effectors of ipilimumab-based treatment regimens.

1.5 The dynamics of tumor/host interaction: Cancer immunoediting

The dual role of the immune system in cancer is now widely acknowledged, as previously discussed. Strong correlations between the phenotype of immune cells and prognosis of cancer have been established (Fridman, Pages et al. 2012), and the successes of immunotherapy has proved beyond doubt that the immune system is, when circumstances are favorable, capable of combating and possibly curing cancer. In addition, the immune elimination of potentially cancer-inducing viruses is a way in which the immune system protects us from cancer, clearly demonstrated by the increased incidence of virus-associated malignancies in severely immunocompromised individuals, notably HIV patients(Cutrell and Bedimo 2013). At the same time, through chronic inflammatory process, immune responses may contribute to mutagenesis, thus supporting tumor development.

One remaining question is what role the adaptive immune system plays in the pathogenesis and clinical emergence of cancer in the individual patient. In 1957, the cancer immunosurveillance hypothesis was formulated in the article of Burnet(Burnet 1957), predicting that the adaptive immune system prevented cancer development in immunocompetent hosts. The hypothesis was called to question by the difficulties in demonstrating significantly increased incidence of cancer in immunocompromised animals(Stutman 1974) and humans. In the 90s, the hypothesis resurfaced based on new experimental data. Allogeneic transplantation had been established, with a demonstrated contribution of anti-tumor immune responses induced by donor-derived lymphocytes (Falkenburg and Warren 2011). Additionally, immunocompromised mice were shown to be more susceptible both to spontaneous and carcinogen-induced tumors (Kaplan, Shankaran et al. 1998). Supplementing this, TCR transgenic mouse models demonstrated the mechanisms acting in vivo (Haabeth, Tveita et al. 2014). The growing number of reports supporting such conclusions, and the development of more robust animal models of immunodeficiency, led to a general acceptance of the pivotal role of the immune system in tumor development (Vesely, Kershaw et al. 2011).

A 2001 study demonstrated that the presence of an intact immune system influenced the immunogenicity of growing tumors, giving rise to the cancer immunoediting hypothesis (Shankaran, Ikeda et al. 2001). In one series of experiments wild-type (WT) and immunodeficient RAG2-/- mice were treated with a carcinogen (methylcholanthrene) and monitored for tumor development. After 160 days 30/52 immunodeficient mice vs 11/57 wild-type mice formed tumors ($p>0,0001$). The same was shown in IFNγ-insensitive mice (25/50 vs 11/57; $p<0,001$). Similar results were published earlier by the same group (Kaplan, Shankaran et al. 1998). Mice were also followed without carcinogen injection, and evaluated by necropsy at 15 months. At this timepoint, all RAG2-/-mice had developed neoplastic lesions, while 9/11 wild-type mice were free of neoplastic disease. Hence, immunodeficient mice are more prone to neoplastic disease, both spontaneous and carcinogen-induced.

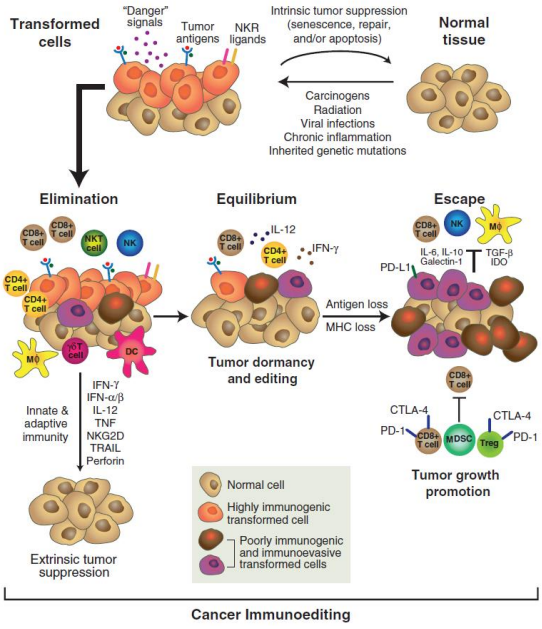

Figure 4 Courtesy of Robert Schreiber, Washington University

To investigate the possible immunoediting of these carcinogen-induced tumors, the tumors were re-injected in new RAG2-/- and WT-mice. When re-injected into RA2-/- mice, both RAG2-/- and WT-derived tumors grew progressively with equivalent rate; this also happened when WT-derived tumors were re-injected into both strains. In contrast, upon reinjection of RAG2-/--derived tumors into naïve WT mice, 8/20 were rejected. The conclusion was that tumors progressed in

immunocompetent hosts, have been immunoedited and lost its immunogenicity. On the other hand tumors developed in immunodeficient mice, still retain immunogenicity and can be targeted by an adaptive immune response when transplanted to a syngeneic naïve host. IFNγ seem to have a prominent role in this process, as reviewed in (Dunn, Koebel et al. 2006).

On the basis of these experiments the relationship between tumors and the hosts immune system is postulated to go through three distinct phases (Vesely, Kershaw et al. 2011) as schematically illustrated in Figure 4 (Schreiber, Old et al. 2011). This model has been formulated as the "Three E" hypothesis of immunosurveillance, and principally involves the following stages:
i) First, the immune system mounts a response against the tumor based on recognition of tumor antigens in conjuncture with danger signals provided by inflammation, dying tumor cells or tissue damage (Guerra, Tan et al. 2008, Sims, Rowe et al. 2010). This phase is referred to as the Elimination phase. Experimentally addressing this issue is inherently challenging, given the lack of strategies to identify the emergence and elimination of transformed cells that do not form tumors. Nonetheless, in theory, such events would be expected to occur, and could account for some of the difference in tumor incidence between immunocompetent and immunocompromised mice.

ii) If the immune system fails in eliminating the tumor, two things can happen. Either the tumor escapes; giving rise to a clinical tumor, or the battle enters a balance where net growth of tumor cells is inhibited by a constant immune attack, without the immune system being able to clear the body of all cancer cells. This is referred to as the Equilibrium phase. In 2007, this phase was shown to be mediated by Th1-cells, keeping fibrosarcoma cells in a dormant phase as long as the tumor remained unedited (Koebel, Vermi et al. 2007). This finding is underscored by the important role of IL-12 in anti-tumor defense, as opposed to the pro-tumor effect of IL23 – a Th17 driver cytokine (Teng, Vesely et al. 2012). Possibly, this phase could last for very long periods, maybe even a lifetime. Some anecdotal cases illustrate this. One patient with malignant melanoma, whose primary tumor was excised, donated a kidney 16 years later resulting in the recipient developing a fatal malignant melanoma with donor chimerism (MacKie, Reid et al. 2003). Whether the immune suppression or the encounter with a new immune system was precipitating the appearance is up for speculation, but the conclusion is anyway that the donor's immune system had controlled this tumor for almost two decades. In animals the harboring of occult cancer cells after low-dose carcinogens, is shown for extended periods, with tumor breakthrough after depletion of T cells and IFNγ (Koebel, Vermi et al. 2007). This implies, as was further analyzed and established in the study, that the adaptive and not the innate immunity is the key element in this equilibrium. In contrast, the elimination phase requires also the action of the innate immune cells.

iii) The equilibrium is maintained by a combination of cytotoxic action and growth inhibitory effects. Over time, this selective pressure selects for cancer cells with immunoevasive properties, leading to the third phase, Escape, after which unrestricted expansion and metastasis of the tumor occurs. The mechanisms underlying the transition to the escape phase appears to be different depending on the nature of the immunosurveillance process. By studying immune escape in various mouse models, several examples of immune escape strategies have been defined, as further discussed below.

This model is based on the combined presence of $CD4^+$ and $CD8^+$ T cells, whereas immunoediting by $CD4^+$ T cells alone has not been studied.

1.6 Mechanisms of tumor escape

With the termination of the equilibrium phase through tumor escape, tumor cells have acquired new abilities through genetic or epigenetic changes. Broadly speaking, this can happen in two ways. Either the tumor cell changes in a way that make it more difficult for the immune system to detect or attack it, or the tumor cells directly modulates the immune response so as to make it less tumoricidal (and in some cases even promote tumor growth). Several specific mechanisms have been identified to facilitate either of these strategies of immune escape.

