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Medizinische Klinik und Poliklinik IV

Klinikum der Universität

Ludwig-Maximilians-Universität München

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The Role of the Chemokine CCL22 in the Interaction of Dendritic Cells and Regulatory T Cells

Dissertation

zum Erwerb des Doktorgrades der Medizin

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

vorgelegt von

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aus Baden-Baden

2017

Mit Genehmigung der Medizinischen Fakultät

der Universität München

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Tag der mündlichen Prüfung:	19.10.2017

Dedicated to my parents Gabriele and Manfred

and to my brother Klaus-Georg

INDEX

1 INTRODUCTION

1.1 Innate and adaptive immunity

Nature developed the human immune system in order to protect our body's integrity from the permanent challenge of invading microorganisms and generation of cancer cells by establishing mechanisms of host defense to eliminate infectious pathogens and tumor cells. The immune system can be conceptually differentiated in innate and adaptive (or acquired) immunity, which are intertwined by a multitude of interactions necessary to cooperatively eliminate pathogens and neoplasms.

The innate immune system dates back very far in phylogenetic terms. This system acts rapidly as the frontline of defense during infections. The cell types involved are capable of phagocytosis, such as dendritic cells and macrophages, as well as detection of invading microorganisms. Subsequent goals are to prevent the spread of the infection and to induce an inflammatory response against it on a systemic level. The innate immune system is not a single entity, but rather an interplay of multiple differentiated subsystems. For example, the mucosa and the skin are functional barriers by producing antimicrobial peptides and mucinous proteins seeking to limit the invasion of pathogens. Another subsystem is represented by the production of acute phase proteins and complement factors that enable opsonization followed by lytic destruction of pathogens. In contrast to original assumptions, the innate immune response is not utterly unspecific as a limited set of genetically encoded pattern recognition receptors (PRR) allow the discrimination between self and foreign. PRRs recognize evolutionary conserved structures of microorganisms that are usually not present in vertebrates, so called pathogen-associated molecular patterns (PAMP). An important connection to the adaptive immune system is the production of chemokines to guide leukocytes to the site of infection. Further links include the processing as well as the subsequent presentation of antigens to adaptive immune cells like T and B cells using the major histocompatibility (MHC) protein family (Iwasaki and Medzhitov 2010).

The adaptive immune system on the other hand makes use of a giant repertoire of antigen-specific receptors that is generated by somatic recombination of genes in T and B cells, producing T and B cell receptors that are expressed on the cell surface. This accounts for its high specificity and enables effective complete elimination of pathogens. At first however, these particular populations of lymphocytes need to replicate in a

mechanism called clonal expansion, which explains why this system acts rather slowly. Another hallmark of the adaptive immune system is the generation of immunological memory, which enables long-lasting immunity for recurrent infections.

However, both of these systems do not function in perfection. Endogenous molecules released after tissue damage or cell necrosis can for example also stimulate the innate immune system through PRRs and the specificities of T and B cells are not explicitly limited to foreign structures. For these potentially harmful situations, nature has evolved mechanisms of immunological tolerance. Nevertheless, this links both parts of the immune system to the pathogenesis of chronic autoimmune and inflammatory diseases.

1.2 Immunological tolerance

During the end of the 19th century, Paul Ehrlich postulated an immunological principle called *horror autotoxicus* (Silverstein 2001). He argued that the body's immune system could only respond to foreign (or non-self-) structures – a reaction to self would be impossible as it would lead to a destruction of the host. Since the discovery of autoimmune diseases, we know that self-antigens are pivotal in their pathogenesis and therefore possible targets of the body's immune system. In order to limit these harmful immune responses, nature has evolved three major mechanisms to provide the vital function of immunological tolerance.

1.2.1 Central tolerance

Central tolerance is a mechanism provided by the thymus, the site of T cell generation. Lymphoid progenitor cells arise from hematopoietic stem cells, populate the thymus and expand locally as immature thymocytes. In a first step called positive selection, thymocytes are tested for their capability to interact with the MHC class I or II complex, which requires a functional T cell receptor (TCR). For this purpose, thymocytes interact with cortical thymic epithelial cells (cTEC) in the cortex of the thymus. The ability to interact results in a survival signal for the thymocyte to promote its further development. In a second step called negative selection, the positively selected thymocytes migrate to the medulla of the thymus to interact with medullary thymic epithelial cells (mTEC) and thymic dendritic cells (tDC). mTEC have the potential to present a large variety of self-antigens on the MHC class I for $CD8^+$ thymocytes. In contrast, tDC phagocytose mTEC

and subsequently present antigens on the MHC class II for $CD4^+$ thymocytes. Thymocytes strongly recognizing self-antigen will receive signals to undergo apoptosis in order to prevent later development of autoimmunity. Although approximately 98% of thymocytes do not survive positive and negative selection, a considerable amount of self-reactive T cells is released into the blood stream as the selection of the thymus is not perfect and therefore considered leaky (Anderson, Venanzi et al. 2002).

1.2.2 Peripheral tolerance

To understand the concept of peripheral tolerance, we first need to recapitulate the basic steps of an immune response, for which the collaboration of innate and adaptive immunity is a necessity. This key process takes place in antigen-presenting cells (APC), that are capable of processing and presenting antigens on their cell surface to other immune cells. The APC with the highest grade of specialization for this task is the dendritic cell (DC). Its characteristic morphology allows efficient cell contacts through multiple branch-like protrusions on the cell surface, for which it received its name. DCs are present throughout almost every tissue in the body and are continuously sampling their surroundings through phagocytosis. Subsequently, the antigen uptake from pathogens, necrotic cells or immune complexes will be processed and presented using the major histocompatibility complex (MHC) molecules.

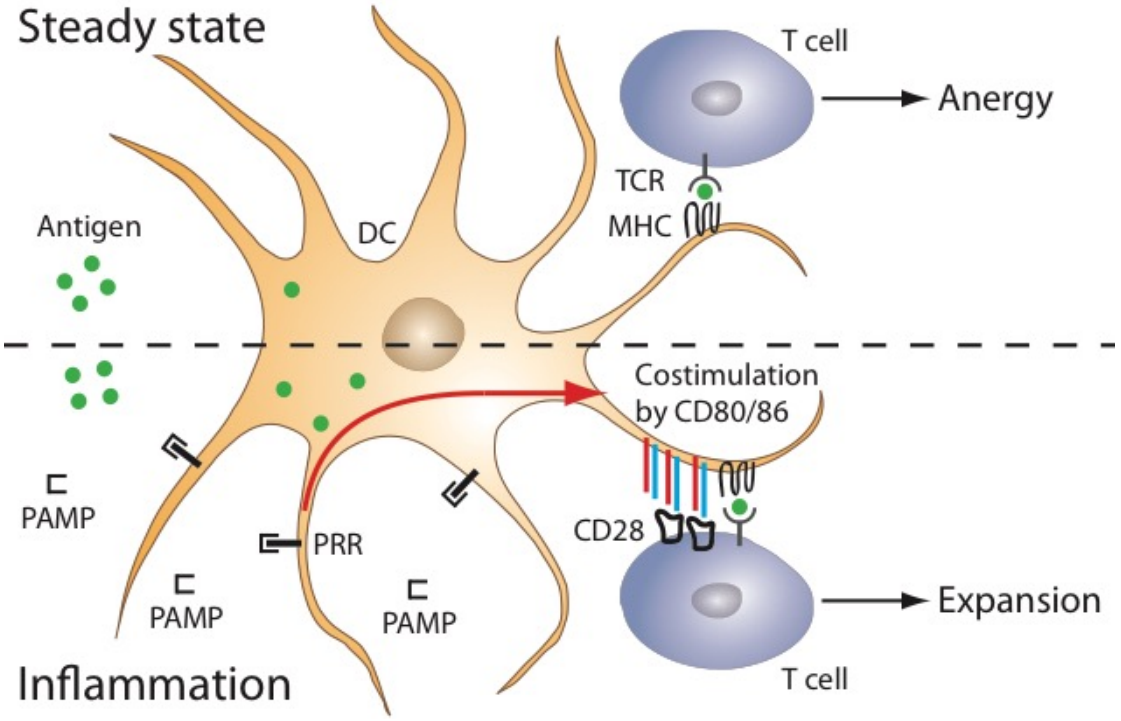

Figure 1.1: Basic illustration of dendritic cell costimulation of T cells
[The illustration is based on a review by Iwasaki and Medzhitov 2010.]

An important feature of DCs is their innate sensing of danger signals through PRRs, which activates them in a process called maturation by inducing the expression of costimulatory molecules, in particular CD80 and CD86. The current state of a DC, either immature (i.e. inactive) or mature (i.e. active), during antigen presentation and recognition is decisive. On the one hand, an immature DC interacting with a T cell recognizing its antigen will induce peripheral tolerance by rendering this T cell anergic, i.e. unable of clonal expansion. On the other hand, a mature DC will induce an effective immune response by signaling this T cell to expand. Therefore, an adaptive immune response requires two specific steps: (1) a danger signal inducing maturation of the DC, and (2) a T cell detecting its cognate antigen on the cell surface of a mature DC. An illustration of the process of costimulation is provided in Figure 1.1.

As in the steady state of the immune system PAMPs are usually absent, DC maturation does not occur and self-reactive T cells are not activated, thereby providing a mechanism of peripheral tolerance. However, endogenous danger signals, e.g. DNA from dying cells as a danger-associated molecular pattern (DAMP), can also induce DC maturation.

