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Regulation of the regulatory T cell-attracting chemokine CCL22 in solid tumors

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"I have no idea what's awaiting me, or what will happen when this all ends. For the moment I know this: there are sick people and they need curing."

Albert Camus - The Plague

Contents

Chapter 1

Introduction: The human immune system

1.1 Discovery

The first description of the existence of the human immune system roots in the accurate documentation of the plague in Athens during the Peloponnesian War by the ancient Greek historian Thukydides. Beside his notable description of signs and symptoms of this particular disease he emphasized that only previously infected and cured persons were protected and could care for the ill citizens (Littman, 2009). Here, by describing immunological memory, Thukydides states one of the four known key features of the body's adaptive immune system (specificity, diversity, recognition of self and nonself, memory), which puts him far ahead of his time. Although further knowledge was gathered over time, the next milestone of immunology was not set until the late 18th century, when Edward Jenner discovered the protective effect of cow pox against small pox infection and thereby invented the first vaccine in history (Smith, 2011). Another hundred years later, Elie Metchnikoff discovered that white blood cells cover and engulf foreign bodies in starfish larvae and proposed that this mechanism (phagocytosis) might be responsible for the elimination of bacteria by leukocytes too (Tauber, 2003). Roughly at the same time, Emil Behring and Kitasato Shibasaburo developed the first antitoxin, an antibody against the diphtheria toxin by inoculating horses with the purified toxin. Thus, Behring and Kitasato unintentionally found the first evidence of the existence of the adaptive humoral immunity (Behring and Kitasato, 1890). During the 20th century, the field of immunological research widely expanded and several highly important discoveries were made all around the world, e.g. the existence of major histocompatibility complexes (MHC) (Snell and Higgins, 1951), the structure of immunoglobulins (Edelman and Gally, 1962), the existence and development of T and B cells (Miller and Mitchell, 1968), the antigen-presentation capacity of dendritic cells (Steinman and Witmer, 1978) and the regulatory potential of certain subsets of immune cells (Sakaguchi et al., 1995).

The current knowledge on the human immune system has allowed us to translate it into clinics not only in terms of prophylactic vaccination and therapeutic immunosuppression but also in treat-

ment of malignancies. For instance, the great improvements in treatment of malignant melanoma and other malignancies by using the so called checkpoint inhibitors (e.g. ipilimumab or nivolumab) show the tremendous potential of therapeutical immunomodulation.

The human immune system is composed by the innate and the adaptive immune system. The innate defense mechanisms act immediately and unspecificly upon activation whereas the delayed adaptive response is highly specific and can generate a long-lasting immunologic memory. Although this separation is somehow arbitrary and does not represent the complexity of the immune system, this systematic approach shall be followed due to its simplicity and lucidity.

1.2 The innate immune system

1.2.1 Cell types

The innate immune system is phylogenetically older than the adaptive one and can be found in almost every higher organism, e.g. plants, fungi, invertebrates and vertebrates (Schulenburg et al., 2004). It consists of several cell types, which are largely mature and functional at time of birth (Table 1.1).

Table 1.1: Innate immune cells

Cell type	Main function	Main localization
Neutrophils	Anti-bacterial activity	Circulating
Eosinophils	Anti-parasitic activity	Circulating
Basophils	Production of cytokines and mediators	Circulating
Mast cells	Production of cytokines and mediators	Tissue-resident
Monocytes	Precursor cells of macrophages	Circulating
Macrophages	Phagocytosis of bacteria and debris	Tissue-resident
Natural killer cells	Anti-viral and anti-tumor activity	Circulating
Dendritic cells	Antigen presentation	Tissue-resident
Epithelial cells	Barrier function	Tissue-resident

Neutrophilic granulocytes represent the biggest fraction of leukocytes in the human blood and show a distinct morphology when observed in the peripheral blood smear: According to their maturation status they contain a rod- or barlike nucleus (less mature), a 3- to 5-fold segmented nucleus (mature) or more than 5-fold hypersegmented nucleus (aged) and largely neutral stained cytoplasmic granules in routinely stained blood smears. Neutrophils combat bacterial infections mainly by phagocytosis and degranulation upon activation releasing antimicrobial enzymes and proteins, e.g. defensins, lactoferrin and lysozyme.

Eosinophils are hardly found in peripheral blood smears , since they account for less than 5% of circulating leucocytes, whereas they compose 20-30% of the intestinal lamina propria-resident cells (Jung and Rothenberg, 2014). Their eosinophilic cytoplasmic granula containing cytotoxic cationic proteins (e.g. major basic protein and eosinophil peroxidase) are the name-giving key property of this particular cell type. Eosinophils are involved in anti-helminthic responses and are commonly associated with hypersensitivity disorders like asthma and eosinophilic esophagitis (Rothenberg and Hogan, 2006).

Basophils and mast cells are often grouped together due to their highly similar morphology and function. Both possess many (basophilic) granules containing histamine, heparin and various eicosanoids and cytokines. They are considered as regulators of innate and adaptive immunity, might contribute to anti-parasitic defense and seem to have a crucial role in inducing a type 2 immune response (see below) (Voehringer, 2013). Furthermore, basophils and mast cells are key effector cells in pathologic conditions such as anaphylaxia and allergic asthma.

Macrophages and their circulating precursor cells (monocytes) are the main phagocytes in the human body. Macrophages are found in essentially every tissue and have - at least in part - tissue specific differentiation represented by tissue specific names, e.g. Kupffer cells, Hofbauer cells or osteoclasts. They ingest not only bacteria, but any foreign bodies, cancer cells and cellular debris, that is detected as non-self or non-intact by their pattern recognition receptors (PRRs). Although the common classification of macrophages in M1 and M2 subtypes has been discussed controversially in literature due to its great simplification and subsumption, it shall be followed in the following (Varol et al., 2015): M1 macrophages are considered to be the "killer" macrophages with pro-inflammatory properties such as secretion of high levels of IL-12, whereas M2 macrophages are believed to be designated to repairing and wound healing due to their immunosuppressive functions, e.g. secretion IL-10 and TGF-β. Interestingly, some tissue-specific macrophages, e.g. cerebral macrophages, the so-called microglia, seem to develop independently from the bone marrow hematopoietic stem cells but originate from yolk sac hematopoietic stem cells and persist throughout life (Schulz et al., 2012).

Natural killer (NK) cells are subsumed among the group of innate immune cells, which originate mostly from the myeloid lineage, although they differentiate from the lymphoid hematopoietic stem cell. In contrast to other immune cells, NK cells do not rely on MHC proteins or antibodies in order to detect infected or dedifferentiated cells due to their unique subsets of activating (e.g. NKG2D) and inhibitory receptors (e.g. CD94/NKG2A heterodimers). This allows a much faster response in case of infection, since no previous immunization is needed (Vivier et al., 2011).