Perhaps the simplest and most intuitive mechanism of tumor escape is the elimination of the epitope(s) recognized by the adaptive immune system. Evolutionary selection of variants with lower or absent production of antigens recognized by $CD8^+$ T cells is well documented to give rise to clinical tumors (Olson and McNeel 2012). This mechanism prevents both the indirect recognition from $CD4^+$ T cells and the direct recognition from $CD8^+$ T cells. Similarly, mutations within immunogenic epitopes may preclude antigen recognition. An alternative way of avoiding recognition by $CD8^+$ T cells is by loss or down-regulation of MHC class I proteins or parts of the processing machinery needed to process peptides for antigen display (Dunn, Bruce et al. 2002, Khong and Restifo 2002), making the tumor difficult to detect for $CD8^+$ T cells. Two recent papers using different models have demonstrated immunoediting with outgrowth of clones able to escape T lymphocyte attack. The outgrowing clones either had lower antigen expression or lower antigen presentation on MHC class I (DuPage, Mazumdar et al. 2012, Matsushita, Vesely et al. 2012). While loss of (display of) tumor-specific antigens is an important immune escape strategy, immune escape does not appear to be fully evolved for all antigens, even in immunocompetent individuals. This is demonstrated by the success of TIL therapy, which relies on endogenous T cells specific for tumor antigens, and the sometimes profound effect seen on treatment with checkpoint inhibitors. When a tumor has arisen, the immune cells residing there are generally functionally impaired, meaning that they are not capable of mounting a fully cytotoxic attack. On the contrary, the environment created around and within a tumor is often favorable for T-cell suppression(Mantovani and Sica 2010). Overcoming the effects of such suppressive influences by inducing inflammation at the tumor site might constitute an important part of both surgical and medical cancer therapy, including chemotherapy and targeted therapy(Balachandran, Cavnar et al. 2011, Zitvogel, Kepp et al. 2011).

Regulatory T cells are recognized by the expression of the surface marker CD25 and the presence of the transcription factor Forkhead box P3 (FoxP3). The potential of such cells to inhibit antitumor immunity has been established in mouse models (Shimizu, Yamazaki et al. 1999). They are mediators of peripheral tolerance to self-antigens (Bala and Moudgil 2006), but are also found within tumors of both epithelial and hematological origin(Shevach 2004). Two major subsets have been described. Natural T_{regs} (nT) develop during recognition of self-antigens within the thymus, whereas inducible T regs (iT_{regs}) arise in the periphery by interaction with APCs (Quezada, Peggs et al. 2011). Both subsets probably play roles in tumor immunology(Zhou and Levitsky 2007). T-regs secrete IL-10 and transforming growth factor beta (TGF-β), two potently immunosuppressive cytokines that reduce proliferation and anti-tumor effects of both $CD4^+$ T cells, $CD8^+$ T cells and NK cells. An unprecedented affinity for IL-2 leads to scavenging of this important stimulator of T cell proliferation from the surroundings of T_{regs}(Fehervari and Sakaguchi 2004). Similar effects on the availability of other cytokines, including IL-7, IL-12 and IL-15 have also been proposed (Gattinoni, Finkelstein et al. 2005).

Tumor cells themselves can also generate immunosuppressive factors. One prominent example is secretion of TGF-β(Yang, Pang et al. 2010) that affect the development and function of APCs, indirectly inducing hyporesponsiveness in several lymphocyte subsets(Li, Wan et al. 2006). TGF-β also leads to down-regulation of both MHC class II and co-stimulatory molecules on APCs (Geissmann, Revy et al. 1999). VEGF causes blockade of maturation and differentiation of antigen presenting cells (APCs) (Zou 2005), and was the first cytokine demonstrated to show this sort of effect (Gabrilovich, Chen et al. 1996). VEGF is produced by multiple cancer types (Geissmann, Revy et al. 1999, Carmeliet and Jain 2000, Li, Wan et al. 2006). IL-10 produced by tumor cells can yield tolerogenic APCs that induce tumor-specific anergy(Roncarolo, Levings et al. 2001), by inducing differentiation of regulatory cells, showing a link between innate and adaptive immunosuppressive effects. IL-10 also inhibits cytotoxicity and IL-12 production by macrophages(Mullins, Martins et al. 2001), skewing the macrophages towards a growth promoting phenotype. Several tumors express high levels of gangliosides – sialic acid containing glycosphingolipids – which can suppress APC differentiation (McKallip, Li et al. 1999). IL-6 from tumor cells differentiates macrophages to a highly phagocytic but poorly presenting phenotype (Duluc, Delneste et al. 2007). Other mechanisms are also present. Tumor-derived chemokine CCL-21 promotes an immunotolerant microenvironment by recruiting regulatory T cells (Shields, Kourtis et al. 2010), and expression of immune checkpoint ligands – like PD-L1 – on cancer cells is associated with poor prognosis (Thompson, Gillett et al. 2004), by an immune evasive mechanism. On the other hand, this poor prognostic can be turned to an advantage when treating with checkpoint inhibitors.

A multitude of immune cells has the ability to facilitate tumor outgrowth. The role of the alternatively activated macrophage and the different Th cells subsets have already been discussed. Additional cell types are commonly present within the tumor microenvironment, including myeloid-derived suppressor cells (MDSCs), dendritic cells, eosinophils, mast cells, platelets and B cells (Kuznetsov, Marsh et al. 2012, Ayari, LaRue et al. 2013, Jordan, Amaria et al. 2013, Yang, Lee et al. 2013, Khatami 2014), with complex effects on tumor progression.

From the above, it follows that the interplay between tumor cells and the immune system is highly complex and dynamic. After initial priming of the immune system caused by the initial encounter of transformed cells, what are the dominant factors that determine the outcome of immunosurveillance?
There is a complex interplay between $CD4^+$ T cell subsets, macrophage and other immune cells, having multidirectional influences on each other, creating positive feedback loops and establishing immune environments with either anti-tumor or pro-tumor effects. The net result of these processes depend on features of the tumor (e.g. expression of immunosuppressive factors and ability to withstand cytotoxic effects), the presence of inflammation and other danger signals, and is influenced by treatment with pharmacological agents and other interventions that affect the tumor cells, stroma cells or immune cells. In a state of equilibrium, changes in the interaction between tumor cells and surrounding stroma can distort the balance and facilitate growth of the tumor. Such mechanisms may orchestrate the process by which tumor cells that are controlled for years by the immune system can start expanding and become clinically relevant.

1.7 Multiple myeloma

Multiple myeloma (from now on referred to as "myeloma") is a common hematological disease, accounting for 12-15 % of hematological neoplasms.(Greenlee, Murray et al. 2000) With an incidence of about 7/100.000(Turesson, Velez et al. 2010) it constitutes about 1% of all malignancies (Greenlee, Murray et al. 2000). The malignant clone originates from a neoplastic post germinal center plasma cell (Matsui, Wang et al. 2008), and most often produces a monoclonal immunoglobulin of non-IgM type. A significant number of cases present with cells secreting only light chains (light chain disease), accounting for about 16 percent.(Kyle, Gertz et al. 2003) In addition, some patients, estimated at < 3 percent, do not secrete any light or heavy chains, most often due to inhibited secretion (Lonial and Kaufman 2013).

In practice, all patients with myeloma are thought to undergo a phase of premalignant clonal plasma cell expansion before developing overt disease (Landgren, Kyle et al. 2009). This condition is referred to as monoclonal gammopathy with unknown significance (MGUS), and is defined by the presence of a monoclonal immunoglobulin (or pathological kappa/lambda ratio) in plasma at a concentration <3g/dL, less than 10% plasma cells on bone marrow examination and no evidence of any end organ damage related to the clone (International Myeloma Working 2003). The plasma cells reside in the bone marrow and depend on the microenvironment to sustain and expand its growth. Identified stromal influences of importance for myeloma survival and growth include factors supporting angiogenesis(Kumar, Fonseca et al. 2003), suppression of cell-mediated immunity (as discussed previously), cytokine stimulation with IL-6 and VEGF(Vacca, Ria et al. 2003) and growth promoting mesenchyme derived exosomes(Roccaro, Sacco et al. 2013).