1.2.3 Dominant tolerance

As central and peripheral tolerance significantly reduce but do not exclude self-reactive immune responses, an additional level of control in the form of dominant tolerance has evolved. A distinct $CD4^+$ T helper cell lineage termed $Foxp3^+$-regulatory T cells (T_{Reg}) can potently suppress immune responses of effector T cells (T_{Eff}). These cells are best characterized by constitutive expression of the transcription factor Foxp3, which is the master regulator of T_{Reg} and controls the expression of proteins that contribute to their function. During negative selection in the thymus, T_{Reg} survive in a specialized niche with a T cell receptor avidity that is thought to lie in between the recognition of foreign and self-antigen. After their initial identification in 1995 (Sakaguchi, Sakaguchi et al. 1995), it was shown that a deficiency in Foxp3 in mice leads to a complete deficiency of the T_{Reg} cell lineage and causes fatal forms of autoimmune disorders (Fontenot, Gavin et al. 2003). The IPEX-Syndrome (Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked) represents a rare disease with multi-organ autoimmunity in humans based on a mutation of the Foxp3 gene, provoking failure of dominant tolerance.

1.3 Regulatory T cell function

Regulatory T cells are guardians of our body's immunological integrity. Preventing autoimmune diseases and restricting overwhelming immune-responses to pathogens are key tasks of these cells, which accounts for their original description as "suppressor T cells". They can be distinguished in naturally occurring (nT_{Reg}) and induced (iT_{Reg}) regulatory T cells. nT_{Reg} arise from the thymus, represent a stable cell lineage under steady state as well as inflammatory conditions and constantly undergo self-renewal in the periphery (Rubtsov, Niec et al. 2010). On the other hand, iT_{Reg} are generated in the periphery from T_{Eff} induced by cytokines like TGFß in immunosuppressive milieus and supplement the function of nT_{Reg} (Haribhai, Williams et al. 2011). Moreover, the presence of T_{Reg} is necessary throughout the lifespan of the host organism (Kim, Rasmussen et al. 2007). Immune suppression by T_{Reg} has been shown to use multiple major mechanisms (Vignali, Collison et al. 2008, Shevach 2009), which can be classified in non-contact- and contact-mediated suppression. An illustrative overview of the main regulatory T cell suppression mechanisms is provided in Figure 1.2.

1.3.1 Non-contact-mediated regulatory T cell function

One mechanism initially proposed is based on IL-2 consumption, a cytokine necessary in low amounts for homeostasis of all T cells and in high amounts for stimulation of clonal expansion. During the initial discovery of this cell lineage, the constitutive expression of the IL-2 receptor alpha-chain (CD25) on the cell surface and its function as a decoy receptor was described. However, this mechanism is disputed nowadays. Nevertheless, T_{Reg} significantly decrease IL-2 levels *in vitro*, most likely by inhibiting IL-2 mRNA induction in T_{Eff} (Thornton and Shevach 1998).

Non-contact-mediated suppression mechanisms are based on the production of soluble factors by T_{Reg} and include the inhibitory cytokines IL-10, TGFß and IL-35. IL-10 is a cytokine known to contribute to T_{Reg} function, but is also produced by many other immune cells, like e.g. monocytes, mast cells and T_H2 cells, and its deficiency preferentially affects autoimmune development in the gastrointestinal tract (Kuhn, Lohler et al. 1993). TGFß is a multi-functional cytokine that is expected to be mainly involved in T_{Reg} differentiation, rather than its suppressive function (Marie, Letterio et al. 2005, Shevach, Davidson et al. 2008). The cytokine IL-35 is contributing to T_{Reg} function by constitutive secretion and its suppressive effect on T cell proliferation. IL-35 has been proven to be

required in the immune control over inflammatory bowel diseases *in vivo* (Collison, Workman et al. 2007), however its absence could be compensated to prevent other autoimmune disorders. Therefore, IL-35 is not essential for T_{Reg} function.

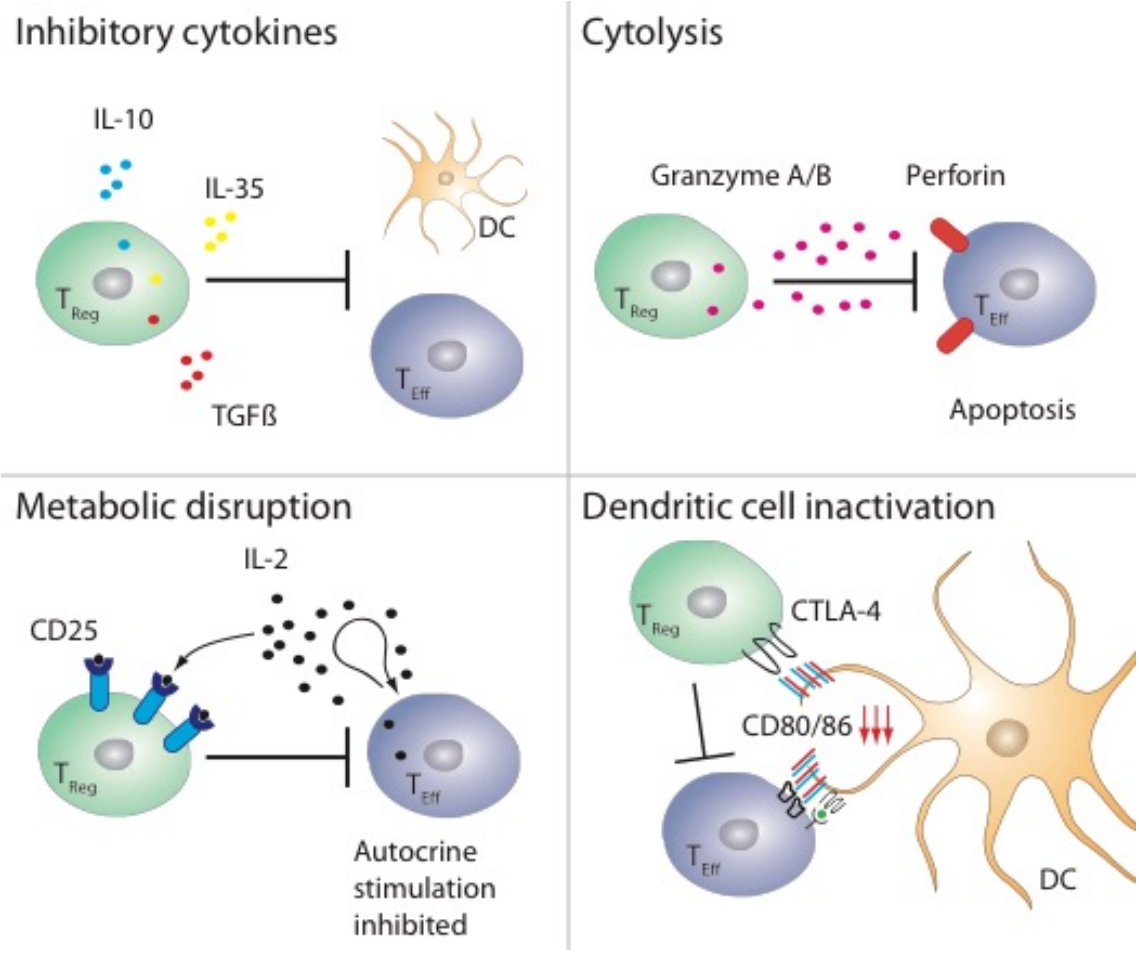

Figure 1.2: Overview of regulatory T cell suppression mechanisms
[The figure is taken from a review by Vignali et al. 2008.]

1.3.2 Contact-mediated regulatory T cell function

Contact-mediated suppression mechanisms either function in contact with DCs by decreasing costimulation or in contact with T_{Eff} by cytolysis. Especially the contact-dependent decrease of costimulatory molecules CD80 and CD86 on DCs through the membrane protein CTLA-4 on T_{Reg} has been proven to be irreplaceable as mice with a conditional knock-out of CTLA-4 in Foxp3-expressing cells (i.e. CTLA-4-deficient T_{Reg}) suffer from fatal autoimmunity similar to overall Foxp3 deficiency (Wing, Onishi et al. 2008). Likewise, mutations in the CTLA-4 gene in humans have been linked to complex immune dysregulation syndromes (Kuehn, Ouyang et al.

2014, Schubert, Bode et al. 2014). The decrease of CD80 and CD86 through CTLA-4 can also be achieved by trans-endocytosis (Qureshi, Zheng et al. 2011). In this contact-dependent mechanism, the immune control by T_{Reg} indirectly suppresses T_{Eff} immune responses by decreasing the costimulation of DCs that are presenting self-antigen. Moreover, inactivated DCs sustain tolerance by rendering circulating self-reactive T cells anergic.

1.4 Cell migration in adaptive immunity

Cell migration is vital in the development and maintenance of multicellular organisms with implications in embryonic development, tissue repair and immune responses. Immune cell migration is as irreplaceable as the cells' effector function during homeostasis as well as during every stage of an adaptive immune response. As the adaptive immune system relies on the near infinite repertoire of T and B cells with each exhibiting a single specificity, a constant patrolling throughout the body is necessary for the recognition of antigens at any particular site. The critical interaction site of antigen, innate and adaptive immune cells is the lymph node. Cell migration involves a multitude of membrane and secretory protein families. For example, the extravasation of T lymphocytes from the blood stream into tissues, also referred to as transendothelial migration or diapedesis, involves cellular adhesion molecules (CAM) like selectins and integrins on the surfaces of endothelial cells and T lymphocytes, as well as cytokines capable of inducing chemotaxis, a mechanism of gradient-based guided cell migration.

Chemokines and their receptors represent a protein family that is primarily responsible for chemotaxis and derived its name from the term chemotactic cytokines. They can be functionally divided into homeostatic and inflammatory chemokines. Homeostatic chemokines are constitutively produced and support steady state leukocyte patrolling. Inflammatory chemokines are induced by pro-inflammatory stimuli, like the cytokines interleukin-1 (IL-1) or tumor necrosis factor (TNF), and attract immune cells to the site of inflammation. Chemokines orchestrate systemic T cell migration in terms of cell homing to specific organs, as well as interstitial T cell migration in the sense of providing migratory pathways within lymphoid tissues.