Dendritic cells (DCs) were discovered in 1973, although the skin-homing subpopulation of DCs, the so-called Langerhans cells, were identified more than 100 years earlier (Steinman and Cohn, 1973). They play a pivotal role in translating an innate into an adaptive immune response by peripheral ingestion of microbes or tumor cells, migration to secondary lymphoid organs and presentation of processed antigens on MHC proteins to T and B cells. Several subtypes of DCs have been described in literature according to specific surface markers and functions, such as plasmacytoid dendritic cells (pDCs) and classical DCs (cDCs) (Merad et al., 2013). Recently, the therapeutic exploitation of the antigen-presentation capacity of DCs has been subject of intensive investigation, since DC-based anti-tumor vaccines seem to be a promising approach in order to enhance the anti-tumor immune response (Radford et al., 2014).

Additionally, epithelial cells are considered as innate immune cells by some authors due to their barrier-function, which helps to repell invading pathogens, and their secretory capacity of antimicrobial proteins such as defensins and lysozyme. Furthermore, endothelial cells mediate diapedesis of leucocytes through the endothelial layer by secretion of cytokines and chemokines (e.g. CXCL8) and the presentation of adhesion-molecules on their surface at the site of inflammation (Kolaczkowska and Kubes, 2013).

1.2.2 Cytokines and complement factors

The innate immune system is not only composed by immune cells but also by soluble factors as cytokines and complement factors. Various soluble molecules are subsumed among the term "cytokines", which are involved in cell differentiation, activation and communication. Important cytokines are listed below (Table 1.2).

Complement factors are plasma proteins produced in the liver that are crucial for anti-bacterial defense and seem to play a role in many auto-immune diseases. Complement factors are able to induce direct lysis of bacteria by forming a so-called membrane-attack complex or by opsonization of pathogens allowing a better and faster clearance by immune cells.

Additionally, the innate immune system provides directly anti-microbial acting proteins and enzymes, e.g. defensins and lysozyme. Defensins are secreted proteins that are capable of immediate lysis of bacteria, fungi and some kind of viruses by disrupting the microbial membrane integrity leading to efflux of ions and nutrients. Lysozyme acts by catalyzing the hydrolysis of glycosidic bonds in specific peptidoglycans forming the bacterial cell wall and is found in many secretions of the human body.

Table 1.2: Important cytokines

Cytokines	Main function	Main producing cell type
IL-1α	Activation of immune cells	Macrophages, neutrophils and epithelial cells
IL-1β	Activation of immune cells	Macrophages and neutrophils
IL-2	Proliferation of activated T cells	Activated T cells
IL-3	Differentiation and proliferation of hematopoietic progenitor cells	Activated T cells
IL-4	Differentiation of Th0 cells into Th2 cells	Activated T cells
IL-6	Activation of immune cells and induction of systemic inflammation	Macrophages and activated T cells
IL-10	Suppression of immune cells	Monocytes and regulatory T cells
IL-12	Differentiation of Th0 cells into Th1 cells	Dendritic cells and macrophages
IFN-α	Induction of an anti-viral response	Lymphocytes, pDC and other immune cells
IFN-γ	Activation of immune cells	NK cells, activated Th1 and CD8+ T cells
TNF-α	Activation of immune cells and induction of systemic inflammation	Activated macrophages and other immune cells
TGF-β	Suppression of immune cells	Regulatory T cells and other immune cells

1.2.3 Toll-like receptors

Toll-like receptors (TLRs) are rather non-specific pattern recognition receptors (PRPs), which are mainly expressed by macrophages, dendritic cells and natural killer cells. These receptors are capable of recognizing pathogen-associated molecular patterns (PAMPs), which are highly conserved among microbes such as bacteria, fungi, parasites and viruses. Thereby, TLRs represent an innate group of first line defense receptors, which are highly important for the initiation and successful execution of an immune response.

There are eleven TLR-genes in the human genome (TLR1-11), although only ten of them are actually expressed, since TLR11 is a so-called pseudogene which is not fully transcribed due to stop codons within the protein coding sequence (Roach et al., 2005). These receptors differ in their respective binding capability of PAMPs, their subcellular localization and their downstream signaling cascade (Table 1.3, adapted and modified from (Takeda et al., 2003; Beutler, 2009; O'Neill

et al., 2013)). TLR10 is the only known anti-inflammatory TLR, whereas all the others drive a strong and immediate inflammatory response upon activation.

Table 1.3: The human toll-like receptor family

Name	Ligands	Localization	Signaling	Dimerization
TLR1	Bacterial peptido-glycan and triacyl lipopeptides	Cell membrane	MyD88/IRAK	TLR1/TRL2
TLR2	Lipoteichoic acid, lipomannan, zymosan and others	Cell membrane	MyD88/IRAK	TLR2/TLR1, TLR2/TLR6
TLR3	Double stranded RNA	Endosomes	TRIF/IRF3	
TLR4	Lipopolysaccharide, fibrinogen, nickel and others	Cell membrane	MyD88/IRAK, TRIF/IRF3	
TLR5	Flagellin, profilin	Cell membrane	MyD88/IRAK	
TLR6	Diacyl lipopeptides	Cell membrane	MyD88/IRAK	TLR6/TLR2
TLR7	Single stranded viral RNA	Endosomes	MyD88/IRAK	
TLR8	Single stranded viral RNA, bacterial RNA	Endosomes	MyD88/IRAK	
TLR9	CpG oligo-nucleotides (unmethylated)	Endosomes	MyD88/IRAK	
TLR10	Unknown	Cell membrane, intracellular	PI3K/Akt	TLR10/TLR2

Although there are other PRRs such as RIG-I-like receptor dsRNA helicase enzymes (RIG-I, MDA5, LGP2), TLRs are an essential part of the human immune system and many diseases have been recently associated to these receptors (Casanova et al., 2011). For instance, missense mutations in the TLR4 gene were found to impair the response to inhaled LPS (Arbour et al., 2000) and may contribute to an increased risk of gram-negative bacterial infections (Agnese et al., 2002), although this finding is somehow controversial (Jessen et al., 2007). Defects in the downstream signaling of TLRs are even more devastating, since they share the same signaling cascade for the most part. Inherited IRAK-4 deficiency leads to greater susceptibility to invasive and noninvasive pyogenic bacterial infections due to multiple TLR malfunction (Picard et al., 2003).