The transformation from MGUS to multiple myeloma is defined by the appearance of end organ damage in the form of osteolytic bone lesions, hypercalcemia, anemia or renal failure. Osteolytic lesions, the most characteristic feature of the clinical myeloma disease, develop in 80% of myeloma patients with pathologic fractures occurring in 40 to 50 percent (Saad, Lipton et al. 2007). The osteolytic lesions are indicative of the uneven distribution of the myeloma bone disease, characterized by areas of bone destruction, soft tissue tumors and areas of apparently unaffected cortical bone. The cause of the osteolysis is the disruptive influence of bone remodeling e xerted by myeloma cells, uncoupling the tight co-operation of osteoclasts and osteoblasts in favor of the former.

Renal failure in myeloma can be multifactorial. Protein deposits, plasma cell infiltration, cryoglobulinemia, hyperuricemia and hypercalcemia are common culprits, but the main cause is light chain cast nephropathy caused by light chains accumulation within the renal tubuli, causing toxic damage to the tubular epithelium. The most common clinical presentation is a reduction of glomerular filtration and often minimal proteinuria. Together with the pronounced breakdown of calcified bone, the reduced capability of the kidneys causes hypercalcemia, which can be symptomatic or discovered by routine testing. The renal impairment is also involved in inducing anemia, with bone marrow replacement often only playing a minor part. Renal failure is sometimes involved, but the most prominent cause is the dyregulated metabolism of "anemia of chronic disease"(Katodritou, Dimopoulos et al. 2009).

Prognosis in myeloma patients is generally poor, with a median survival of about 5-7 years(Liwing, Uttervall et al. 2013), although there are pronounced inter-individual variability, with survival ranging

from <2 years to more than 20 years. There are different ways of calculating prognosis in a particular patient, using characteristics of the patient, the tumor or of the organs affected. However, the dominant prognostic determinant is the presence of certain high-risk karyotypes within the malignant clone.

Current treatment regimens are mainly based on different combinations of chemotherapeutic agents along with glucocorticoids and, for the younger patients, autologous stem cell transplant. The goal of treatment is to eliminate as much as possible of the malignant plasma cell pool to facilitate long-term remission. The most widely used traditional myeloma drug is the alkylator melphalan, discovered in the 1950s. Together with cyclophosphamide and the more recently introduced bendamustin, it formed the alkylator backbone in myeloma treatment for decades, and is still considered elemental in myeloma treatment. Within the last decade, several drugs with other mechanisms of action have been introduced in myeloma treatment, collectively referred to as novel drugs. This includes the resurgence of the anti-angiogenic compound thalidomide, and its derivative lenalidomide and a family of proteasome inhibitors. Within the next couple of years, antibodies(van der Veer, de Weers et al. 2011) and histone deacetylase inhibitors(Dimopoulos, Siegel et al. 2013), currently undergoing phase III clinical studies, are expected to be added to the clinical armamentarium.

1.7.1 Immunoglobulin synthesis and light chain secretion

Antibodies consist of two identical heavy chain polypeptides and two identical light chain polypeptides, of either κ or λ type(Pilstrom 2002). The light chain production is 10-40% in excess of heavy chain production, leading to free light chains secreted into the circulation(Hopper and Papagiannes 1986), and rapidly cleared from the blood by the kidney(Waldmann, Strober et al. 1972). The free light chains has been regarded as having no known biological function(Pilstrom, Lundqvist et al. 1998).

In the bone marrow, the V(D)J-regions of heavy and light chains undergo gene rearrangement as part of ensuring diversity in antibody repertoire (reviewed in (Maizels 2005)). First, the heavy chain segments recombine, and the translated protein bind to invariant chain selectively expressed in progenitor/precursor B-cells(Melchers 2005), before the complex moves to the surface of the cell. If this is not successful, the pre-B cells die; if it is, invariant chain expression turns off and light chain rearrangement begins (Melchers, ten Boekel et al. 2000). Upon light chain rearrangement and production, heavy and light chains assemble in the endoplasmatic reticulum (ER), form complete immunoglobulins, and are transferred to the plasma membrane where they form B cell receptors (BCR)(Askonas and Williamson 1966). Excess light chains are secreted. Heavy chains not paired with light chains, are retained in the (ER), and eventually degraded by proteasomes (Fagioli, Mezghrani et al. 2001). The constant intracellular pool of excess light chains mediates assembly, secretion and hence elimination of heavy chains, and prevents toxic heavy chain aggregates (Askonas and Williamson 1966, Haas and Wabl 1984).

After functional recombination has succeeded, the gene is transcribed as a long primary mRNA, yielding differently cleaved and spliced mRNA molecules according to the maturity of the B cell. Alternative RNA processing generates immunoglobulin either with or without the transmembrane domain that anchors it to the plasma membrane. In B cells that differentiate into antibody-producing plasma cell, most transcripts are of the secreted type.

In myeloma, light chain escape – relapse of cells with only light chains secreted – is observed in approximately 3% of relapsed patients (Kuhnemund, Liebisch et al. 2009). The first observation was done in 1969(Hobbs 1969), but the incidence may be increasing as a result of new biological treatments(Dawson, Patil et al. 2007, Qu, Zhang et al. 2010). The mechanistic basis of this phenomenon is largely unknown, one theory is a proliferative advantage of light-chain-only clones (Ayliffe, Davies et al. 2007).

1.8 Murine models of myeloma

The microenvironment of the bone marrow is an important player in the pathogenesis(Anderson and Carrasco 2011), immune evasion and drug resistance(Tassone, Tagliaferri et al. 2009) of myeloma, and must be fully appreciated to evaluate the immunotherapeutic opportunities in this disease. To understand the pathogenesis, drug resistance mechanisms and the possibilities of immunotherapeutic interventions against myeloma, it is therefore desirable to gain further insight into the interplay between malignant cells and the microenvironment of the bone marrow and extramedullar tumors in myeloma patients. To facilitate such studies, a number of animal models have been developed. Several murine models are currently in use, with different strengths and weaknesses. The 5TMM line is a serially transplanted tumor line from spontaneous myeloma in C57BL/KaLwRij aging mice (Radl, Hollander et al. 1978), resembling many of the clinical features from human myeloma, including monoclonal gammopathy, renal involvement, hypercalcemia and bone lesions (Asosingh, Radl et al. 2000). This cell line has contributed considerably to pathophysiological understanding (Heath, Vanderkerken et al. 2007), but its biology is still different from humans, it lacks genetic heterogeneity, and the microenvironment of these tumor cells does not resemble that of human myeloma cells.

Other models have been devised by genetical engineering, with the insertion of myeloma oncogenes and B-cell lineage tumor suppressor genes. Vk*myc mice overexpress c-myc under the control of the kappa light chain promoter in post-germinal B cells. Mice of this strain develop a monoclonal gammopathy, decreased bone mineral density and anemia (Chesi, Robbiani et al. 2008). The model can be used to predict clinical drug efficacy (Chesi, Matthews et al. 2012). Another transgenic model, the pEμXBP-1, with spliced X-box-binding protein (XBP)-1 targeted to the B cell lineage, exhibit features resembling MGUS with progression to myeloma in a certain number of animals (Carrasco, Sukhdeo et al. 2007). The model demonstrates dysregulation of genes commonly affected in myeloma patients, and can be used to study the evolution of genetic lesions during the progression of disease. Also here the stroma has obvious differences to the

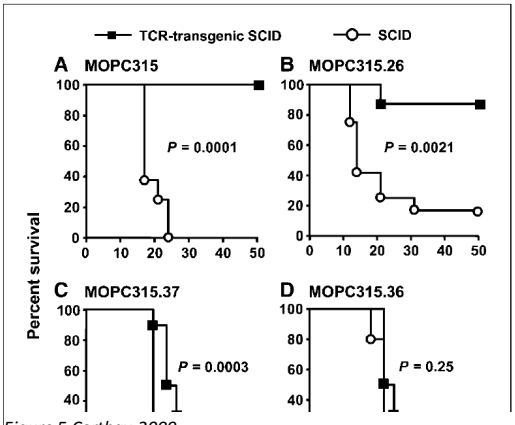

Figure 5 Corthay 2009.