1.4.1 Systemic T cell migration among lymphoid and non-lymphoid tissues

During homeostasis, constant circulation of immune cells is provided via blood and lymphatic vessels. Naive T cells circulate through our body using the blood stream to lymph nodes and the efferent lymph via the thoracic duct back to the blood stream. This process is merely random but supported by constitutive production of the chemokines CCL19/21 by high endothelial venules (HEV), which supports lymph node entry of naive T cells based on their expression of the chemokine receptor CCR7 (Bromley, Mempel et al. 2008). In contrast, the circulation of memory T cells is sophistically orchestrated. Memory T cells can be divided in central memory T cells recirculating lymph nodes and effector memory T cells recirculating the organs of their generation, i.e. the organ in which the initial immune response took place. On the one hand, central memory T cells preserve their CCR7 expression to allow further circulation of lymph nodes. On the other hand, effector memory T cells are characterized by loss of CCR7 expression and acquisition of organ-specific chemokine receptor profiles, a feature provided by the interaction with DCs called imprinting (Mora, Bono et al. 2003). This important feature of cell migration is referred to as homing. For example, the recirculation of T_{Eff} to the skin is (among others) characterized by CCR4 expression, whereas recirculation to the gut is sustained (among others) by CCR9 expression.

DCs, the antigen platform of lymph nodes, either transport antigens as peripheral DCs to the lymph node or take up lymph borne antigens as lymph node-resident local immature DCs (von Andrian and Mempel 2003). After peripheral DCs receive danger signals by a PAMP, they acquire expression of the chemokine receptor CCR7 in the process of maturation. This is an essential trigger for these DCs to enter afferent lymphatics and migrate to draining lymph nodes, guided by the homeostatic chemokine production of CCL19 and CCL21 by afferent lymph vessel endothelial cells (Forster, Schubel et al. 1999).

Chemokines are also involved in the migration of T_{Reg}. Lymph node homing of T_{Reg} equally requires CCR7 expression, a property they share with T_{Eff} (Schneider, Meingassner et al. 2007). The chemokine receptor repertoire of T_{Reg} compared to T_{Eff}, however, appears to be more diverse, allowing different T_{Reg} subsets to home to a large variety of organs and sustain immune tolerance (Mailloux and Young 2010, Campbell 2015). As a distinct difference, T_{Reg} show significantly higher expression of CCR4 and CCR8 along with stronger migratory response towards the respective chemokines

CCL17/22 and CCL1 (Iellem 2001). An overview of chemokine receptor expression, migratory properties and implied function for T_{Reg} is provided in Table 1.1 (Campbell and Koch 2011).

Receptor	Site of migration	Pathological implication
CCR2	Inflamed tissues	Allograft rejection
CCR4	Skin Other inflamed tissues	Skin and lung tolerance Inflammatory bowel disease Malignant tumor
CCR5	Inflamed tissues	Allograft rejection *Leishmania* infection Inflammatory bowel disease
CCR6	T_H17 cell-mediated inflammation	T_H17 cell-mediated autoimmune diseases
CCR7	Lymph node Spleen	Inflammatory bowel disease Allograft rejection
CCR8	Skin T_H2 cell-mediated inflammation	Not yet characterized
CCR9	Small intestine	Not yet characterized
CCR10	Mucosal tissues Skin	Skin infection Inflamed liver Malignant tumor
CXCR3	T_H1 cell-mediated inflammation	Inflamed liver
CXCR4	Bone marrow Peyer's patches	Malignant tumor
CXCR6	Liver	Not yet characterized

Table 1.1: Overview of chemokine receptors expressed by regulatory T cells
[The table is adapted from a review by Campbell and Koch, 2011]

1.4.2 Interstitial T cell migration within lymphoid tissues

The lymph node (LN) is a highly organized subunit of the immune system, integrating all available pieces of information for adaptive immune cells to make the fateful decision to either tolerate an antigen or launch an immune response against it. The interstitial migration of T cells within LNs is not stochastic but rather strategic to optimize the encounter with their cognate antigen (Krummel, Bartumeus et al. 2016). Microanatomic studies of LNs revealed specific distributions of immune cells in functionally separated areas, in particular T cell and B cell zones. This substructure in interstitial tissues depends on migration mechanisms – e.g. T cell zones show high CCL3 and CCL19

levels attracting CCR5$^+$ and CCR7$^+$ naive or central memory T cells, B cell zones show high CXCL13 levels attracting CXCR5$^+$ B cells. The importance of these homeostatically produced chemokines in the functional organization of a lymph node is exemplified by various pathogens, like *influenza virus, vaccinia virus* and *Listeria monocytogenes*, which disrupt these interstitial migratory processes as an immune evasion mechanism to boost their pathogenicity (Mueller, Hosiawa-Meagher et al. 2007). A simplified illustration of lymph node homing and interstitial organization is provided in Figure 1.3.

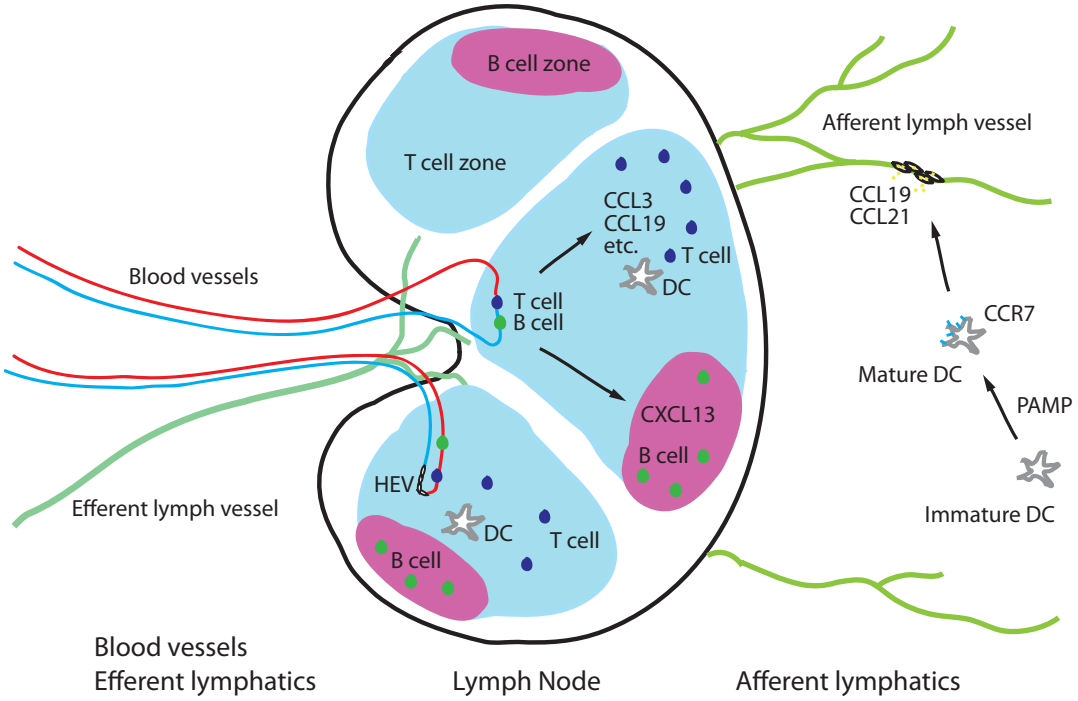

Figure 1.3: Basic illustration of lymph node homing and interstitial organization

The T cell zone itself is a dynamic conglomerate of CD4$^+$ effector (T_{Eff}) and CD8$^+$ cytotoxic T lymphocytes (CTL), DCs and stroma cells. Specific guidance mechanisms are also essential to the function of this subunit, in particular if the interaction of three specialized subsets of immune cells is required.

As an example, to initiate adaptive immune responses using T_{Eff} and CTL, both first need to interact with DCs. To facilitate these tricellular encounters, chemokines are induced during antigen-specific interaction of DCs and T_{Eff} to attract CTLs (Castellino, Huang et al. 2006), in this case CCL3, CCL4 and CCL5 for the chemokine receptor CCR5. This sophisticated mechanism could be identified in other functionally connected subpopulations of immune cells, e.g. DCs, T_{Eff} and NK cells (Semmling, Lukacs-Kornek et al. 2010), and highlights the importance of interstitial cell migration.

1.5 Objectives

The main objective of this thesis was to investigate chemokine-based migration systems that could impact the interstitial interaction of DCs and T_{Reg} and thereby represent a potential mechanism involved in immune tolerance. As outlined in the chapters 1.1 to 1.4, DCs integrate various pieces of information in the process of starting or inhibiting an immune response, such as the presence of pathogen-associated molecular patterns (PAMP) as a signal of danger for the host. The effectors of an adaptive immune response are T and B cells, which require the interaction with the dendritic cell as the central antigen-presenting platform of the host. As the interaction of DCs and T_{Reg} is necessary in the steady state to maintain immune tolerance (Wing, Onishi et al. 2008), a chemokine-based migration system could be sustaining the interaction of these two cell types within the lymph node. Through comprehensive literature review and previous results of our research group, we hypothesize the CCL22-CCR4 axis to be involved in such a process. The chemokine CCL22 is homeostatically produced in large amounts by immature DCs (Vulcano, Albanesi et al. 2001), and known to be a ligand for the chemokine receptor CCR4 (Imai, Chantry et al. 1998). CCR4 is a chemokine receptor expressed by a variety of immune cells, with a very high expression as well as chemotactic response reported for T_{Reg} (Iellem 2001). Interestingly, CCR4 expression is driven by Foxp3, the transcriptional regulator of T_{Reg} (Yuan, Bromley et al. 2007). Therefore, I investigated the constitutively expressed chemokine CCL22 in DCs and the constitutively expressed CCL22-responding chemokine receptor CCR4 on T_{Reg} in interstitial migration of these cell types. Following questions were addressed:

1. Do CCL22 and CCR4 play a role in cell-cell attraction of dendritic cells and regulatory T cells *in vitro* and *in vivo*?
2. Do innate or adaptive immune responses influence the expression of CCL22 in dendritic cells and of CCR4 in regulatory T cells?
3. Do CCL22 and CCR4 influence regulatory T cell suppression *in vitro*?