Furthermore, the pro-inflammatory properties of TLRs might contribute to tumorigenesis by maintaining inflammation and TLR-mediated anti-apoptotic effects. Reversely, TLRs have also been considered as a promising target in tumor immunotherapy and tumor vaccination strategies over the last years because of their strong immunostimulatory effect. TLRs are known to enhance antigen-internalization and -presentation on dendritic cells (West et al., 2004), to promote Type-I interferon release (Kadowaki et al., 2001) and subsequent NK cell activation and to directly inhibit Treg functioning (Pasare and Medzhitov, 2003). After many years of basic research, Imiquimod was the first direct TLR agonist approved by the FDA for treatment of condylomata (Arican et al., 2004) and basal cell carcinoma (Geisse et al., 2004). By now, other TLR agonist have made their way into clinical trials either for treatment (e.g. MGN1703 (Schmoll et al., 2014)) or as adjuvants for peptide vaccinations (e.g. resiquimod (Sabado et al., 2015)).

However, despite encouraging preclinical results, many recent trials using TLR agonists for the treatment of cancer have shown disappointing results either due to tumor-promoting effects or severe side effects of these drugs (Guha, 2012). Therefore, using synthetic TLR agonists for the treatment of malignancies is still in its infancy and requires further basic and clinical research to fully exploit the therapeutic potential of these receptors without facing adverse effects.

1.3 The adaptive immune system

1.3.1 T and B lymphocytes

The adaptive immune system represents a highly complex and elaborate interplay between cells and soluble factors allowing specific and long-lasting immune responses upon activation by antigens. Among others, two main cell types constitute the adaptive immune system: T and B cells.

T and B cells originate from the hematopoietic stem cells in the bone marrow but follow distinct paths of differentiation. Whereas B cells largely remain in the bone marrow for maturation and selection processes, T progenitor cells leave the bone marrow in an immature state and home in the thymus, where they are henceforth called thymocytes. Here, in the cortex of the thymus, the positive selection process of the double positive thymocytes ($CD4^+/CD8^+$) takes place. The positive selection ensures that only thymocytes capable of binding MHC survive. Interestingly, their affinity to the two classes of MHC determine the cell's fate: thymocytes with a high affinity to MHC might get activated by MHC itself and therefore undergo apoptosis. Cells binding MHC Class I lose the CD4 surface protein (CD8 single positive) whereas thymocytes interacting with MHC Class II loose their CD8 protein (CD4 single positive). Cells without or with very weak MHC binding capacity are of no further use and undergo apoptosis (Palmer and Naeher, 2009).

Positively selected MHC-restricted single positive thymocytes migrate into the medullary stroma of the thymus and are negatively selected by tissue-resident medullary thymic epithelial cells expressing the transcription factor AIRE (autoimmune regulator) allowing the presentation of almost every self-antigen found in the body. Thymocytes strongly binding the MHC-presented self-antigens are negatively selected and undergo apoptosis due to their auto-reactive behavior. This process is essential for the maintenance of the central tolerance. Cells with intermediate affinity to the presented self-antigens differentiate into regulatory T cells (Treg), which mainly suppress other T and B cell populations and are responsible for peripheral tolerance (Hsieh et al., 2012). Cells without any binding to self-antigens survive and leave the thymus in a functional state.

B cell selection takes place in the bone marrow. Immature B cells express IgM as B cell receptors followed by IgD during their maturation process. The selection process is performed in the IgM single positive state by clonal deletion or receptor-editing: B cells recognizing self-antigens on the surface of bone marrow-homing cells either rearrange the antigen-binding fragment of their B cell receptor in order to lose affinity to self-antigens or undergo apoptosis when receptor editing is not successful (Halverson et al., 2004).

Mature B and T cells circulate in the blood and lymphatic vessels and home to the secondary lymphatic organs, like tonsils, lymph nodes and spleen. There, they rest in a naive state ready for antigen recognition and activation. Although the activation processes of B and T cells are completely different, they share the requirement of two activation signals and are dependent on each other. Whereas B cells can recognize unprocessed and non-MHC-presented antigens, T cells rely on antigen-processing and -presentation on MHC Class I and II. When a specific, but naive $CD4^+$ T cell (Th0) finds its antigen presented on MHC Class II of an antigen-presenting dendritic cell (first signal) and receives a costimulatory signal (second signal) simultaniously, it differentiates into a antigen-primed and therefore activated helper T cell. Costimulation usually occurs by activation of CD28 on T cells by CD80/86-binding provided by antigen-presenting cells. Depending on the cytokine milieu, the $CD4^+$ T cell will either differentiate into a Th1, Th2, Th3, Th9, Th17, Tfh or Treg and expand clonally by autocrine IL-2 signaling. The characteristics of each helper T cell subtype are depicted below (Table 1.4).

$CD8^+$ cytotoxic T cells are activated by the TCR-mediated recognition of their specific antigen presented on MHC Class I on antigen-presenting cells (first signal) and - by analogy to $CD4^+$ T cells - by costimulatory ligands, e.g. CD80/CD86 (second signal). This second signal can be modified or even replaced by soluble factors released by activated T helper cells. Once activated, $CD8^+$ T cells undergo clonal expansion and circulate through the body until they encounter their specific antigen once again. If an activated $CD8^+$ T cell recognizes its specific antigen on a potential target cell, it either induces apoptosis intrinsically by releasing granules containing perforin

Table 1.4: Characteristics of helper T cell subtypes

Helper T cell subtype	Main function	Biomolecular characteristics	Inductive cytokine milieu
Th1	Secretion of IFN-γ, activation of macrophages and $CD8^+$ T cells, antibody class switch to IgG	T-bet, STAT4	IL-2, IL-12
Th2	Secretion of IL-4, IL-5, IL-9, IL-10 and IL-13, activation of Eosinophils, Basophils and B cells, antibody class switch to IgE	GATA3, STAT6	IL-4
Th3	Secretion of TGF-β and IL-10, promotion of tolerance against nonpathogenic non-self antigens in the intestinal mucosa	Unknown	TGF-β
Th9	Secretion of IL-9, IL-10 and IL-21, promotion of allergic inflammation	STAT6, PU.1, IRF4, GATA3	IL-4, TGF-β
Th17	Secretion of IL-17A, IL-17F, IL-21 and IL-22, maintenance of mucosal barrier function and pathogen clearance	STAT3, RORγ, RORα	TGF-β, IL-6, IL-21, IL-23
Tfh	Expression of CD40L, secretion of IL-4 and IL-21, promotion of selection and survival of B cells in germinal centers of lymph follicles	CXCR5, Bcl-6	IL-21
Treg	Secretion of IL-10 and TGF-β, depletion of IL-2 by its high affinity receptor chain CD25, suppression of immune responses by CTLA-4 expression	FoxP3	IL-2, TGF-β

and granzymes or extrinsically by Fas-interaction. Since $CD8^+$ cytotoxic T cells bind MHC Class I bound antigens, their most common target cells are virus infected somatic cells, transplants and tumor cells.