Antigen secretion by myeloma cells is required for cancer immunosurveillance mediated by $CD4^+$ T cells. Id ($\lambda 2^{315}$)–specific TCR-transgenic SCID mice or control non-transgenic SCID mice (8–12 mice per group) were injected s.c. with $3x10^5$ of the indicated tumor cells: A, MOPC315, which secretes complete IgA with $\lambda 2^{315}$ chain; B, MOPC315.26, which secretes free $\lambda 2^{315}$ L-chains only; C, MOPC315.37, which produces but does not secrete free $\lambda 2^{315}$ L-chains; and D, MOPC315.36 which has lost expression of the $\lambda 2^{315}$ L-chain (antigen loss variant). Tumor growth was followed over time by palpation.

human microenvironment, and the tumor does not develop in a natural milieu.

Another approach is to inject cell lines of human or mouse origin, subcutaneously or otherwise, into immune compromised mice. Especially with subcutaneous injection, monitoring is easy by a simple caliper, and some models have fluorescent or bioluminescent markers, making it possible to follow tumor growth both subcutaneously and systemically (Mitsiades, Mitsiades et al. 2003, Hofgaard, Jodal et al. 2012). Certain lines are homing to the bone marrow developing osteolysis resembling myeloma (Hofgaard, Jodal et al. 2012). One limitation to the relevance of such cell lines is the fact that most of them are derived from extramedullar disease, and hence do not depend on the stroma to the extent that normal human myeloma does. They tend to change phenotype when in culture, grow rapidly when injected, but they can however be a valid model for studying drug - or immunotherapy. The use of human cell lines is also restricted to immunocompromised mice, precluding studies of host immune responses.

The SCID-hu mouse model was developed to study human hematopoiesis by implantation of intact human bone marrow, and engraftment of human myeloma cells in fetal bone chips have been shown with clinical signs like monoclonal gammopathy, hypercalcemia and bone lesions resembling human myeloma (Urashima, Chen et al. 1997). This model probably better recapitulates the human disease, and can possibly be used to evaluate drug combinations in samples from a particular patient. Still, limitations include the introduction of allogeneic fetal bone chips, their limited availability and the lack of immunologic activity seen in wild type animals. Using rabbit bones circumvents the availability problem (Yata and Yaccoby 2004). A synthetic variant of the SCID-hu model was recently developed, in which the inserted "bone" is artificial, reproducing the microarchitecture of human bone. It manages to support engraftment of human myeloma cells (Calimeri, Battista et al. 2011), is not allogeneic and availability is not a problem. The weakness is that the model is not studying the immunological component of myeloma elimination, immunoediting, growth and treatment.

1.8.1 The MOPC315.4 model

Intraperitoneal injection of mineral oil reliably induces plasmacytomas, designated Mineral Induced plasmacytomas (MOPC), in certain susceptible mouse strains (Potter 1972). The mineral oil appears to act as a local irritant within the peritoneum, causing recruitment of macrophages and B cells, and inducing high levels of IL-6 secretion (Salwa 1980). These tumors have been utilized in a number of immunological tumor studies, often after serial transplantation after subcutaneous, intraperitoneal or intravenous injection (Lauritzsen, Weiss et al. 1994, Dembic, Schenck et al. 2000, Corthay, Skovseth et al. 2005, Corthay, Lundin et al. 2009, Haabeth, Lorvik et al. 2011). They grow well in culture, and home to the bone marrow when injected intravenously, although with different kinetics depending on the originating cell line and subclone (Hofgaard, Jodal et al. 2012). MOPC315 is a cell line that secreted a monoclonal IgA designated M315 (Lauritzsen and Bogen 1993). The MOPC315.4 variant was developed by two sequential subcutaneous transplantations and clonal selection of cells with high M315 production to obtain a cell line with reproducible *in vivo* growth characteristics (Lauritzsen and Bogen 1993). The cell line is found to be MHC class II negative (Lauritzsen and Bogen 1993, Dembic, Schenck et al. 2000), even in the presence of high levels of IFN-γ (Corthay, Skovseth et al. 2005).

By immunizing BALB/c mice with the $\lambda 2^{315}$ light chain and complete Freund's adjuvant, $CD4^+$ T cells specific for an epitope within the M315 light chain were isolated and cloned (Bogen, Malissen et al.

1986). These cells were found to recognize an epitope within the CDR3 region of $\lambda 2^{315}$ containing amino acids 94, 95 and 96, which result from somatic mutations in the germ-line V lambda2 315 locus (Bothwell, Paskind et al. 1982, Bogen, Malissen et al. 1986). These T cell clones were shown to confer anti-tumor activity when injected with tumor cells in a Winn assay (Lauritzsen, Weiss et al. 1993), and the TCR (α/β) sequence from one of them (4B2A1) was used to generate a T cell receptor transgenic (TCR-Tg) mouse model on a BALB/c background (Bogen, Gleditsch et al. 1992). The TCR-Tg mice were protected against subcutaneous challenges with MOPC315.4 cells (Bogen, Munthe et al. 1995). Later, this transgene was backcrossed with homozygous SCID mice to generate a model with ensured Id-reactivity in all T cells and which did not involve $CD8^+$ T cells and B cells (Bogen, Munthe et al. 1995). These TCR-Tg SCID mice were also protected against MOPC315 tumor development, demonstrating the direct ability of idiotype (Id)-specific $CD4^+$ T cells to mediate immunosurveillance (Bogen, Munthe et al. 1995). Both types of mice were protected against challenge with the light chain-only producing MOPC315.26 variant, but not against the non-Id-producing MOPC315.36 or the MOPC315.37 variant, which produces but does not secrete M315 (Corthay, Lundin et al. 2009).

1.9 Id-driven $CD4^+$ T cells immunoprotection

Idiotypes are genuine tumor-specific antigens with unique determinants formed within the variable regions during somatic hypermutation. The secreted antibody is endocytosed and processed by APCs within the tumor and draining lymph nodes, and presented to $CD4^+$ T cells in the context of MHC II molecules (Bogen, Munthe et al. 1995). (A discussion on $CD4^+$ T cell anti-tumor mechanisms in this situation is found in chapter 1.2.) Whether it is transported there in the lymph fluid or by migrating dendritic cells is still an unsolved issue. Naïve T-cells recognizing the antigen are thereby activated, eventually returning to the circulation and the tumor (Lauritzsen, Weiss et al. 1994). If the egress from the lymph node is prevented by the drug fingolimod, the tumor protection is disrupted, indicating that the T cells need to be activated in the lymph node, and need to return to the tumor post-activation (Lorvik, Bogen et al. 2012). Secretion of Id by the tumor is required for T cell activation and tumor rejection, see figure 5(Corthay, Lundin et al. 2009) and (Corthay, Lundin et al. 2009). Within the tumor the T-cells re-encounter Id-derived epitopes presented by tumor-infiltrating macrophages. Cognate interaction leads to macrophage activation and skewing towards a tumoricidal phenotype, competent of killing tumor cells nearby (Dembic, Rottingen et al. 2001) with mechanisms still under investigation. It is important that T cell activation start when the tumor load is small, because increasing amounts of antigen will eventually induce T cell tolerance, leaving tumor growth uninhibited (Lauritzsen, Hofgaard et al. 1998).

Recently the molecular mechanisms behind this immunoprotection was elaborated in papers from our group. By introducing the matrigel cytokine assay, tumor-infiltrating immune cells and locally secreted cytokines was qualitatively and quantitatively assayed on different time points during the immune response. A set of nine cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-6, IFNγ, CXCL9 and CXCL10) was associated with effective cancer immunosurveillance, resembling a profile of Th1-driven inflammation (Haabeth, Lorvik et al. 2011). Histological phenotypes of activated macrophages in the tumor infiltrate correlated with gene expression microarrays. Based on these data, a biochemical signature for successful immunoprotection was derived, showing that macrophages and Th1-cells work together in an antigen-restricted manner, inhibiting tumor growth. IFNγ from Th1-cells

activates macrophages in a classical way, to inhibit or kill tumor cells, and to secrete anti-angiogenic chemokines, preventing tumor growth and survival.

In another paper, the $CD4^+$ T cells in activated lymph nodes and at the tumor site were extensively characterized by means of flow cytometry and gene-expression microarray, to widen the knowledge of the molecular mechanisms behind successful immunoprotection by $CD4^+$ Th1 cells. During Th1-cell activation, 29 cell surface molecules and 609 genes were up-regulated, while five surface molecules and 284 genes were down-regulated(Lorvik, Haabeth et al. 2013).