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Technical equipment

Amaxa Nucleofector II	Lonza, Basel, Switzerland
Balance (LP 6209)	Sartorius, Göttingen, Germany
Cell culture CO2 incubator (BD 6220)	Heraeus, Hanau, Germany
Cell culture laminar flow	Heraeus, Hanau, Germany
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Centrifuge 5424	Eppendorf, Hamburg, Germany
Confocal Leica TCS SP5	Leica Microsystems, Wetzlar, Germany
DynaMag 15/50 magnet	Invitrogen Dynal, Carlsbad, USA
FACSCanto II	Becton Dickinson, San Jose, USA
LightCycler 2.0 System	Roche, Mannheim, Germany
Microscope Axiovert 25	Zeiss, Jena, Germany
MiniMACS, QuadroMACS	Miltenyi, Bergisch Gladbach, Germany
Mithras LB940 multilabel plate reader	Berthold, Bad Wildbad, Germany
Multifuge 3L-R	Heraeus, Hanau, Germany
Nanodrop ND-1000	NanoDrop, Wilmington, USA
BX50WI fluorescence microscope	Olympus, Tokyo, Japan
Omnifuge 2 ORS	Heraeus, Hanau, Germany
pH meter	WTW, Weilheim, Germany
Power Supply 200/2.0	Biorad, Munich, Germany
Refrigerators (4°C, -20°C, -80°C)	Thermo Scientific, Waltham, USA
Shaker	NeoLab, Heidelberg, Germany
Thermocycler T3	Biometra, Göttingen, Germany
Thermomixer	Eppendorf, Hamburg, Germany
Vortex VF2	Janke & Kunkel, Staufen, Germany

2.1.2 Chemicals, reagents and buffers

Aqua ad injectabilia	Braun AG, Melsungen, Germany
Bovine serum albumine (BSA)	Sigma Aldrich, Steinheim, Germany
Collagenase D	Roche, Mannheim, Germany
Chloroform	Sigma Aldrich, Steinheim, Germany
DNase II	Roche, Mannheim, Germany
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, Steinheim, Germany
Dulbecco’s PBS (1x)	PAA, Pasching, Austria
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, Steinheim, Germany
FACSFlow, FACSSafe	Becton Dickinson, San Jose, USA

Heparin-Natrium 25,000 I.E./5 ml	Braun AG, Melsungen, Germany
Isoflurane (Forene®)	Abbott, Zug, Switzerland
Isopropanol (70 Vol%)	Apotheke Innenstadt, LMU Munich
Paraformaldehyde (PFA)	Sigma Aldrich, Steinheim, Germany
Sodium azide (NaN3)	Sigma Aldrich, Steinheim, Germany
Sodium chloride (NaCl 0.9%)	Apotheke Innenstadt, LMU Munich
Trypan blue	Sigma Aldrich, Steinheim, Germany
Trypsin (10x)	PAA, Pasching, Austria

MACS buffer
2 mM EDTA
2% FCS
in PBS

Cell fixation buffer
2 % PFA
in PBS

ELISA coating buffer
in PBS
pH 7.2-7.4

ELISA assay diluent
10% FCS or 1% BSA
in PBS
pH 7.2-7.4

ELISA wash buffer
0.05% Tween 20
in PBS

2.1.3 Cell culture materials, reagents and media

β-Mercaptoethanol	Sigma Aldrich, Steinheim, Germany
5-Bromo-2′-deoxyuridine (BrdU)	Sigma Aldrich, Steinheim, Germany
DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-3N-trimethylammonium-methylsulfate	Roche, Mannheim, Germany
Dulbecco's modified Eagle´s medium (DMEM), high glucose	PAA, Pasching, Austria
Fetal calf serum (FCS)	GibcoBRL, Karlsruhe, Germany
Hank's balanced salt solution (HBSS)	PAA, Pasching, Austria
Iscove's modified Dulbecco's medium (IMDM)	PAA, Pasching, Austria
L-glutamine 200mM	PAA, Pasching, Austria
MEM-NEAA (non-essential amino acids)	GibcoBRL, Karlsruhe, Germany
Phosphate-buffered saline (PBS)	PAA, Pasching, Austria
Penicillin / streptomycin (100x)	PAA, Pasching, Austria
Roswell Park Memorial Institute (RPMI) 1640 medium	PAA, Pasching, Austria
Sodium pyruvate	PAA, Pasching, Austria

RPMI complete medium	IMDM complete medium
10% FCS	10% FCS
2 mM L-glutamine	2 mM L-glutamine
100 IU/ml penicillin	100 IU/ml penicillin
100 µg/ml streptomycin	100 µg/ml streptomycin
1 mM sodium pyruvate	1 mM sodium pyruvate
1% non-essential amino acids	1% MEM-NEAA
(MEM-NEAA)	in IMDM
3.75 x 10-4 % β-mercaptoethanol	
in RPMI 1640	

DMEM complete medium	Cryo medium
10% FCS 2 mM L-glutamine	50% DMEM complete medium
100 IU/ml penicillin	40% FCS
100 µg/ml streptomycin	10% DMSO

Cytokines and OVA peptides	
Granulocyte-macrophage colony-stimulating factor (GM-CSF), recombinant murine	PeproTech, Rocky Hill, USA
IL-4, recombinant murine	PeproTech, Rocky Hill, USA
OVA 323-339	InvivoGen, San Diego, USA

Chemokines and blocking antibodies	
Mouse CCL22 Antibody, monoclonal	R&D Systems, Minneapolis, USA
Recombinant Mouse CCL22	R&D Systems, Minneapolis, USA

Disposable plastic materials for cell culture experiments were purchased from Becton Dickinson (Heidelberg, Germany), Bibby Sterilin (Stone, Staffordshire, Great Britain), Corning (Corning, USA), Eppendorf (Hamburg, Germany), Falcon (Heidelberg, Germany), Nunc (Rochester, USA) or Sarstedt (Nürnbrecht, Germany).

2.1.4 Oligonucleotides, TLR ligands and other stimuli

CpG 1826 (CpG)	Coley, Langenfeld, Germany
Curdlan	Roche, Mannheim, Germany
Flagellin	InvivoGen, San Diego, USA
Lipopolysaccharide (LPS) (*Salmonella enterica ssp. enterica*)	Sigma, St. Louis, USA
PAM3CysSerLys4 (Pam3CysK)	tebu-bio, Offenbach, Germany
Peptidoglycan	InvivoGen, San Diego, USA
Poly (dA)	InvivoGen, San Diego, USA

Poly (I:C) HMW	InvivoGen, San Diego, USA
R848	InvivoGen, San Diego, USA
ssPoly U Naked	InvivoGen, San Diego, USA
5'-triphosphate 2.2ds RNA (3pRNA)	Eurogentec, Köln, Germany
9.2s double right RNA	CureVac, Tübingen, Germany

Poly (dA), 3pRNA and Poly (I:C) HMW were transfected using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions.

2.1.5 Kits

Cell labeling

PKH26 Red Fluorescent Cell Linker Kit	Sigma Aldrich, Steinheim, Germany
PKH67 Red Fluorescent Cell Linker Kit	Sigma Aldrich, Steinheim, Germany
CellTracker Green CMFDA Dye	Thermo Fisher Scientific, Waltham, USA
CellTracker Orange CMTMR Dye	Thermo Fisher Scientific, Waltham, USA

RNA isolation, reverse transcription, qRT-PCR

High pure RNA isolation kit	Roche, Mannheim, Germany
Transcriptor first strand cDNA synthesis kit	Roche, Mannheim, Germany
LightCycler TaqMan Master kit	Roche, Mannheim, Germany
Universal ProbeLibrary	Roche, Mannheim, Germany

Magnetic-activated cell sorting

CD4+CD25+ T Cell Isolation Kit, murine	Miltenyi, Bergisch Gladbach, Germany
CD8a Micro Beads, murine	Miltenyi, Bergisch Gladbach, Germany
CD11c Micro Beads, murine	Miltenyi, Bergisch Gladbach, Germany
CD45R/B220 Micro Beads, murine	Miltenyi, Bergisch Gladbach, Germany

Cytokine ELISA sets

CCL17 murine + human	R&D Systems, Minneapolis, USA
CCL22 murine + human	R&D Systems, Minneapolis, USA
IL-2 murine	BD Biosciences, San Diego, USA
IL-10 murine	BD Biosciences, San Diego, USA

2.1.6 FACS antibodies

Description	Clone	Distributor
anti-BrdU	BU20A	BioLegend, San Diego, USA
anti-CCR4	2G12	BioLegend, San Diego, USA
anti-CD3	17A2	BioLegend, San Diego, USA
anti-CD4	RMA4-5	BioLegend, San Diego, USA
anti-CD8a	53-6.7	BioLegend, San Diego, USA
anti-CD11c	HL3	BioLegend, San Diego, USA
anti-CD25	7D4	BioLegend, San Diego, USA
anti-CD69	H1.2F3	BioLegend, San Diego, USA
anti-CD80	16-10A1	BioLegend, San Diego, USA
anti-CD86	GL1	BioLegend, San Diego, USA
anti-Foxp3	FJK-16S	eBioscience, San Diego, USA

2.1.7 Software

Adobe Illustrator CS4	Adobe System, San Jose, USA
Endnote X7	Thompson Reuter, Carlsbad, USA
FlowJo 8.7	Tree Star, Ashland, USA
Imaris 7.0	Bitplane, Zurich, Switzerland
Microsoft Office	Microsoft, Redmond, USA
Prism 5	GraphPad, La Jolla, USA

2.2 Methods

2.2.1 Fluorescence-activated cell sorting (FACS)

Fluorescent-activated cell sorting (FACS) - also called flow cytometry - is a method to count, investigate and sort cells. Single-cell suspensions need to be prepared for FACS analysis. A variety of antibodies conjugated with fluorescent molecules (fluorochromes) can be bound to an antigen. The cell suspension is taken up through a thin capillary into a flow cell, where the cells run through a focused laser beam one at a time. When hitting a cell, the light is either absorbed or scattered. Absorbed light of the appropriate wavelength will be re-emitted as fluorescence in case the cell has an attached fluorescent molecule or the cell is labeled with an antibody conjugated with a fluorochrome. The scatter of the light depends on intracellular structures and the cell's configuration and dimension. Light scatter at low angles depends on the relative cell dimension and is reported as forward scatter (FSC). Light scatter orthogonal to the capillary depends on the cell's granularity and surface configuration, which is recorded

as side scatter (SSC). Several photodiodes detect and amplify the fluorescence and light scatter. Optical filters permit light of a specific wavelength of fluorescence to reach the photodiode.