B cells comprise the second axis of the adaptive immune system and are capable of recognition of unprocessed and non-presented antigens by their B cell receptor (BCR), which consists of a membrane bound immunoglobulin (IgM or IgD) in complex with an Igα/β sheath containing immunoreceptor tyrosine activation motifs (ITAMs). Antigen engagement induces BCR cross-linking and ITAM phosphorylation in B cells, which are the initial events of the intracellular signal transmission cascade leading to the activation of the cell (first signal) and presentation of

the processed antigen on MHC Class II. The latter allows the recruitment of a cognate $CD4^+$ T cell, that is specific for the same antigen and contributes to the full activation of the B cells. Interestingly, the kinetic segregation model (size-based exclusion of inhibitory phosphatases upon peptide binding to the TCR or BCR) established for T cell activation seems to be transferrable to B cell activation considering the strong requirement of the phosphatase activity of CD45 (T cells) and CD148 (B cells) for TCR- or BCR-mediated activation, respectively (Harwood and Batista, 2010). Both the location of an activated B cell in secondary lymphatic organs and the cytokine milieu conditioned by surrounding T helper cells modify its activation process and differentiation.

When activated, a B cell either starts producing high amounts of low-affinity immunoglobins (mainly IgM) or enters the germinal center of a lymph follicle in a secondary lymphatic organ. Inside the germinal centers B cells undergo a process called somatic hypermutation, that leads to increased affinity of the variable fragment of the produced antibodies by random mutation of the germline VDJ rearrangements and subsequent selection of clones that successfully performed this process (Victora and Nussenzweig, 2012). Thereafter, B cells differentiate either in long living memory B cells or high-affinity antibody-producing plasma cells. The following table shows key characteristics of the five different subclasses of immunoglobins (Table 1.5).

Table 1.5: Characteristics of immunoglobin subclasses

Immuno-globin	Main function	Distribution	Molecular structure
IgA	Antibody-dependent cellular cytotoxicity	Serum and secretions (e.g. salivary, breast milk)	Dimer fused by a J chain
IgD	B cell receptor	Membrane-bound	Monomer
IgE	Activation of mast cells, antibody-dependent cellular cytotoxicity	Serum	Monomer
IgG	Activation of complement, antibody-dependent cellular cytotoxicity, neutralization	Serum	Monomer
IgM	Activation of complement, neutralization and B cell receptor	Serum and membrane-bound	Pentamer fused by a J chain

1.3.2 Regulatory T cells

Regulatory T cells (Treg) comprise about 5-10% of peripheral $CD4^+$ T cells and are molecularly characterized by the expression of their hallmark-transcription factor Forkhead box P3 (FoxP3). These cells act suppressive on other lymphocytes and APCs by various mechanisms and naturally

antagonize overwhelming inflammation and autoimmunity. First evidences for the existence of T cells with suppressive capacity came from early thymectomy experiments performed by Nishizuka and Sakaguchi (Nishizuka and Sakakura, 1969; Sakaguchi et al., 1982). They could show that neonatal thymectomy between 2 and 4 days after birth resulted in severe tissue-inflammation, which was mediated by auto-reactive T cells. Further studies revealed, that mice having a dysfunctional FoxP3 gene suffered from early onset, T cell-mediated autoimmune diseases like diabetes, dermatitis and thyreoiditis stressing the relevance of Treg for the maintenance of the immune homeostasis (Brunkow et al., 2001).

Several mechanisms contribute to the suppressive capacity of Treg: first, Treg constitutively express the high-affinity IL-2 receptor-α-chain (CD25), which is usually expressed only upon activation on other T cell subsets. Therefore, Treg are capable of scavenging soluble IL-2 due to their higher affinity for this cytokine and subsequently compromise the growth of IL-2-dependent T cell subsets, e.g. $CD8^+$ memory T cells (Sharma et al., 2007). Second, Treg induce a tolerogenic micro-milieu by directly producing suppressive cytokines like IL-10, IL-35 and TGF-β. These cytokines seem to be of great importance for the maintenance of immune homeostasis especially at immunological barriers like the lungs, the skin and the gut, since mice with a Treg-specific knockout of IL-10 develop severe auto-immune disorders at these sites (Rubtsov et al., 2008). Third, Treg highly express CTLA-4, a cell surface receptor for CD80/86, which is known as a cell-intrinsic immune checkpoint, that induces T cell anergy upon receptor-ligand interaction. However, CTLA-4 seems to act on Treg differently, since mice lacking CTLA-4 specifically on Treg showed a reduced suppressive capacity of $FOXP3^+$ Treg, whereas the activation and expansion of these cells was enhanced under inflammatory conditions. Qureshi *et al.* could reveal that CTLA-4 down-regulates CD80/86 on APC via trans-endocytosis *in vivo* and *in vitro* (Qureshi et al., 2011). Two other cell surface molecules involved in adenosine metabolism, CD39 and CD72, contribute to the tolerogenic characteristics of Treg by converting extracellular cAMP into adenosine, which has a suppressive effect on $CD4^+$ effector T cells (Deaglio et al., 2007). Furthermore, several other cell surface molecules (e.g. LAG3, TIGIT and GITR) were found to be required for optimal Treg function by independent research groups (Josefowicz et al., 2012). Finally, Treg are capable of direct lysis of target cells (e.g. APC or antigen-specific T cells) by induction of apoptosis in a granzyme-dependent manner upon CD3 and CD46 stimulation (Grossman et al., 2004; Cao et al., 2007).

The importance of Treg for maintaining immune tolerance in peripheral tissues is stressed by the observation that people bearing a mutation in the *FOXP3* gene develop a rare but severe syndrome called immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (short IPEX), which is linked to type I diabetes, various allergies, thyroiditis and inflammatory bowel disease with an onset in early childhood (Bennett et al., 2001). Furthermore, the development or progression

of autoimmune diseases can be influenced not only by mutations in the *FOXP3* gene, but also by other Treg-related alterations. For example, in 2017 an Australian research group was able to show that a certain HLA subtype (HLA DR1) can favor Treg differentiation over conventional T cell differentiation. This observation was made in patients with Goodpasture disease, who were subsequently protected against the development of this fatal autoimmune disease by the dominant suppressive character of this polymorphism (Ooi et al., 2017). Since defective Treg functioning is considered to be frequently involved in various autoimmune reactions, Treg based cell therapy seems to be a promising strategy in order to re-establish the immune homeostasis, that is broadly discussed in literature (Miyara et al., 2014).