The direct killing mechanism executed by the macrophage to kill tumor cells is still unknown. When activated, the macrophage is able to kill susceptible cells in its vicinity. Theoretically, it should be equally able to kill tumor cells whether or not they have antigens recognizable to the adaptive immune system. Antigen-loss bystander cells, should thus be eliminated similarly to antigen-expressing tumor cells, if this antigen provides cognate recognition by $CD4^+$ T cells when presented by tumor macrophages in the vicinity of both cell clones. This will depend on over wh at distance macrophages are able to display cytotoxicity, and on other factors affecting the macrophage phenotype. On the other hand, if no recognized antigen is present, the macrophages will possibly be tumor-promoting, as discussed earlier in this introduction.

Whether Id-driven T cells responses act primarily by inhibition of tumor cell growth or by direct killing of tumor cells is still not fully determined. As discussed earlier, there are examples of the immune system being able to suppress tumor cells for extended periods, without being able to eliminate it. In this dormant – or equilibrium – situation, the continuous possibility of tumor escape endures, upon clonal evolution of the tumor or tolerizing interaction with the immune system. Preliminary observations of late tumors have suggested that such mechanisms might be at work in our system. This thesis set out to elucidate these facets of the T-cell-macrophage-tumor-cell interaction, focusing on bystander killing, tumor escape, and the phenotype of tumor-associated macrophages.

2. Aims of the study

The aim of the current work was to investigate characteristics of indirect $CD4^+$ Th1 T-cell-induced, macrophage-mediated, tumor control in a mouse myeloma model. Previous studies have demonstrated the robust protection provided by Id-reactive T cells against Id-peptide-secreting MOPC315 mouse myeloma cells, and that the nature of this protection is indirect via T-cell induced activation of macrophages present in the tumor stroma.

The indirect mechanism of tumor elimination, effectuated by cells that do not themselves recognize tumor cells directly, led to three important questions, which we have addressed in this work.

1. Will antigen-negative tumor cells interspersed with antigen-positive tumor cells also be eliminated by the unselective killing mechanism executed by macrophages, and if so, what are the limitations regarding ratios and distances?

2. When there is no direct link between tumor cell recognition and killing, is it possible to eradicate all tumor cells from an individual animal, or will there be an eventual escape of tumor cells?
3. In the absence of interaction with Th1 cells, are tumor-infiltrating macrophages growth-supportive in vivo? Is it possible to therapeutically reduce such tumor-supportive effects?

3. Summary of results

Paper 1: Indirect CD4^{+} T-cell-mediated elimination of MHC II^{NEG} tumor cells is spatially restricted and fails to prevent escape of antigen-negative cells, Schjesvold & Tveita et al. Eur J Immunol, 2014

In this paper, we investigated the immune response dynamics when injecting antigen-negative tumor cells mixed with antigen-positive tumor cells. The lack of specificity of macrophage-mediated cytotoxicity could facilitate killing of antigen-negative escape variants of the original tumor. Surprisingly, in all ratios possible in the experiment, the antigen-negative cells grew unimpeded, forming tumors with normal growth dynamics. We further demonstrated that this was due to spatial restriction of the tumoricidal activity to areas dominated by antigen-positive tumor cells. In conclusion, the macrophages do not confer clinically relevant bystander killing in vivo.

Paper 2: Tumor escape from CD4+ T cell-mediated immunosurveillance, Tveita, Schjesvold et al. Cancer Research, 2015

In this paper, we followed fluorescently marked tumor cells injected subcutaneously in TCR-transgenic protected mice, to see what would happen with the tumor cells over time. As expected, the signal decreased after the initial growth spurt. More surprisingly regarding our previous experience with this model, the signal persisted at a low intensity over time, and eventually and gradually manifested itself as a tumor. When transplanted to other TCR-transgenic mice, the protection was lost, and tumor growth equaled growth in un-protected mice.

Further investigations demonstrated that, despite the presence of the relevant CD4+T cell epitope in both complete immunoglobulin and in free, non-complexed light chains, the latter constitutes the predominant source of antigen for presentation to T cells. Accordingly, the basis of immune escape was found in a diminished secretion of surplus free light chain, without change in secretion of the complete immunoglobulin. In conclusion, the tumor was never eradicated and eventually escaped by impairing indirect presentation of tumor antigen.

Paper 3: CSF1R-inhibition delays growth of myeloma cells in a non-T-cell-dependent manner, Schjesvold et al. Submitted (PLOS One)

In this paper, we studied the significance of macrophage phenotype and macrophage presence in our subcutaneous myeloma model. We treated TCR-transgenic murine tumor recipients with inhibitors of CSF-1 during expected tumor immunosurveillance, and saw a pronounced reduction in macrophage numbers, while the phenotype of residual macrophages remained predominantly M1-like. This reduction in macrophage abundance did not affect successful T-cell-mediated tumor killing. When treating wild-type tumor recipients, we showed the same reduction of macrophages (but in this mice with M2-like features) correlating with a significant delay in tumor growth. In conclusion, macrophages can have both tumor-promoting and tumor-killing, depending on interactions with the adaptive immune system. Reduction of macrophage numbers can reduce the tumor-promoting ability without abrogating tumor immunosurveillance.

4. Methodological considerations

Detailed descriptions of methods are provided in the respective papers included in this thesis. General aspects of specific methods, their advantages and limitations are discussed below.

4.1 Validity of the model system

The complexity of the adaptive immune system, with its diversity in terms of antigen specificities makes studies of antigen-specific T cell responses challenging. This is particularly true when addressing issues related to the dynamics of immune responses. Working with T cell receptor-transgenic mice offers a valuable reductionist approach to this aspect of immunobiology, and allows unique opportunities to dissect T cell-driven immune responses. A the same time, it is obvious that results obtained in such mice cannot be directly extrapolated to humans, and it has become increasingly clear that aspects of both the innate and adaptive immune system in mice differs from that in humans (Mestas and Hughes 2004). Because of the large diversity in T cell repertoire in wild-type mice, the number of naïve T cells specific for different epitopes is very small, with results ranging from a clone size that might be as small as one for many clonotypes, to 1500 in the most abundant ones (Casrouge, Beaudoing et al. 2000, Jenkins and Moon 2012, Tubo, Pagan et al. 2013). The difference to the transgenic mice where all (SCID mice) or most of the T-cells (BALB/C) are specific for the tumor antigen is large. It is perhaps most appropriate to interpret results from experiments done in transgenic TCR models as representing the full potential of an immune response initiated by a single antigen.

Studying patient derived myeloma cells is challenging. Plasma cells and myeloma cells die rapidly after aspiration from their microenvironment in the bone marrow, and in vitro culturing even with cytokine additions is difficult beyond a few days. Patient derived myeloma cell lines that grow autonomously is mainly from patients with advanced disease (Matsuo, Drexler et al. 2000), sometimes in extramedullary sites, distinguishing them from most myeloma cells. The MOPC315 murine myeloma cell line was originally generated by Eisen and co-workers in 1968 by injecting mineral-oil (Eisen, Simms et al. 1968) into the peritoneum of the inbred mouse strain BALB/c. The derivative MOPC315.4 was obtained by *in vivo* passaging to attain cells displaying more aggressive growth properties. The cells are independent from the bone marrow, with a rapid growth pattern both in vitro and subcutaneously in vivo. With a well characterized tumor antigen (Id) and a transgenic mouse model recognizing this antigen in a CD4/MHCII-restricted manner, the model is well adapted to study facets of this immune response. While these experiments were performed, our lab also refined the cell line to (MOPC315.BM) have characteristics more similar to the clinical myeloma disease (Hofgaard, Jodal et al. 2012).