In this thesis, all studies were performed with a FACSCanto II. This device used three lasers with a wavelength of 405 nm, 488 nm and 633 nm. A multi-color capability system detected the following fluorochromes: Pacific Blue, Pacific Orange, AmCyan, FITC, PE, PerCP, PerCP-Cy5.5, PE-Cy7, APC, APC-Cy7.

2.2.1.1 Analysis of cell surface antigens

For evaluation of antigens on the cell surface, the cells were diluted at 1-2 x 10^6 in PBS with 10% FCS. Up to seven flourochrome-conjugated antibodies targeted against antigens of interest were added at a 0.5 µl/ml concentration. Cells together with the antibodies were incubated for 30 minutes at a temperature of 4°C. During the staining, light exposure of cells was kept as low as possible. Cells were washed two times with PBS and re-suspended in PBS supplemented with 10% FCS for the FACS analysis.

2.2.1.2 Analysis of intracellular antigens

The intracellular transcription factor Foxp3 was analyzed to characterize regulatory T cells using the Treg staining Kit (BD Biosciences). At first, surface antigens were stained as described in the previous chapter. Antibodies bound to the cell surface were fixed and the cellular membranes were permeabilisized to enable the anti-Foxp3 antibody to enter the cytosol. For this, the cells were incubated in the fixation/permeabilisation buffer for 30 minutes at a temperature of 4°C. After one washing step with buffer, the cells were incubated with 0.5 µl anti-Foxp3 antibody at a temperature of 4°C for another 30 minutes. At last, the cells were washed two times and suspended in PBS with 10% FCS for the following analysis. Furthermore, the intracellular uptake of BrdU was analyzed to measure the proliferation of cells. The staining with the anti-BrdU antibody was performed equal to anti-Foxp3.

2.2.2 Magnetic-activated cell sorting (MACS)

Magnetic-activated cell sorting (MACS) is a method to isolate live and functional cells with minimal interference for the following experiments. Cells are labeled with superparamagnetic particles, so-called magnetic beads. These are biodegradable,

therefore there is no need to remove them from the isolated cells after the sorting procedure. The magnetic beads are linked with antibodies specific for surface antigens of interest and attach to the cells expressing this specific antigen. Labeled cells are subsequently put into a plastic column that is placed in a strong magnetic field induced by a permanent magnet. The magnet retains cells attached to the magnetic beads in the column while unlabeled cells pass and are collected. After removing the column from the magnetic field, the retained cells can be washed out of the column and collected. Therefore, cells can be sorted positively or negatively. Positive selection is performed by isolating the cells of interest that are magnetically labeled and retained. Negative selection means depleting a cell suspension of unwanted cells and using the cell fraction passing through the magnet.

In this thesis, all reagents were used from Miltenyi Biotec. T_{Reg} and T_{Eff} were isolated from splenocytes in a two-step process. In a first step, negative selection of $CD4^+$ cells was performed by using an antibody cocktail against non-$CD4^+$ cells. In a second step, positive selection using CD25 beads was performed. In line with the manufacturer's instructions, cells were washed with MACS buffer and incubated together with the microbeads at a temperature of 4°C for 15 min and then washed two times. LD columns were rinsed with 3 ml MACS buffer, loaded with the incubated cells diluted in 2 ml of buffer and then positioned in the provided MACS separator magnet. The columns were washed in three steps with 3 ml MACS buffer. The effluent was collected as the negative fraction. After removal from the magnetic field, the elution was performed by using a plunger to flush the labeled cells of the column with 2 ml MACS buffer. For the smaller fractions of the $CD4^+$ isolated cells MS columns were rinsed, loaded, washed two times and eluted with 1ml MACS buffer each. Splenic DCs were separated from splenocytes using CD11c beads and LD columns.

2.2.3 Enzyme-linked immunosorbent assay (ELISA)

The Enzyme-linked immunosorbent assay (ELISA) is a technique used to quantitatively analyze an antigen of interest in a sample by specific antibodies linked to enzymes. As an antibody binds to an antigen, this subsequently leads to the conversion of a substrate to a colored substance by the enzyme that is coupled to the antibody.

In this thesis, chemokines and cytokines from cell culture supernatants were analyzed by sandwich ELISA. ELISA detection plates were coated with antibodies that bind the desired antigen. Fixed antigens were treated with a second, biotinylated antibody. In a next step, this antibody was bound by streptavidin coupled with the enzyme peroxidase that catalyzes the oxidation of tetramethylbenzidine with hydrogen peroxide to a fluorescent molecule. The analysis of every studied antigen was done with openly available detection kits in line with the company's' instructions.

The detection plates were coated overnight at 4°C with capture antibody in coating buffer. Unspecific binding on the plates was minimized by diluent, applied for 60 minutes at room temperature. Coated plates were washed several times with washing buffer, then the samples and reference protein dilutions were put on the plates and again incubated. After several washing steps, horseradish peroxidase was used to identify the plate-bound detection antibody. After the incubation at room temperature, tetramethylbenzidine with hydrogen peroxide were used as substrates for the enzyme. For this, substrate solution was put into each well after the plates were washed. The enzymatic reaction was terminated by adding sulfuric acid. Readout of all ELISA assays was performed at 450 nm wavelength with correction through subtraction at 590 nm.

2.2.4 Chemokine knock-down by RNA interference

RNA interference (RNAi) is a technique that enables to modulate the expression of specific proteins by inhibiting the translation of the corresponding messenger RNA (mRNA). To achieve this, a specific RNA sequence is produced, e.g. in the form of short-interfering RNA (siRNA), that binds the corresponding mRNA. For this purpose, it has to be located in the cytosol to be in proximity to the mRNA. The binding of siRNA to the mRNA is mediated by the protein RNA-induced Silencing Complex (RISC), which subsequently leads to the cleavage and degradation of the mRNA. As a result, the mRNA cannot be translated and its corresponding protein not be produced.

In this thesis, this method was used to inhibit the production of the chemokine CCL22 in bone marrow-derived dendritic cells (BMDC). BMDC from C57BL/6 mice were transfected (Amaxa nucleofector system; Y-001 immature DC program) at a cell number of 10×10^6 with 10 ng CCL22-siRNA (Mm_Ccl22_3 FlexiTube siRNA; Qiagen),

CCL17-siRNA (Mm_Ccl17_1 FlexiTube siRNA; Qiagen) or control-siRNA (AllStars Negative Control siRNA; Qiagen). After transfection DC were rested for 2 hours.

2.2.5 T cell proliferation assay

T cell proliferation was assessed by measuring the incorporation of 5-Bromo-2′-deoxyuridine (BrdU) into the DNA of the cells during replication. Herefore, BrdU was added to the culture medium during the whole time of the incubation at a concentration of 10 μM. The BrdU uptake was assessed by flow cytometry analysis of intracellular BrdU (chapter 2.2.1.2.) using an anti-BrdU antibody (BioLegend).

2.2.6 Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from sorted single cell suspensions using High Pure RNA Isolation Kit (Qiagen) according to the manufacturer's instructions. 1 μg of RNA was converted into cDNA using the Revert Aid First strand cDNA Synthesis Kit (Fermentas). Quantitative real-time PCR amplification was performed with the Light Cycler TaqMan Master (Roche Diagnostics) on a LightCycler 2.0 instrument (Roche Diagnostics) together with the Universal Probe Library System (Roche Diagnostics; CCL22 probe #84; HPRT probe #69). Relative gene expression is shown as a ratio of the CCL22 mRNA expression level to the expression level of hypoxanthine phosphoribosyltransferase (HPRT) mRNA. The primers for CCL22 (left: tcttgctgtggcaattcaga; right: gagggtgacggatgtagtcc) and HPRT (left: ggagcggtagcacctcct; right: ctggttcatcatcgctaatcac) were obtained from Metabion.

2.2.7 Confocal microscopy

In all experiments, static and dynamic visual data were achieved using a Leica TCS SP5 laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). Magnification was set to 40x unless indicated otherwise. For general cell membrane labeling fluorophores PKH26 (Sigma, red dye) and PKH67 (Sigma, green dye) were used according to the manufacturer's protocol. A xyz-positioning table enabled time-lapse video microscopy with intervals of 60 seconds unless indicated otherwise. In order to provide cell culture conditions during imaging, all samples were incubated at 37°C and 5% CO_2 using a heating stage (Leica, Microsystems, Wetzlar, Germany).