On the other hand, the suppressive capacity of Treg is hijacked by different tumors in order to prevent immune rejection and to allow immune escape. The negative prognostic effect of high intratumoral Treg burden was shown for ovarial (Curiel et al., 2004), gastric (Perrone et al., 2008; Wu et al., 2018) and hepatocellular (Gao et al., 2007) among others.

1.4 Chemokines

1.4.1 Chemokine families

Chemokines represent a group of chemo-attractant cytokines, which feature chemotactic migration of immune cells, fibroblasts, endothelial cells, osteoclasts and many others, and are thereby involved in multiple physiological and pathological processes such as immune response, T cell selection, wound healing, tissue remodeling, neoangiogenesis and even metastasis (Rossi and Zlotnik, 2000). Chemokines are most commonly sub-divided according to their molecular structure, that depends on the frequency and the orientation of disulfide bonds within their tertiary structure (Table 1.6).

The so-called CC chemokine ligands (CCLs), which represent the largest sub-group among all chemokines having 27 distinct members (CCL1-CCL28, CCL9 and CCL10 are considered to be the same molecule), have two directly adjacent cysteins (C) forming disulfide bonds near the amino terminus of the protein. There are CC chemokines with four or six cysteine residues, respectively. These chemokine ligands interact with their corresponding chemokine receptors (CCRs) and induce migration in many different cells, e.g. CCL1 and CCL2 in monocytes and dendritic cells, CCL5 in T cells, CCL9 in osteoclasts and CCL17 and CCL22 in regulatory T cells.

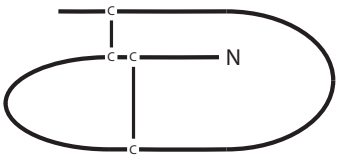
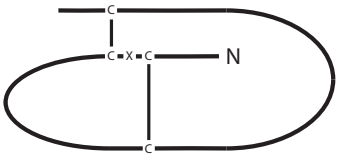
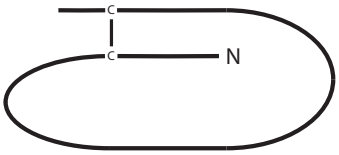
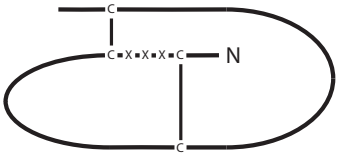
The second largest group of chemotactic cytokines are the CXC chemokines (CXCLs) comprising 17 known members. In this family, the two N-terminal cysteine residues or disulfide bonds, respectively, are separated by one other amino acid. The CXC chemokines are widely involved in the attraction of monocytes, dendritic cells and T cells but are also found to have either pro- or

anti-angiogenic properties depending on the presence or absence of the amino acid motif ELR being located right before the first cysteine of the CXC sequence (Vandercappellen et al., 2008). Their corresponding receptors are called CXC receptors (CXCRs).

The C chemokines (XCLs) represent another group of chemokines containing only two cysteines in their molecular structure leading to the formation of one disulfide bond. This group has two members, which are called XCL1 (lymphotactin-α) and XCL2 (lymphotactin-β). Both chemokines are capable of attracting T cells via the interaction with their mutual receptor XCR1.

CX_3CL1 forms the last and smallest chemokine family having only one member. In this protein the two disulfide bonds are separated by three other amino acids. Interestingly, CX_3CL1 combines the features of chemokines and adhesion molecules since it is not only secreted into the extracellular space but also tethered to the membrane of the cell (Bazan et al., 1997).

Table 1.6: Chemokine families

Family	Members	Molecular Structure
CCL	27	
CXCL	17	
XCL	2	
CX_3CL	1	

1.4.2 The chemokine CCL22

CCL22 is a chemokine of the CC chemokine family. It has been first described as a macrophage derived chemokine (MDC) by Godiska *et al.* in 1997 and was found to have chemoattractive properties on macrophages, monocyte-derived dendritic cells and natural killer cells (Godiska et al., 1997). Only one year later, researchers from the same group identified the chemokine receptor CCR4 as the functional receptor of CCL22 (Imai et al., 1998). Since then, many new aspects concerning the role of this particular chemokine on immune response and homeostasis have been revealed.

The first hint that CCL22 might not only have effects on the innate immune system and its cells but also play a role in orchestrating adaptive immune responses came from a Japanese group: they showed that dendritic cells and $CD4^+$ T cells form clusters in human skin and secondary immune tissues in a CCL22-CCR4-dependent way (Katou et al., 2001). Although the distinct T cell subsets, which were found to interact with dendritic cells in this study, haven't been further characterized by the researchers, results from other groups suggest that regulatory T cells (Iellem et al., 2001) and Th2 cells (Bonecchi et al., 1998) express CCR4 predominantly. Although CCR4 might not be the only receptor for CCL22 (Struyf et al., 1998), the association of CCL22 and regulatory T cells was highlighted by several following studies in various ways (Eby et al., 2015; Yang et al., 2012; Ménétrier-Caux et al., 2012).

In recent years, even therapeutic strategies hijacking the CCL22-CCR4 axis have been proposed. In 2011, Montane *et al.* published a novel and successful strategy to delay or even prevent the onset of murine autoimmune diabetes by CCL22-mediated recruitment of Treg into pancreatic islets (Montane et al., 2011). In this study the authors injected CCL22-encoding adenoviruses into the pancreatic duct of non-obesity diabetes (NOD) mice and achieved long-lasting insulin production of the pancreatic islets due to Treg infiltration and subsequent protection from autoimmune-mediated destruction.

Additionally, CCL22 has been considered as a potential target for therapeutic approaches in tumor therapy. Over the past years, several studies revealed a role of CCL22 in establishing and maintaining an immune-suppressive micromilieu within tumors by attracting regulatory T cells. Therefore, Rapp *et al.* tried to exploit this finding by stably transducing tumor-specific $CD8^+$ T cells with CCR4, shifting the CCL22-mediated preferential recruitment of Tregs towards anti-tumor directed cytotoxic T cells (Rapp et al., 2016). Through this approach, the number of tumor-specific T cells could be strongly enriched and tumor growth was repressed significantly in a murine subcutanous pancreatic carcinoma model. Most recently, an American group could restrict the growth of melanomas and lung metastases of melanomas by repetitive gen gun injections of CCL22-encoding plasmids into the skin of mice by trapping Treg at the injection site and preventing tumor homing

of these cells (Klarquist et al., 2016). Furthermore, the authors could show that not only tumor growth was reduced in treated mice, but also autoimmune disorders affecting the skin (e.g. vitiligo), which are common side effects of checkpoint-inhibitors, were remarkably attenuated by this strategy.

Thus, CCL22 remains to be a promising candidate for future therapeutic approaches for both autoimmune and malignant diseases and needs to be further evaluated in clinical studies in order to translate the recent findings from bench to bedside.