There are also several other murine myeloma models with different advantages. The xenograft models, where human cell lines or patient derived myeloma cells are injected into either fetal, lapine or synthetic bone embedded subcutaneously into SCID mice, makes a very relevant model for human disease, but the immune suppression implicated in the model, makes them unsuitable for immunological studies. The 5T series is like our model a murine "myeloma-like" model, originally a spontaneous tumor in a small fraction of C57BL/KalwRij mice (Radl, Hollander et al. 1978). In that series the 5TGM1 is the most similar to our model, displaying autonomous growth (not present in the 5T2MM), osteolytic lesions (not present in the 5T33MM), and with a quite rapid tumor take. Being a spontaneous model homing and growing orthotopically, it probably presents a better option for describing the myeloma disease, but the available transgenic mice targeting the Id tumor antigen

secreted from the MOPC cells are essential for our immunological studies. The available transgenic models are hampered by both the latency of tumor development and lack of known tumor epitopes (Cheung, Kim et al. 2004, Carrasco, Sukhdeo et al. 2007, Chesi, Robbiani et al. 2008). The extensive latency will also pose challenges in studying a continuously evolving immune system.

To facilitate monitoring of tumor growth, the s.c. route is commonly utilized for the introduction of tumor cells in murine studies. Although myeloma cells are most commonly localized in bone marrow, the MOPC315 grows readily within the s.c. space. The limitations of such non-orthotropic systems need to be taken into account, especially when addressing interactions between tumor-infiltrating T cells and stromal cells such as tumor-associated macrophages. On the other hand, the need for cells to grow readily *in vitro* will inadvertently require adaptations in cellular characteristic that are likely divergent from those seen in a tumor growing in its natural environment.

In the end, artificial systems like this can still give insight into the mechanisms of action and interplay between stromal and immune cells, and cancer cells. The results from studies like these, while not accurately reflecting the situation neither in normal mice nor in humans, provide hypotheses on key "real world" interactions, paving the road for studies on strategies to exploit the immune system in cancer treatment, which is the ultimate goal in tumor immunology.

4.2 Tumor specific (Id-specific) T cell receptor transgenic mice

Tumor-specific (Id-specific) TCR-Tg SCID mice were generated by Bogen *et al.* as described in the introduction part of the thesis (1.8.1). Importantly, in these mice allelic exclusion of endogenous TCRα and TCRβ chains is complete, ensuring a single specificity of all T cells (Bogen, Munthe et al. 1995). Severe combined immune deficiency (SCID) is a rare congenital disease syndrome that results in loss of B and T cell immunity (Rosen, Cooper et al. 1984). The condition is due to a rare recessive mutation on Chromosome 16 responsible for deficient activity of the protein kinase Prkdc that is activated by DNA, and is a catalytic polypeptide enzyme involved in DNA repair. Cancer immunosurveillance studies done in TCR Tg SCID are un-physiological in the sense that they exclude the potential contributions of B cells, γδ T cells, NKT cells, and $CD8^+$ T cells to antitumor immunity (Bogen, Munthe et al. 1995). Moreover, the high frequency of T cells with a single specificity is highly unlikely to appear in a normal immune system. Nevertheless, SCID mice have proven very useful in basic research of normal and diseased conditions (reviewed in (Bosma and Carroll 1991)). Importantly, we are fully aware that $CD8^+$ T cells, B cells, NKT cells, and γδ T cells may play pivotal roles in antitumor immunity, although we have repeatedly shown that tumor-specific $CD4^+$ T cells may also mediate successful antitumor immunity in the absence of such cell types (Lundin, Hofgaard et al. 2003, Corthay, Skovseth et al. 2005, Haabeth, Lorvik et al. 2011).

The high frequency of tumor-specific $CD4^+$ T cells in tumor-specific TCR Tg mice renders it resistant to immediate tumor growth after s.c. injections of syngeneic MOPC315 tumor cells, while non-transgenic mice develop fatal tumors (Lauritzsen, Weiss et al. 1994, Bogen, Munthe et al. 1995). Protection is dependent on the total number of tumor cells injected. While TCR Tg SCID mice are temporarily completely protected when injected with cells in the range of $10^5 – 2x10^5$ per mouse, increasing cell numbers cause a dose-dependent loss of protection (Bogen, Malissen et al. 1986). Berge *et al.* showed that in non-SCID tumor-specific TCR Tg mice injected with 2×10^6 MPOC315 cells 10 out of 11 mice developed tumors (Berge, Gronningsaeter et al. 2012). Moreover, the injection of 2×10^6 cells consistently resulted in the development of palpable tumors in nearly all tumor-specific

TCR Tg SCID after 60 days (B. Bogen, unpublished data). Although the inflammatory reaction is at its strongest about one week after injection, earlier studies have observed a persistent antitumor immune response lasting for more than 60 days (unpublished data). Quantification of the tumor-specific Id peptide in serum provides a surrogate marker of tumor load. Studies published by Corthay *et al.* in 2005 showed that the Id peptide concentration in serum of TCR Tg SCID mice increases during the first 6-8 days after injection, and on day 12-14 declines to levels below the detection limit in most mice (Corthay, Skovseth et al. 2005). In 2011 Haabeth *et al.* confirmed that this inflammatory response is driven by tumor-specific Th1 cells (Haabeth, Lorvik et al. 2011).

The Id-specific TCR Tg SCID model enables us to study in detail the response driven by the $CD4^+$ T cells in cooperation with the innate immune system. But the system also precludes studies of $CD8^+$ T cells and B cells in collaboration with the $CD4^+$ T cells, making the experiments increasingly non-physiological. Data from such studies still provide proof-of-concept for actions and effects provided by the cell types under study, expanding our knowledge of their capabilities and modes of action.

To overcome some of these challenges, we also performed experiments in Id-specific TCR-Tg Balb/c mice, the original model. This model is different from the SCID model in many aspects. They express normal numbers of B cells and antibodies (Bogen, Gleditsch et al. 1992). They also contain $CD8^+$ cells, most of which express endogenous α (α_E) chains in addition to the transgenic α (α_T) chain. In such mice, the $CD4^+$ cells often co-express $\alpha_T\beta_T$ and $\alpha_E\beta_T$ receptors (Bogen, Munthe et al. 1995). As a result, the mice are also capable of recognizing antigens other than M315, and potentially also recognize other epitopes in M315. The mice are at most only slightly immunodeficient, and as such they constitute a less artificial model system, serving as a necessary control for the results in our experiments (paper 3).

4.3 Matrigel as tissue surrogate; in vivo and in vitro

Matrigel is a soluble basement membrane extract of the Engelbreth-Holm-Swarm (EHS) murine tumor (Kleinman, McGarvey et al. 1982). This extract resembles the complex extracellular environment found in many tissues. The Matrigel is liquid at 4°C, but when incubated at 37°C, the contained constituents polymerize, forming a solid plug. This results in a well-defined tumor bed that can be excised from the mouse and processed as a whole allowing analysis of both the cellular and extracellular compartment of the tumor. Matrigel is quite stable *in vivo* thus providing a basis for the investigation at various time points during the antitumor immune response. Especially when the tumor is small, it is easier to illuminate and characterize the immune cell migration and interactions with the tumor cells.

Matrigel in itself, although processed for the elimination of growth factors, contains several different growth factors and cytokines, and could therefore potentially have immunogenic properties that might influence these experiments. Earlier papers have eliminated this as a problem in our model (Haabeth, Lorvik et al. 2011), not showing a strong immune response when measuring macrophage infiltration and cytokine concentration in "empty" matrigel. Cytokines are generally unstable proteins with relative short *in vivo* half-lives (Finkelman and Morris 1999). We therefore assume that the cytokines present are rapidly degraded after deployment of the Matrigel.

Another issue is the potential of the Matrigel substance to support the initial survival and proliferation of injected tumor cells by providing growth factors and spatial niches. The use of Matrigel may limit the immune cell access to the tumor hence representing a barrier to the immune

system. In our experiments though, we see equal protection whether tumor cells are injected mixed with Matrigel or just as dissolved in PBS.

Matrigel, when solidified, constitutes a 3-dimensional framework in which tumor cells, immune cells and stromal cells can interact. To be able to follow this interaction more closely in real-time we established an *in vitro* Matrigel platform, to study growth inhibition (Paper 1). The Matrigel containing various mixtures of cells were allowed to solidify in 16-well CultureWell chambered cover glass. This allowed us to follow their growth patterns with a confocal microscope, in different combinations of cells and added antigen. The lack of complexity in this model makes the results difficult to generalize. Its best usage is probably to confirm and further elaborate findings originally seen in the in vivo models. In our studies the findings from the in vitro experiments confirmed theories from in vivo studies, but added mechanistic insights and illustrative advantages.