2.2.8 Cell lines and culture

The murine immortalized DC line DC2.4 was kindly provided by K. Rock (University of Massachusetts, USA). DC2.4 and primary immune cells were cultured in RPMI1640 medium supplemented with 10% FBS, 1% penicillin and streptomycin, 1% L-glutamine, 1% sodium pyruvate, 1 mM HEPES and 50 μM β-mercaptoethanol (all from PAA).

2.2.9 Statistical Analysis

All data are presented as mean +/- SEM and the statistical significance of differences were determined by the two-tailed Student's t-test. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). p values < 0.05 were considered significant.

2.3 Animal experimentation

2.3.1 Animals

Female C57BL/6 mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Mice transgenic for a chicken OVA 323–339-specific T cell receptor (OT-II) were purchased from the Jackson laboratory (Bar Harbor, USA). OT-II-Foxp3-GFP mice were a gift from Vijay Kuchroo (Boston, USA). Mice were 5 to 10 weeks of age at the onset of experiments. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

2.3.2 Organ preparation

2.3.2.1 Isolation of splenocytes and lymph node cells

The mice were anesthetized with isoflurane and sacrificed. Spleens were removed and tissues were enzymatically digested with collagenase D (1 mg/ml) and DNase I (0.05 mg/ml) in complete medium at 37°C and moderate stirring for 25 to 35 minutes. The digested tissues were passed through a 40 µm-pore cell strainer to disintegrate tissue structure. Single cell solutions were washed with PBS and centrifuged at 400 G for 7 minutes and resuspended in erythrocyte lysis buffer. Red blood cell debris was removed by a second centrifugation step. Inguinal and axillary lymph nodes were resected and directly pressed through a 40 µm-pore cell strainer.

2.3.2.2 Generation of bone marrow-derived dendritic cells

Bone marrow cells of wild type (WT) mice were isolated from the femur and tibia bones. After incubation with red blood lysis buffer cells were diluted to $1x10^6$ cells/ml in RPMI medium supplemented with 10 % FBS (Gibco BRL), 1 % L-glutamine, 1 U/ml penicillin, 0.1 mg/ml streptomycin (all PAA), 20 ng/ml GM-CSF and 20 ng/ml IL-4 (both PeproTech). Bone marrow-derived DCs (BMDC) were harvested on day 7.

2.3.3 *In vivo* TLR stimulation assay

Mice were injected subcutaneously with 100 µg fully phosphorothioated CpG oligodeoxynucleotide 1826 (59-TCCATGACGTTCCTGACGTT-39; Coley Pharmaceutical) diluted in 100 µl PBS or 100 µl PBS alone. Mice were sacrificed 24 hours after injection.

2.3.4 *In vivo* infection assay

Mice were infected orally with the Salmonella enterica serovar Typhimurium (S. typhimurium) strain SB300. The strain was cultured in 0.3 M NaCl Luria–Bertani medium to allow for the activation of the Salmonella type III secretion system before oral infection. 24 hours' prior, mice were orally treated with 20 mg streptomycin to ensure consistent infection conditions. Water and food were withdrawn 4 hours before the mice were orally immunized with 10^3 colony forming units (CFU) of S. typhimurium in PBS by using round-bottom gavage needles. Afterwards, drinking water was offered immediately and food was provided 2 hours later. Mice were sacrificed 24 hours after infection.

2.3.5 Intravital microscopy

Intravital microscopy is an umbrella term for the microscopic study of biological processes *in vivo*. In the field of immunology, the observation of leukocytes has long relied on *ex vivo* static analyses, which did not provide enough information about the physiologic movement of leukocytes. The research on the cell recruitment cascade was revolutionized by intravital microscopy. In these experiments, the blood vessels of mice were examined in paper-thin muscular structures, like e.g. the cremaster muscle, as this setup enables to visualize the blood vessels and the leukocytes by transillumination. This, however, limits the imaging studies to tissues with a maximum depth of

approximately 50 μm, thereby the study of secondary lymphoid organs such as the lymph node would not be possible by this technique.

Multi-photon intravital imaging (MP-IVM) relies on the excitation of fluorophores by multiple uniform photons, which together meet the necessary energy, in contrast to a single high energy photon. In most applications, two uniform photons are generated by specialized pulsed lasers and used to excite the fluorophores. The wavelength is usually ranging in the infrared spectrum as this reduces the scattering significantly compared to photons of shorter wavelengths. The reduced scattering allows to excite fluorophores in deeper tissues of up to 1,000 μm. This technique therefore enabled us to study *in vivo* cell migration and interaction in secondary lymphoid tissues such as the lymph node.

2.3.5.1 Animal preparation

In the field of immunology, MP-IVM is often performed using the lymph node in the popliteal region of mice, as it lies superficial and is accessible for preparation. Prior to the preparation and during the whole imaging time period, the mice were anaesthetized by an injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) into the peritoneum. The hair covering the right popliteal region was shaved off. The popliteal lymph node was prepared with microsurgical techniques, paying particular attention to not injure adjacent blood vessels or lymph vessels. The prepared lymph node was subsequently dipped in saline using a custom-built construction, which was covered by glass. This construction was connected to a thermocouple adjacent to the lymph node to maintain a temperature around 36 to 37°C. The right popliteal lymph node was then analyzed for 1 hour by two-photon intravital imaging on an Olympus BX50WI fluorescence microscope equipped with a 20×, 0.95 numerical aperture objective.

In the experiments of this thesis, pretreated BMDC were used as experimental groups. The BMDC were prepared from bone marrow of C57BL/6 mice and transfected with either control- or CCL22-siRNA. Subsequently DC were pulsed with OVA323–339 peptide and labeled for 20 min at 37°C with 10 mM 5-(and 6-)-([(4-chloromethyl)benzoyl] amino) tetramethylrhodamine (CellTracker CMTMR; Invitrogen) or 7-amino-4-chloromethylcoumarin (Cell Tracker CMAC; Invitrogen). Control-siRNA- and CCL22-siRNA-treated DC (each at a number of $2x10^6$) were co-injected in 20 μl IMDM

(with 10% FCS) containing 10 ng *E. coli* LPS (Sigma) into the right hind footpad of C57BL/6 OT-II-Foxp3-GFP mice.

2.3.5.2 Data acquisition & analysis

For four-dimensional analysis of cellular migration, stacks of six squared x–y sections with 6 μm z spacing were acquired every 20 s with electronic zooming up to 4 times to provide image volumes 30 μm in depth and 300 μm in width. All image analysis was performed blinded by two independent investigators using Imaris 7.0 software (Bitplane). Cells were manually tracked to calculate instantaneous velocities. The number of contacts and the contact time between DCs and T_{Reg} were manually measured. Cellular interactions that were shorter than 2 minutes or incompletely depicted spatially or temporally were excluded from the analysis. The contact time was manually measured in each case as number of frames during the interaction multiplied by the time interval of image acquisition.

3 RESULTS

Chemokine guidance of immune cells in interstitial tissues has been demonstrated to increase the interaction between distinct immune cell subgroups and amplify subsequent immune responses (Castellino, Huang et al. 2006, Semmling, Lukacs-Kornek et al. 2010). We hypothesized that a chemokine-mediated mechanism may be involved in the formation of contacts between dendritic cells (DC), regulatory T cells (T_{Reg}) and effector T cells (T_{Eff}). Review of the literature and previous work of our research group identified the homeostatically produced chemokine CCL22 by DCs as a possible candidate, supported by the fact that the corresponding chemokine receptor CCR4 is highly expressed on T_{Reg}. This thesis investigates the *in vitro* and *in vivo* roles of CCL22 and CCR4 in interstitial migration of DCs and T_{Reg}, the regulation of CCL22 and CCR4 by innate and adaptive immunity and the role of CCL22 in *in vitro* T_{Reg} function.

3.1 Static *in vitro* analysis of dendritic cell - regulatory T cell interaction

In order to investigate whether the CCL22-CCR4 axis is involved in interstitial migration of DCs and T_{Reg} we studied static *in vitro* interactions of these cells in two independent experimental interaction setups as well as in the absence or presence of cognate antigen of T_{Reg}.

3.1.1 CCL22 production favors antigen-independent dendritic cell - regulatory T cell interaction

One method of studying *in vitro* cell interactions is coincubation of cells on fibrogen-coated flat bottom dishes, which allow 2-dimensional cellular migration. As a simplified model of chemokine-producing DCs we used the immortalized dendritic cell line DC2.4 (provided by K. Rock). This cell line is unable to secrete CCL22 due to the mutations leading to immortalization. As comparison, we used DC2.4 cells transfected with CCL22 (DC2.4-CCL22) that spontaneously and continuously produce CCL22. We set up a coincubation experiment of 100,000 MACS-sorted $CD4^+$-$CD25^+$ wild type (WT) T_{Reg} cells with either 50,000 DC2.4 or DC2.4-CCL22 cells on fibrogen-coated dishes. Prior to coincubation, the T_{Reg} cells and the DC2.4 cells were labeled using the

fluorophores PKH26 and PKH67. Colocalization analysis by confocal microscopy was used as readout after 15 and 30 hours of coincubation. Figure 3.1a shows the CCL22 amounts in the supernatants of coincubated cells measured by ELISA, validating the CCL22 production of the transfected DC2.4 cell line. Figure 3.1b provides colocalization frequencies of T_{Reg}-DC per DC per high-power field that were measured by two independent blinded readers (representative of three independent experiments).