1.5 Chemokine receptors

There are two types of chemokine receptors, namely typical and atypical chemokine receptors. Whereas typical chemokine receptors are seven-transmembrane G-protein coupled receptors, that induce migration and activation after intracellular calcium release, atypical chemokine receptors, that seem to be structurally homologous to their counterpart, lack conventional downstream intracellular signaling. For many years, the existence and the function of these atypical receptors have been unknown.

In recent years, research on atypical chemokine receptors such as CCRL1 suggested that these receptors could hold evidence for an almost forgotten hypothesis, namely the *source and sink* hypothesis (Ulvmar et al., 2014). According to this hypothesis, which was introduced by Francis Crick in the field of embryology for the first time in 1970, the development of functional gradients based on diffusion requires both a source producing the diffusing molecule and a sink locally removing the molecules out of the system and thereby shaping the gradient (Crick, 1970). Following this hypothesis, the atypical chemokine receptors act as scavenger receptors allowing the development of functional chemokine gradients, which are in turn recognized by typical chemokine receptors and can then be translated into migration of cells.

In analogy to the classification of chemokines, there are four families of typical chemokine receptors. The first group, the so-called CC chemokine receptors (CCRs), comprise a family of 11 members, which all bind CCL chemokines. This family covers some of the most famous members of all chemokine receptors including CCR5, CCR7 and CCR11, which have been intensively investigated over the last couple of years. CCR5, for instance, was shown to be a co-receptor for HIV-1, the virus causing acquired immunodeficiency syndrome (AIDS), and was subsequently identified as a promising therapeutic target for HIV treatment (Deng et al., 1996; Fätkenheuer et al., 2005). Moreover, CCR7 was found to mediate CCL19- and CCL21-dependent lymphocyte trafficking and homing to secondary lymphoid organs such as lymph nodes and to be a hallmark of residual memory T cells (Baekkevold et al., 2001; Sallusto et al., 1999). The ability of

CCR11 to bind CCL19, CCL21 and CCL25 was responsible for its allocation to the CCR family, although subsequent research could show that CCR11 acts as atypical chemokine receptor. Therefore, CCR11 was renamed as CCRL1 and remained as a historical remnant in this family. Another member of the CC chemokine receptor family is CCR4. CCR4 is capable of recognizing CCL2, CCL4, CCL5, CCL17 and CCL22 but seems to have different affinities and to react differently depending on its ligands (Mariani et al., 2004). Apart from its predominant expression on Treg, CCR4 was described to have major effects in regulating innate immune responses upon LPS-induced endotoxic shock (Chvatchko et al., 2000).

The CXC chemokine receptors (CXCRs) represent the second largest family of chemokine receptors. The seven members of this family (CXCR1-CXCR7) are bound by CXC chemokines and are equally relevant in terms of immune homeostasis and response as the CC chemokine receptors. Among these chemokine receptors CXCR4 is the most famous one due to its high clinical relevance. Notably, CXCR4 was found to be another important co-receptor for HIV-1 in addition to CCR5 (Feng et al., 1996) and seems to regulate hematopoietic stem cell homing to the bone marrow and stem cell quiescence (Möhle et al., 1998). Exploiting this property, a drug blocking CXCR4 is now officially approved to efficiently mobilize stem cells for stem cell transplantation in combination with G-CSF after the substantial enhancement of stem cell mobilization by CXCR4 blockage was described earlier in the literature (Dar et al., 2011).

The two remaining families of chemokine receptors contain only one particular member. XCR1, the only member of the C chemokine receptor family, is known to recognize both XCL1 and XCL2, and was found to be a hallmark of a specific subset of dendritic cells. These $XCR1^+$ $CD11c^+$ $CD141^+$ dendritic cells are highly specialized in cross-presenting antigens to $CD8^+$ T cells and seem to resemble murine $CD11c^+$ $CD8^+$ dendritic cells in humans (Dorner et al., 2009; Bachem et al., 2010).

In turn, CX_3CR1 comprises the last chemokine receptor family (CX_3C chemokine receptor family) and is predominantly expressed by lymphocytes and monocytes. In the central nervous system CX_3CR1^+ monocytes were identified to modulate learning abilities in the context of systemic inflammation by releasing TNF-α and inducing subsequent dendritic spine remodeling (Garré et al., 2017).

Since the existence of atypical chemokine receptors is a very recent discovery, there is no such classification as for the typical chemokine receptors. However, the inconsistent nomenclature for these receptors were partially reversed when four of them were renamed in ACKR1-4 by the Human Genome Organization Gene Nomenclature Committee and Nomenclature Committee of the International Union of Pharmacology in 2013 (Table 1.7, adopted and modified from Bachelerie

et al. (Bachelerie et al., 2014)). The confirmation of two more receptors (CCRL2 and PITPNM3) as atypical chemokine receptors is still pending.

Table 1.7: Atypical chemokine receptors

Name	Synonyms	Ligands	Expression Pattern	Function
ACKR1	DARC, Duffy antigen, CD234	CCL2, CCL7, CCL8, CCL13, CCL14, CCL16, CCL17, CCL22, CXCL1, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL11, CXCL13	Erythrocytes, neurons, vascular endothelial cells	Ligand transcytosis and presentation, ligand sink and reservoir
ACKR2	D6, CCR9, CCR10	CCL2, CCL3, CCL3L1, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, CCL22	Lymphatic endothelial cells, syncytiotrophoblast	Ligand scavenging
ACKR3	CXCR7, RDC1	CXCL11, CXCL12	Hematopoietic cells, neurons, mesenchymal cells, endothelial cells, cancer cells	Ligand scavenging, gradient shaping
ACKR4	CCRL1, CCR11	CCL19, CCL21, CCL25, CXCL13	Lymphatic endothelial cells, thymic epithelial cells, bronchial epithelial cells, keratinocytes	Ligand scavenging, gradient shaping

As mentioned above, the common feature of atypical chemokine receptors is the scavenging of chemokines. Nevertheless, the distinct receptors differ in the exact mechanism of scavenging and some of them seem to influence chemotaxis in other, more elaborate ways (Nibbs and Graham, 2013). For instance, ACKR1 is able to transport chemokines from the blood through endothelial cells and thereby facilitates transcytosis of chemokines into the subendothelial tissue (Pruenster et al., 2009). Furthermore, atypical chemokine receptors were also found to influence the downstream signaling of the conventional chemokine receptor CXCR4 by heterodimerization (Levoye et al., 2009).

However, the understanding of the function of atypical chemokine receptors, their involvement in immune regulation and their expression profile is still very poor and further experimental work is required to elucidate the biological role of this interesting receptor family.