4.4 In vivo imaging

When injecting two different tumor lines in the same site, as well as when tumors were controlled in the dormant phase in live animals, we wanted to monitor tumor load whilst following potential tumor growth by palpation. To be able to do this, the lab utilized an IVIS Spectrum in vivo imaging system from PerkinElmer. Different tumor lines were transfected with different fluorescent labels, to be followed as separate entities while growing in the same site. This was pivotal to experiments in paper 1, where two lines were injected in a mixture, and in paper 2, where fluorescence was used to confirm the presence of tumor cells in the dormant phase with no palpable tumor.

The use of fluorescence imaging technology has some intrinsic technical limitations. All tissue harbors some level of autofluorescence, which is most pronounced in the lowest wavelength range. Hair on the skin is especially prominent in this respect. We therefore shaved the mice at the start of every experiment, in an area around the injection site and wide enough to contain the growing tumor. With such preparations, we were able to visualize tumor loads down to about 2.5-5x10^4 cells. Titration experiments have revealed these numbers of injected cells closely match the minimum number required for consistent tumor take upon s.c. injection, so this detection limit seemed acceptable. We used the Living Image software to mark an area around the injection site, for evaluation of fluorescent signals. The cell lines did not have the same fluorescence, the GFP signal in antigen loss variants being much stronger than the mCherry signal in the parental line. Fortunately, this decreased the challenges posed by autofluorescence, since this is most pronounced in the GFP range. Some variation was observed between consecutive measurements. This was probably not reflecting the tumor load, but rather natural variations in many premises for the measurement. Especially with low tumor loads this could be difficult to interpret, but control mice followed with imaging without tumor cells provided good control. We also saw that the signal from dead cells disappeared completely within 24 hours. Within each experiment, the autofluorescence would increase marginally because of hair regrowth, unevenly distributed between individual mice , but not to an extent that caused any problem in longitudinal monitoring. When tumor growth eventually appeared, the fluorescence marker was still produced, with infrequent exceptions. All in all, the in vivo imaging approach provided a real-time picture of the tumor growth situation, and gave a good impression of tumor load when correlated to palpable tumors and serum levels of secreted myeloma protein.

4.5 Statistics

For tumor challenge experiments differences in survival rate were calculated using the log-rank test with GraphPad Prism 5 software. The log-rank test is a hypothesis test to compare survival distribution of two samples. It is a non-parametric test, and is more powerful than analyses based simply on proportions, and is appropriate to use when the data are censored, such as in the case for *in vivo* experiments in which some mice survive beyond the planned duration of the experiment. It compares survival across the whole timeframe, not just at one or two points.

The Mann-Whitney U-test was utilized to analyze flow cytometry data. The Mann Whitney test is a nonparametric hypothesis test for assessing whether one of two samples of independent observations tends to have larger values. All results are stated with mean ± SD, sample size and the significance level (P-value). The rational for not choosing students t-test, is mainly that Mann-Whitney can be used for both normally (Gaussian distributed) and non-normally distributed data. Moreover, student t-test compares means in a normally (Gaussian) distributed dataset, whereas the Mann Whitney test compares the sum of ranks, and is thus less prone to be affected by outliers.

5. Discussion and conclusions

Through the herein presented work, we have addressed different aspects of the mechanistic basis and limitations of $CD4^+$ T-cell induced macrophage tumor killing in a mouse model where MHC II-restricted T-cells in a TCR transgenic mice recognize an epitope of a secreted antigen, a lambda light chain from the myeloma cell line MOPC315. Spatial limitations (Paper 1), downregulation of secreted antigen (Paper 2), and the tumor microenvironments effect on the macrophage phenotype all affect the interplay between tumor and immune cells.

In Paper 1, we demonstrated that tumor-associated macrophages failed to prevent the outgrowth of antigen-negative tumor cells when mixed with antigen-positive cells before tumor challenge, even at high Ag^{POS}/Ag^{NEG} ratios favoring a strong immune reaction towards the antigen-positive cells in the mix. Antigen-negative cells were used as a substitute for the in vivo setting of a tumor losing its antigen by clonal evolution. The result was that in areas of antigen-positive cells, Th1 and M1 cells infiltrated and eliminated the tumor cells, while areas dominated by antigen-negative cells were infiltrated by less T cells and macrophages without signs of classical (M1) activation. The idea behind the experiments was to show so-called bystander killing, where activated unspecific macrophage killing would kill most cells in its close vicinity, and at least delay the outgrowth of antigen-negative cells, providing some protecting against antigen loss by clonal evolution. In our experiments there was no sign of bystander killing. While puzzling at first, several factors may contribute to explain this phenomenon.

Secreted tumor antigen, which we know is essential for this immune protection, will be higher the shorter distance from the secreting MOPC315 cell. Macrophages interacting with $CD4^+$ T-cells within such areas will become activated, increase their engulfment of more antigen, interact more with T cells, and at the same time scavenge the extracellular fluid for antigen, limiting diffusion to near areas with antigen-negative myeloma cells. Effective killing of myeloma cells, will lead to large amounts of antigen from the dying cells, increasing the inflammatory activity in that area, because of antigen uptake and presentation and hence activation of tumor infiltrating macrophages. In areas

dominated by antigen-negative cells, dying cell will not similarly contribute extra antigen for increased presentation. Another possible factor is that the continuous interaction with T cells might make the macrophage less mobile (Egeblad, Ewald et al. 2008), thus stabilizing its presence in the vicinity of the antigen-positive cells. The cognate interaction with IFNγ secreted by T cells into the immunological synapse might not be sufficient to induce activate neighboring macrophages which do not harbor the tumor-specific antigen, and thus do not directly interact with T cells (Huse, Lillemeier et al. 2006).

In the in vitro Matrigel assay we could confirm that in fact there is a very strict spatial limitation to the effects of an activated macrophage, with cells only a few hundred microns away from the macrophage growing unimpededly. From a physiological perspective, this is probably a requirement for continued tissue integrity when faced with the detrimental potential of activated macrophages. As of now our group has not been able to pinpoint the exact mechanism of macrophage -mediated cytotoxicity, but it is of great interest in our present work. The mechanism must work over short distances, cannot be transferred by supernatant, and does not need cell-to-cell-contact. Reactive oxygen species are possible candidates fulfilling these criteria.

Schietinger et al. (Schietinger, Philip et al. 2010) demonstrated bystander killing in their model using tumor cells containing both a CD4-restricted and a CD8-restricted tumor antigen in the same tumor cells. When both antigens were present in the tumor, but not within the same cells, tumor outgrowth was seen, most profoundly of the tumor cells carrying the CD4-restricted tumor antigen. When antigen-negative cells, or cells carrying one of the tumor antigens, were mixed with cells carrying both tumor antigens, there was a clear reduction in the probability of tumor outgrowth. The mechanistic basis of these findings is still unknown. One possibility is a macrophage-mediated tumor killing as in our model. The requirements for $CD8^+$ activity for effectiveness can be due to the lack of secretion of these antigens. Another possibility is that the stroma cells themselves are targeted, with secondary loss of tumor support, but experimental proof of this is lacking.

Even though we failed to observe bystander killing in our experiments, the artificial setup and small ratios between positive and negative tumor cells does not negate the possibility that with in vivo clonal evolution singular cells with antigen loss would be susceptible to $CD4^+$ T-cell-induced macrophage mediated killing. In spontaneous tumors in normal individuals there will also be a more heterogeneous T-cell response, facilitating recognition of various combinations of antigens on tumor clones. Whether this will increase the likelihood of bystander killing is not certain, but difficult to test in our reductionist approach. Our results indicate that this type of immune response will have difficulties containing clonal evolution with loss of recognized antigens. Furthermore, it tempers a widespread notion that macrophage activation induces widespread killing of surrounding cells in a more or less arbitrary manner. This may have implications for other types of cancer therapy in which macrophage-mediated effects play a role.

The results of Paper 2 builds on and extends the understanding of the results of Paper 1. In paper 1 we demonstrated that the tumor cell eliminating potential of activated macrophages demands interaction on very short distances, probably an appropriate limitation to the macrophage's lethal arsenal. It would be logical to assume that this indirect mode of action would make it difficult to eliminate every single cell from a malignant clone. Indeed, this is exactly what is observed though the phenomenon of tumor escape, which forms the basis of Paper 2.