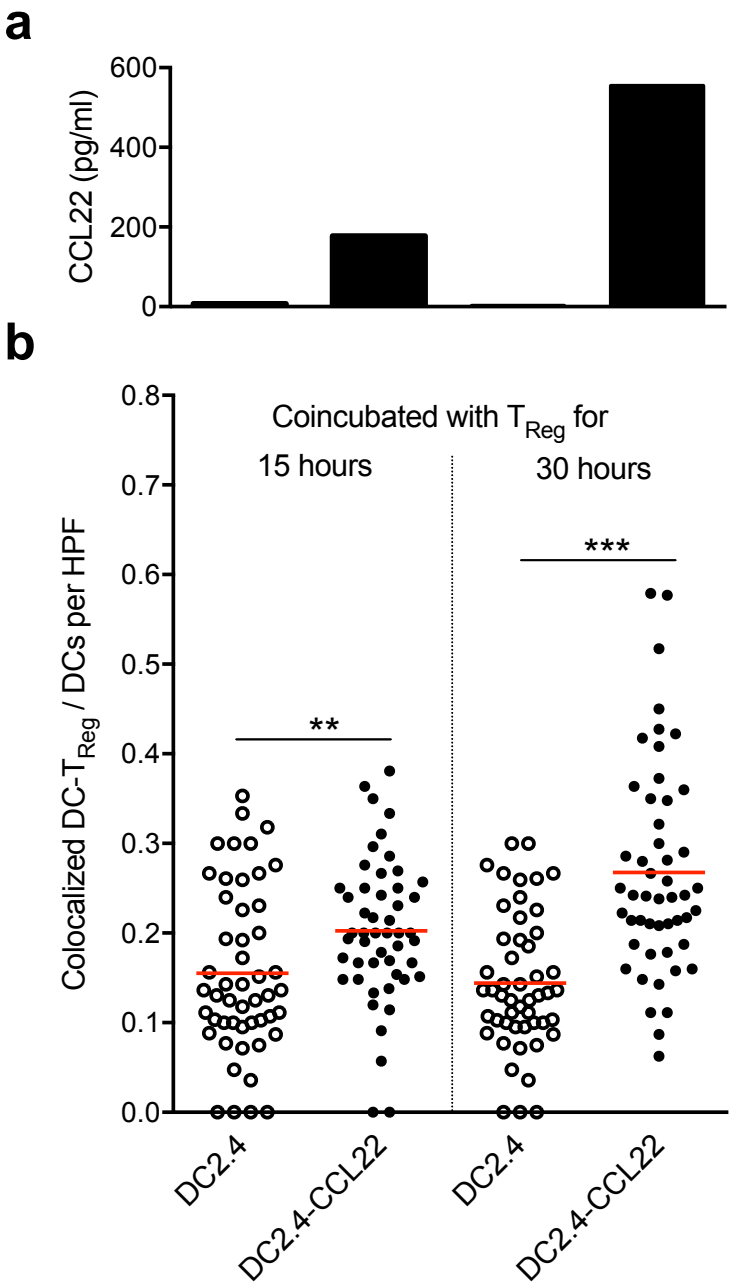

Figure 3.1: Colocalization of coincubated T_{Reg} and immortalized DCs of the cell lines DC2.4 and DC2.4-CCL22 after 15 and 30 hours

50,000 DC2.4 or DC2.4-CCL22 cells were coincubated with 100,000 $CD4^+$-$CD25^+$-sorted WT T_{Reg} and analyzed by confocal microscopy after 15 and 30 hours. (a) CCL22 levels were measured in the corresponding supernatants by ELISA. (b) The number of colocalized DC-T_{Reg} divided by all DCs in one high-power field (HPF) was assessed for 50 HPF in each condition. P values were calculated comparing DC2.4 and DC2.4-CCL22 (** $p < 0.01$; *** $p < 0.001$).

It shows that DC2.4-CCL22 have a significantly higher colocalization rate with T_{Reg} at the two different time points, 15 hours and 30 hours. Furthermore, the difference between the DC2.4 and DC2.4-CCL22 condition increased following longer coincubation time. These data suggest that CCL22 favors DC-T_{Reg} interactions *in vitro* in a time (or respectively dose)-dependent manner.

3.1.2 CCL22 production favors antigen-dependent dendritic cell - regulatory T cell aggregate formation

The formation of an immunological synapse between DCs and T cells is sustained by the presentation and recognition of cognate antigen and the interaction of costimulatory molecules. Onishi et al. investigated the setting in which T_{Eff} and T_{Reg} are competing for the interaction with a DC presenting their cognate antigen (Onishi, Fehervari et al. 2008). They demonstrated that DCs preferentially form aggregates with T_{Reg}, which therefore strongly outcompeted T_{Eff}. To study the role of CCL22 production by DCs in the process of DC - T cell aggregate formation *in vitro* we set up a coincubation experiment in round bottom dishes allowing 3-dimensional cellular interactions. An antigen-specific setting was established using T cells isolated from OT-II transgenic mice, which produce $CD4^+$ T cells recognizing the specific peptide OVA 323-339 of the protein ovalbumin. 50,000 MACS-sorted $CD4^+$-$CD25^+$ OT-II T_{Reg} cells together with 50,000 $CD4^+$-$CD25^{neg}$ OT-II T_{Eff} and 25,000 MACS-sorted OVA 323-339 pulsed $CD11c^+$ DCs were used. T_{Reg} and T_{Eff} cells were labeled prior to coincubation using the fluorophores PKH26 and PKH67. To study the presence and absence of CCL22, we added either anti-CCL22 blocking antibody or a control antibody at a concentration of 2 μg/ml. After 24 hours, the cells were gently transferred to glass bottom dishes to preserve the formed cell aggregates. Consecutively, the readout was performed using confocal microscopy and the contact ratio of T_{Reg} to T_{Eff} was measured per aggregate. The results of one of three independent experiments is shown in Figure 3.2a, representative confocal microscopy images are provided in Figure 3.2b. The T_{Reg} / T_{Eff} contact ratio is significantly lower in the condition with added anti-CCL22 blocking antibody compared to the control antibody. These data suggest that the absence of CCL22 decreases the likelihood of *in vitro* T_{Reg} aggregate formation with DCs compared to T_{Eff} and support the hypothesis that CCL22 promotes preferential encounters between DCs and T_{Reg}.

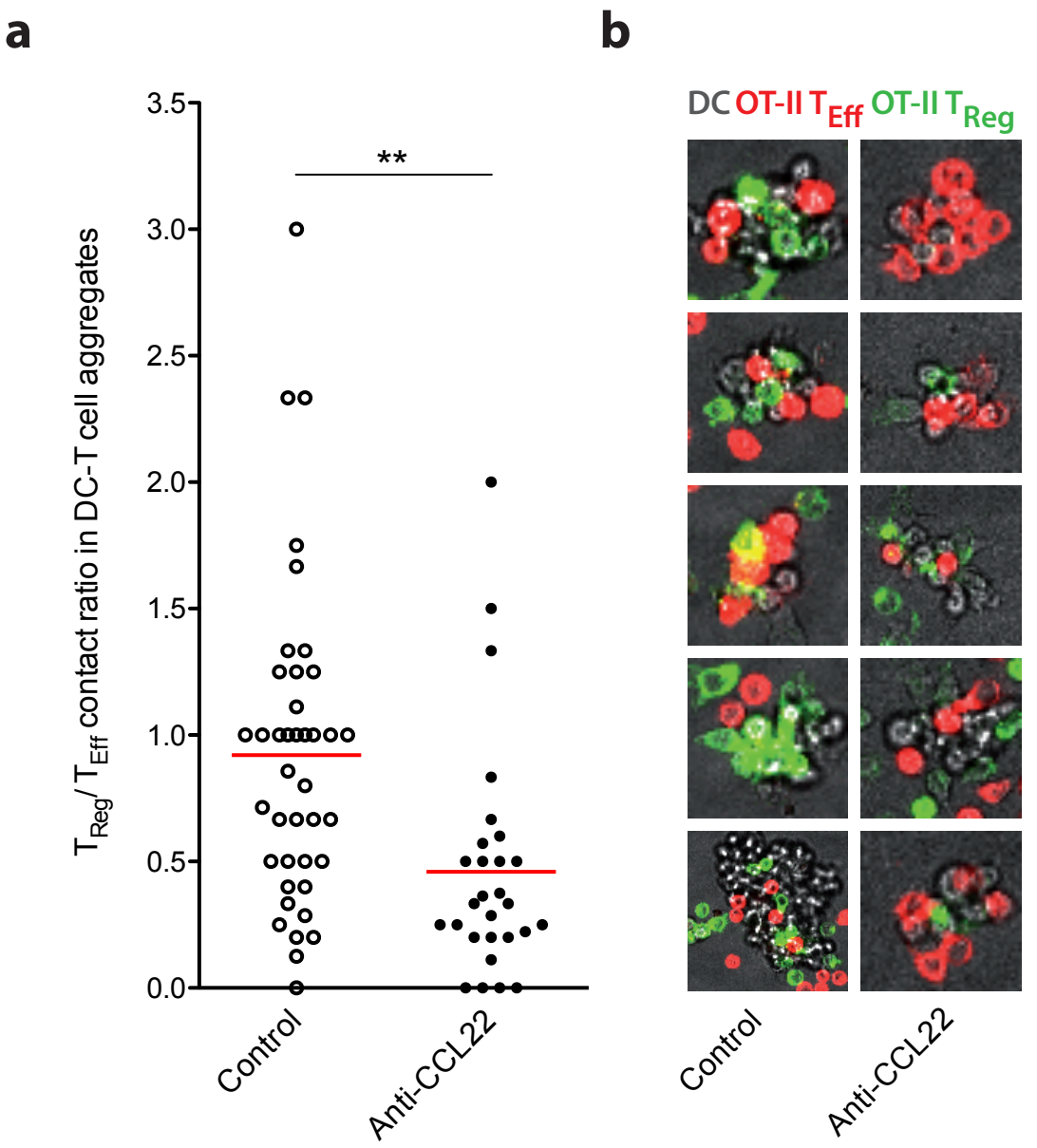

Figure 3.2: Contact ratio of OT-II T_{Reg} and OT-II T_{Eff} in antigen-dependent DC-T cell aggregate formation in the presence of control or anti-CCL22 blocking antibody

25,000 OVA 323-339 pulsed $CD11c^+$-sorted DCs were coincubated for 24 hours with 50,000 $CD4^+$-$CD25^+$-sorted OT-II T_{Reg} and 50,000 $CD4^+$-$CD25^{neg}$-sorted OT-II T_{Eff} with control or anti-CCL22 blocking antibody (2 μg/ml) and subsequently gently transferred to glass bottom dishes for analysis by confocal microscopy. (a) The graph shows the ratio of OT-II T_{Reg} to OT-II T_{Eff} in DC-T cell aggregates. (b) Representative confocal microscopy images of DC-T cell aggregates for each condition. P value was calculated relative to the anti-CCL22 condition (** $p < 0.01$).