Chapter 2

Definition of the project

The chemokine CCL22 seems to play a pivotal role for intratumoral recruitment of regulatory T cells and subsequent immune escape in many solid tumor entities. However, immunohistochemical staining revealed that only in a few tumor entities the tumor cells themselves are capable to produce CCL22. In contrast, immune cells infiltrating the tumor frequently show a strong staining for CCL22. Thus, CCL22 production seems to be a tightly regulated process that is highly dependent on tumor-immune cell crosstalk.

However, the regulatory mechanisms leading to CCL22 expression in bystander immune cells have not been investigated so far and may offer a promising therapeutic target for cancer treatment strategies in the future.

This thesis aims at understanding of the regulatory mechanisms that influence the expression of the Treg-attracting chemokine CCL22 in the tumor micromilieu. In particular, our research group was interested in (1) the cell type that is responsible for the intratumoral production of CCL22, (2) the factors that are produced by tumor cells inducing the expression of CCL22 in immune cells and (3) the evaluation of potential pharmacologic targets in this cascade.

Chapter 3

Results

The publications covered by this thesis elucidate two important regulatory mechanisms concerning the expression of CCL22, a Treg-attracting chemokine. The first paper shows that many tumor cell lines are capable of inducing CCL22 expression and that IL-1α produced by human pancreatic adenocarcinoma cells is an important positive regulator of CCL22 production. IL-1α is a mainly membrane-bound but also secreted cytokine with many known immunoregulatory effects. Its role on tumor growth and disease progression has been investigated extensively. The findings published in this paper add another role of tumor-derived IL-1α that can directly be linked to Treg migration and Treg-mediated immunosuppression. Notably, anakinra, a recombinant IL-1 receptor antagonist was able to fully inhibit the CCL22-induced Treg migration *in vitro* and may represent a promising therapeutic strategy to reduce Treg-infiltration in solid tumors.

The second paper focuses on novel mechanisms and pathways, that directly suppress CCL22 production within tumors and might also be used therapeutically in the future. In detail, treatment of tumor-bearing mice with Toll-like receptor agonists such as the oligodesoxynucleotide CpG and the small molecule R848 as well as with the RIG-I-like helicase agonists poly(I:C) was found to reduce the intratumoral number of Treg and to slow down tumor growth significantly. Interestingly, CpG indirectly suppressed the migration of Treg into the tumor by reducing the expression of the Treg-attracting chemokines CCL17 and CCL22. In contrast, other pro-inflammatory chemokines such as CCL5 were induced upon TLR9 treatment. Confocal microscopy revealed that intratumoral macrophages and dendritic cells are the CCL22 producing source within human tumors and respond with downregulation of CCL22 expression when CpG is administered. This effect was mediated by type-I interferons (especially IFN-α), which is released due to TLR stimulation by immune cells. Furthermore, when CCL22 production was maintained in CCL22 over-expressing cell lines, CpG treatment failed to repress tumor growth *in vivo* due to maintained Treg infiltration.

Chapter 4

Summary

Regulatory T cells and their contribution to tumorigenesis and tumor growth are of high interest both for basic and translational medical research. By now, it is well accepted that regulatory T cells play an essential role in establishing and maintaining the intratumoral immunosuppression. They may therefore represent a favorable target for immune-modulating cancer therapies. At the same time, Treg physiologically control overwhelming immune responses and autoimmunity by directly inhibiting self-directed T and B cells. Thus, treatment strategies that alter their function or even deplete the respective cell population using Treg-specific antibodies bear the danger of inducing severe autoimmune side effects. In order to circumvent these limitations, novel Treg-based strategies are urgently needed to overcome the Treg-induced intratumoral immunosuppression. Inhibiting the infiltration of tumors by Treg without altering the numbers or the function of Treg in other parts of the organism may represent a promising approach to selectively change the immunological milieu within a tumor without putting the patient's health at risk.

Taken together, both papers stress the relevance of the chemokine CCL22 for Treg migration into tumor tissue and reveal two novel strategies for the blockage of the CCL22-Treg axis in order to overcome Treg-mediated immunosuppression within solid tumors (see Fig. 4.1 for a graphic summary).

Regulatorische T Zellen und ihr Beitrag zu Tumorigenese und Tumorwachstum sind sowohl für die Grundlagenforschung als auch die translationale medizinische Forschung von großem Interesse. Mittlerweile gilt es als weithin akzeptiert, dass regulatorische T Zellen eine essentielle Rolle für die Etablierung und Aufrechterhaltung einer intratumoralen Immunsuppression spielen. Deshalb könnten sie ein vielversprechendes Ziel von immunmodulierenden Therapieoptionen bei Krebs darstellen. Gleichzeitig aber kontrollieren Treg in ihrer physiologischen Funktion überschießende Immunantworten und Autoimmunität durch direkte Inhibition von aktivierten oder autoimmun wirksamen T und B Zellen. Daher bergen Therapiestrategien, die die Funktion oder die Anzahl von Treg z.B. mittels Treg-spezifischen Antikörpern verändern, die Gefahr,

schwerwiegende autoimmun-vermittelte Nebenwirkungen zu induzieren. Um diese therapeutischen Limitationen zu umgehen und gleichzeitig die intratumorale, Treg-vermittelte Immunsuppression zu durchbrechen, werden neuartige Treg-basierte Therapieoptionen dringend benötigt. Somit könnte eine Therapie-induzierte, verminderte Infiltration des Tumors durch Treg ohne Veränderung der Funktion und Anzahl der regulatorischen T Zellen im sonstigen Organismus eine vielversprechende Maßnahme sein, selektiv das intratumorale Milieu zu verändern, ohne dabei die Gesundheit des Patienten zu gefährden.

Zusammengefasst betonen beide Originalarbeiten die Relevanz des Chemokins CCL22 für die Migration von regulatorischen T Zellen in Tumore und zeigen zwei neuartige Strategien zur Blockade der CCL22-Treg-Achse und der durch sie vermittelten intratumoralen Immunsuppression auf (siehe auch Fig. 4.1 für eine graphische Zusammenfassung).