In Paper 2 we observe that the tumor cell killing is not complete. Following T-cell mediated killing of the bulk of injected tumor cells, residual cells persist dormant in a state of equilibrium. This steady-state situation may form either through an equal number of cells being killed and being produced by proliferation, or by a subset of cells that remain in a non-dividing, quiescent state. In either case, the net result is a stable residual tumor burden that persists as long as the tumor cells carry the antigen recognized by the T cell. The fewer cells present, the less inflammation in the surrounding area maybe diminishing the migration of T cells and monocyte derived cells, creating a tumor small enough to avoid the immune system. If the tumor would have grown more, the increased inflammation might benefit the destructive immune response until the equilibrium again is reached. Subsequent analyses suggest that the majority of the residual tumor cells remain in a quiescent state which is gradually reversed with regained proliferation upon isolation an *ex vivo* culturing (A. Tveita, unpublished observations).

Eventually this equilibrium ends with escape of a tumor that bypasses full activation of an adaptive immune response. Upon isolation and re-injection into Id-specific TCR-transgenic mice, such escape tumor cells, even after cloning and prolonged culture, still retain their ability to resist T-cell mediated killing. These stable changes intrinsic to the tumor cells did not seem to be a result of clonal evolution, and could not be traced to pre-existing clones within the original tumor inoculum. Since the changes in antigen secretion by the tumor cells occurred in practically every single experiment, and at a fairly consistent time point, we hypothesize that the development of this escape phenotype may be the result of a stereotypic response to the inflammatory stress imposed by the activated macrophages.

Mechanistically, the basis for the loss of immune recognition was a dramatic reduction in the surplus secretion of free light chains. This notion represents a change in our understanding of this tumor antigen. Whereas the previous assumption was that the complete Ig molecule carrying numerically most of the epitopes was the major antigen variant responsible for T cell activation, careful evaluation of the immunogenicity of Ig fragments revealed that free light chains were much more efficiently displayed by macrophages and dendritic cells. Our speculation from these observations was that the assembled Fab region might prevent processing of the complete Ig molecules, in line with previous published evidence (Grey, Colon et al. 1982). These findings could be relevant for other tumor antigens forming complexes. By excluding mutations and reintroducing the light chain expression to induce rejection, we proved a causal link between the presence of free light chains and successful tumor rejection.

Another speculation from this paper relates to the mechanism linking the immunological stress, the dormancy state and the escape of a tumor cell with downregulated free light chains. As supported by mRNA expression analyses and FLC measurements from cell lysates, the down-regulation appears to occur at a transcriptional level. Normally there is a surplus of light chains to prevent heavy chain cytotoxic effects (Kohler 1980), while the high protein turnover in this cells through endoplasmatic reticulum (ER) stress (White-Gilbertson, Hua et al. 2013) makes the cells vulnerable for macrophage attack by reactive oxygen species.

We propose that a tradeoff between avoiding heavy chain cytotoxic effects, balanced by a reduced immunogenicity from reduction of free light chain availability, may serve as the basis for the Ig secretion phenotype observed in escape tumor cells. One theory could be that the immune response

which eliminates the bulk of injected tumor cells may allow for the survival of a small number of tumor cells in a quiescent state. Resumption of growth of such cells may require a metabolic adaptation, which may involve alterations in signaling pathways that also affect light chain transcription.

In Paper 1 and Paper 3 we investigated the important role of macrophages of different phenotypes in tumor biology, both as tumor-promoting cells or as part of immune protection. An M2-like phenotype, which is the typical state of a tumor-associated macrophage (Mantovani, Sozzani et al. 2002, Biswas, Gangi et al. 2006), generally conveys a negative prognostic impact (Pollard 2004, Mantovani, Allavena et al. 2008). On the flip side, the potential cytotoxicity of normally activated (M1) macrophages is well documented (Solinas, Germano et al. 2009). In Paper 1 we observed that Id-specific tumor-infiltrating Th1 cells increased the abundance of intratumoral M1 macrophages. The same type of skewing towards M1 phenotype was demonstrated by treatment with agonistic CD40-mAb, with successful tumor protection as result. This effect of stromal modification has also been seen with other tumor types (Beatty, Chiorean et al. 2011). One interesting aspect of the CD40-mAb experiments is that it bypasses the need of cognate recognition of tumor antigens, and could perhaps be an adjunctive measure enforcing T cell tumor therapy.

In Paper 3 we investigated another approach; medically removing an M2-phenotype stimulator in tumor-infiltrating macrophages, either by blocking CSF1R antibodies, or by utilizing a small-molecular-weight inhibitor (PLX3397) inhibiting the intracellular kinase of the receptor. In experiments with no tumor-antigen-specific T cells present, inhibition of CSF1R decreased the proliferation of tumor cells and hence the growth of the tumor. These results underline the dependence of myeloma growth on microenvironmental supportive factors, and reiterates the observations from Paper 1, where tumor cells were found to grow more rapidly when co-incubated with non-stimulated macrophages. Also, in the TCR-transgenic setting where cognate antigen recognition leads to macrophage stimulation, there was a comparable reduction in the total number of macrophages, but to an extent that did not prevent the indirect immune response underlying tumor control. If we could deplete the macrophages completely, immune control would probably be lost, and the tumor would grow in a fashion resembling that of PLX3397-treated wild type mice.

In conclusion, our model demonstrates the potential of $CD4^+$ T-cell-induced, macrophage-mediated immune responses as an immunotherapeutic strategy. In order to take full advantage of this potential it is important to understand the limiting mechanisms that can prevent the clinical effect. It is our hope that the results of this thesis may contribute to a further optimization of immunotherapeutic interventions that may fully exploit the cytotoxic effects of tumor-associated macrophages.

6. Future directions

6.1 How tumor cells are killed

Results in these papers, especially Paper 1, have brought new insight into the potential mechanistic basis of tumor killing. The experiments have suggested a non-cell-contact-dependent mode of action, still requiring a very short distance acting effector. Potential macrophage-derived factors, notably including nitrogen- and/or oxygen radicals fit this description, warranting further investigation.

6.2 Inflammatory stress and the link to downregulation of free light chain transcription

In Paper 2 we are speculating about a link between reduced free light chain production and a reduction in cellular stress. The next step would be to confirm this link and to find the mechanistic link between the stress the cells are put under and the reduction of free light chains. We have preliminary data indicating a role for the transcription factor NF-KB, and this should be investigated further.

6.3 Can the findings be replicated in a model of disseminated disease?

The subcutaneous model used in this thesis, although resembling an extramedullary plasmacytoma, has extensive differences to the real-life scenario where the myeloma cells are living in bone marrow, a totally different environment, with a number of microenvironmental stimuli supporting tumor growth. An important extension of these findings will therefore be to replicate and expand these experiments in more clinically relevant models, including mice harboring orthotopic tumors and in models of disseminated disease. Based on the MOPC315 model, our group has developed a version of the cell line; MOPC315.BM, which displays bone marrow-homing properties and recapitulates key elements of human myeloma, including osteolytic lesions and dissemination throughout the axial skeleton and long bones (Hofgaard, Jodal et al. 2012). Similar experiments in this model would strengthen the physiological relevance of our present findings and may allow us to address the impact of CD4+ T cells in advanced-stage myeloma.

6.4 The role of MHC class II

These experiments have been performed in a model with MHC II^{neg} cells, depending on tumor-associated MHC II^{pos} cells for antigen presentation. In this CD4^{+} model it would be important to more completely elucidate the role of antigen presentation in a model where the tumor cells also carry MHC class II. The group has a lymphoma model producing the same Id which may be used for this purpose (Lauritzsen, Weiss et al. 1994).

6.5 Clinical application

Further on, a local clinical trial using the knowledge gained about CD4^{+} T cell immune protection and the role of tumor-associated macrophage phenotype should be established at Oslo University Hospital. One option is autologous adoptive transfer of in-vitro cultured T cells collected and selected before autologous stem cell transplant, and re-infused after reconstitution of the haematological system in a situation with low tumor load. Although details in this approach should still be tested out pre-clinically, such a trial should be a long-term goal in our institution.

7. References

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