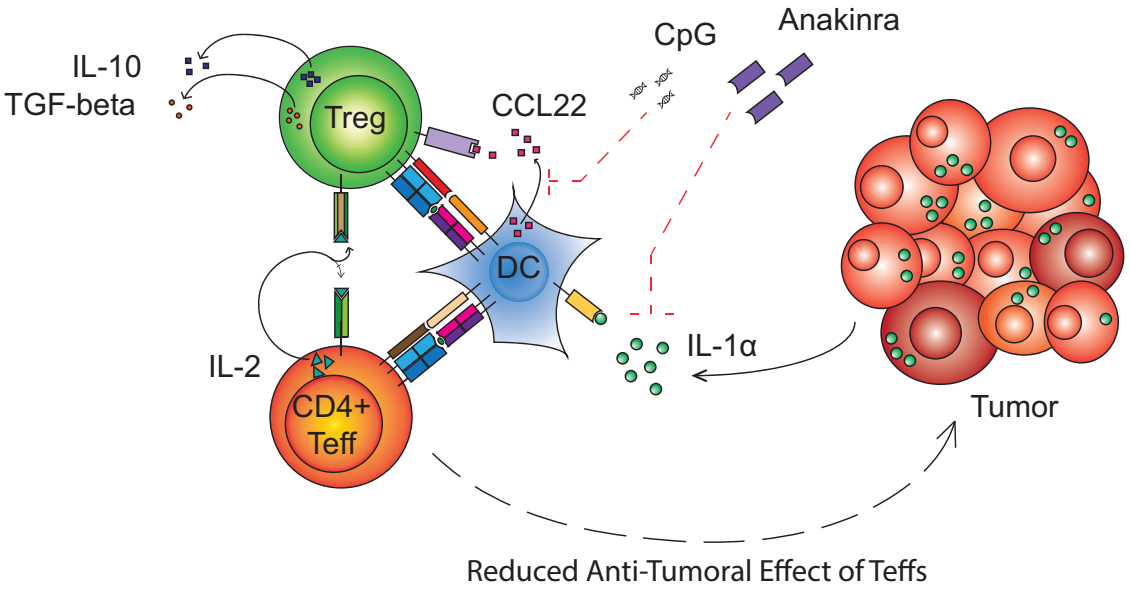


Figure 4.1: Regulation of CCL22 production in solid tumors

Chapter 5

Publications

5.1 Cancer cell-derived IL-1α induces CCL22 and the recruitment of regulatory T cells

5.1.1 Own contribution to the original article

The paper "Cancer cell-derived IL-1α induces CCL22 and the recruitment of regulatory T cells" focuses on the mechanism responsible for the up-regulation and the cellular source of the chemokine CCL22 in solid tumors. My experiments revealed that dendritic cells isolated from human blood respond to the co-incubation with tumor cell supernatants with the up-regulation of CCL22, whereas the DC-depleted cell fraction or $CD14^+$ monocytes didn't show this phenotype (Figure 3C and unpublished data). Using qRT-PCR analysis I could show that IL-1α and IL-1β are heterogeneously expressed in human and murine cancer cell lines, which were tested to induce CCL22 *in vitro* (Figure 4B and Supplemental figure 2C). I validated this finding in two cell lines at protein level using ELISA (Figure 4A). In order to prove that IL-1 was responsible for the inductive effect of the tumor-conditioned media in our system, I blocked IL-1 signaling by the addition of a recombinant IL-1 receptor antagonist called anakinra (Figure 4C) and tested if recombinant IL-1α was able to induce CCL22 in PBMC cultured in standard medium (Supplemental Figure 2D). Furthermore, I could demonstrate that transcriptional regulation was the underlying mechanism for the increased CCL22 production using a luciferase reporter assay in HEK cells (Supplemental Figure 4E). Moreover, I evaluated the functional relevance of the expression of IL-1 by tumor cells using a transwell migration assay and found increased migration of regulatory T cells towards the supernatants of PBMC that were co-incubated with tumor-conditioned medium (Figure 5). My contribution to this paper was rewarded with a shared first authorship. All supplemental figures can be accessed via the official homepage of *Oncoimmunology*.

5.1.2 Original article

The original article "Cancer cell-derived IL-1α induces CCL22 and the recruitment of regulatory T cells" can be accessed at `https://www.tandfonline.com/doi/full/10.1080/2162402X.2016.1175794` and is cited as follows:

Wiedemann G.M., Knott M.M.L., Vetter V.K., Rapp M., Haubner S., Fesseler J., Kühnemuth B., Layritz P., Thaler R., Kruger S., et al. Cancer cell-derived IL-1α induces CCL22 and the recruitment of regulatory T cells. Oncoimmunology. 2016;5:e1175794.
doi: 10.1080/2162402X.2016.1175794.

5.2 Suppression of intratumoral CCL22 by type I interferon inhibits migration of regulatory T cells and blocks cancer progression

5.2.1 Own contribution to the original article

The second publication "Suppression of Intratumoral CCL22 by Type I Interferon Inhibits Migration of Regulatory T Cells and Blocks Cancer Progression" addresses the mechanism of TLR agonist-mediated tumor growth inhibition. The results published in this article convincingly show that treatment of subcutaneous tumors with the TLR9 agonist CpG leads to significant reduction of tumor size and complete response in some animals. This effect was highly dependent on the suppression of CCL17- and CCL22-mediated Treg infiltration by Type I interferons secreted in response to CpG treatment. Constitutive expression of CCL22 by the tumor cells was able to fully revert this effect.
I substantially contributed to the review process of this paper by performing a time-course CT26 tumor model with or without CpG treatment and compared the tumor growth, the infiltration by Treg and other immune cells after 4 and 8 days. My experiments revealed that CpG treatment had a long lasting effect on the immunological micromilieu in subcutaneous solid tumors (Supplemental Figure 1C). Furthermore, I evaluated the effect of IFN-α (the putative downstream mediator of CpG) treatment of tumor-bearing mice in two different tumor models. I could observe a trend towards reduced levels of CCL22 on protein level in IFN-α treated mice and a reduced infiltration by Treg in CT26 and Panc02 tumors. Both findings were not significant though, suggesting a superior effect of CpG versus IFN-α administration (Supplemental Figure 6B). Interestingly, my experiments also showed that successful CpG treatment induced memory (Supplemental Figure 7A) in CT26 tumor bearing mice. Moreover, $CD8^+$ T cells showed an increased specificity towards the tumor specific antigen AH1 (Supplemental Figure 7B) after CpG treatment, most likely due to the reduced number of intratumoral Treg. My contribution to this paper was rewarded with a co-authorship. All supplemental figures can be accessed via the official homepage of *Cancer Research*.

5.2.2 Original article

The original article "Suppression of intratumoral CCL22 by type I interferon inhibits migration of regulatory T cells and blocks cancer progression" can be accessed at `https://cancerres.aacrjournals.org/content/75/21/4483.long` and is cited as follows:

Anz D, Rapp M, Eiber S, Koelzer VH, Thaler R, Haubner S, Knott M, Nagel S, Golic M, Wiedemann GM, Bauernfeind F, Wurzenberger C, Hornung V, Scholz C, Mayr D, Rothenfusser S, Endres S, Bourquin C. 2015. Suppression of intratumoral CCL22 by type I interferon inhibits migration of regulatory T cells and blocks cancer progression. Cancer Res 75(21):4483–4493. doi: 10.1080/2162402X.2016.1175794.

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