3.2 Dynamic *in vivo* analysis of dendritic cell - regulatory T cell interaction

3.2.1 Experimental setup for *in vivo* analysis of the influence of CCL22 on dendritic cell - regulatory T cell interactions

After establishing a role of CCL22 in static *in vitro* migration between DCs and T_{Reg} we wanted to investigate this hypothesis *in vivo*. Intravital microscopy of popliteal lymph nodes enables to visualize *in vivo* dynamic cell interactions. We set up an experiment using OT-II-Foxp3-GFP mice, in which OT-II T_{Reg} constitutively express a green-fluorescent protein. To track the movements of dendritic cells we injected *ex vivo*-labeled bone marrow-derived dendritic cells (BMDC) into the hind footpads of OT-II-Foxp3-GFP mice. In order to study the importance of CCL22 we used RNA interference to selectively downregulate the CCL22 production in one of two differently labeled coinjected groups of BMDC. To accomplish active homing of the injected BMDC to the draining lymph node we stimulated them by coinjection of the TLR4 ligand LPS. Figure 3.3 illustrates the experimental setup.

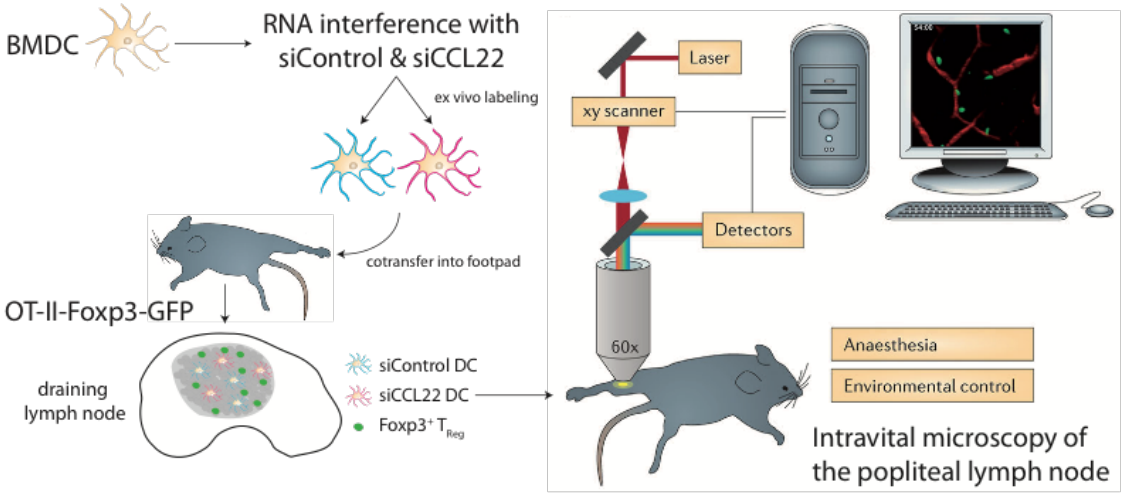

Figure 3.3: Experimental setup for *in vivo* analysis of DC-T_{Reg} interactions and the influence of CCL22-CCR4-mediated attraction
Intravital microscopy enables to study fluorescent cells in deep tissues like the T cell zones of lymph nodes. Regulatory T cells were detected by green-fluorescent protein (GFP) expression in OT-II-Foxp3-GFP mice. In order to study the influence of CCL22, bone marrow-derived dendritic cells were grouped and knock-down of CCL22 was performed. Subsequently the cells were labeled *ex vivo* with dyes developed for intravital microscopy and injected into the footpad. [The figure was partly taken from a review by (Fackler, Murooka et al. 2014) and adapted to the setup.]

3.2.2 CCL22 knock-down in dendritic cells and characterization of maturation status and lymph node homing

We analyzed siControl- and siCCL22-treated BMDC in their immature state and after LPS-induced maturation for production of CCL22 and CCL17 by ELISA (Fig. 3.4a-b) and for expression of the maturation markers CD86 (Fig. 3.4c) and CD80 (not shown) by flow cytometry. Furthermore, we studied if the knock-down influences dendritic cell homing to the draining lymph node (Fig. 3.4d), as this could be a potential confounder in assessing DC-T_{Reg} interactions. The ELISA results confirm substantial decrease of the CCL22 production after siRNA knock-down. For maturation as well as lymph node homing we could show that there is no significant difference between the two groups of siControl- and siCCL22-treated BMDC as equal amounts of transferred BMDC were recovered from the draining lymph nodes.

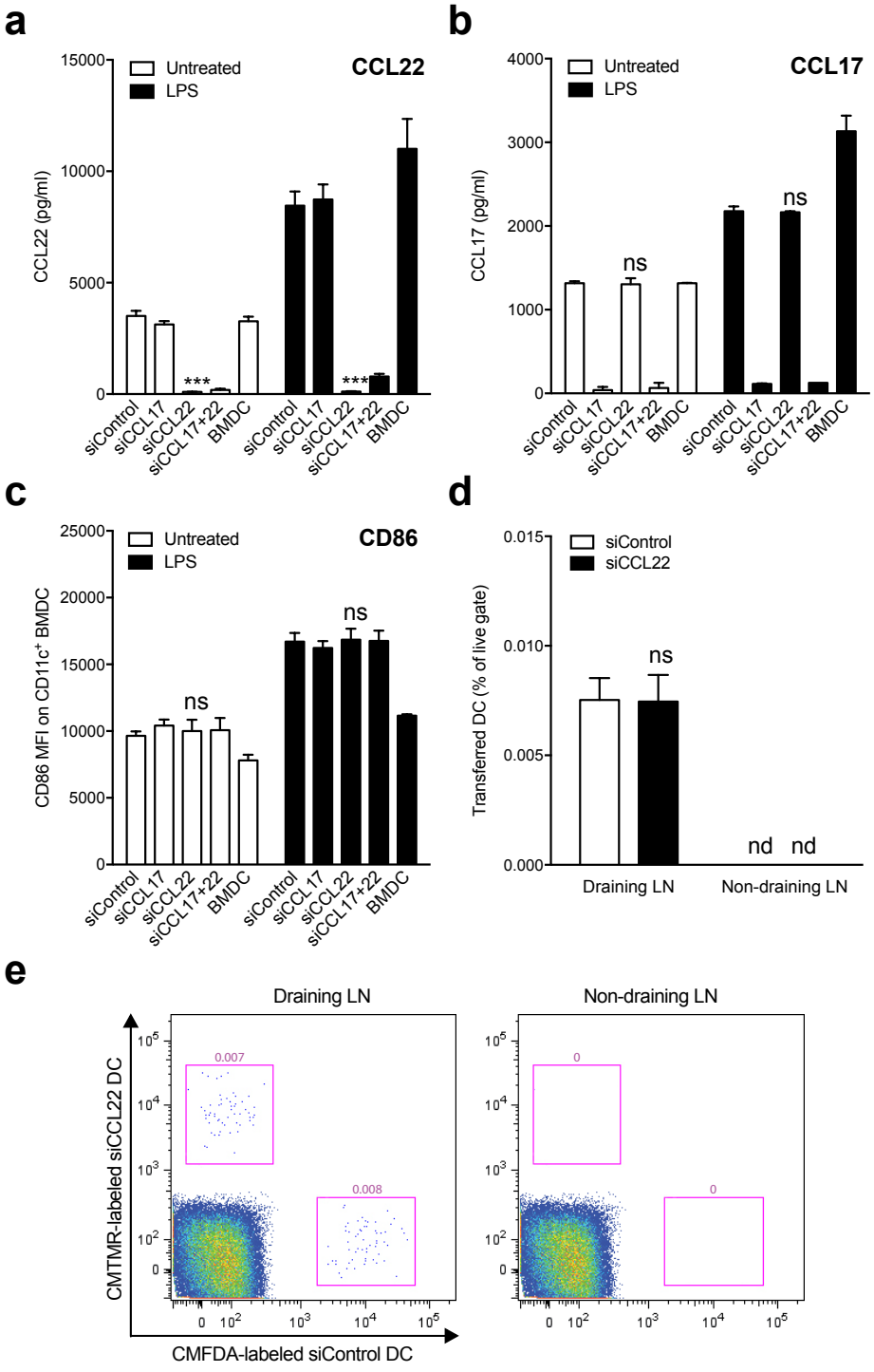

Figure 3.4: CCL22 knock-down in BMDC and characterization of maturation status and lymph node homing

(a-c) 4,000,000 BMDCs were either not treated or transfected with siControl, siCCL17, siCCL22 or siCCL17 and siCCL22. Thereafter 200,000 BMDCs were incubated in the presence of absence of LPS (1 μg/ml) for 18 hours. Subsequently supernatants were analyzed by ELISA for CCL22 levels (a) and CCL17 levels (b) and BMDCs were analyzed by flow cytometry for CD86 expression (c). (d/e) C57BL/6 WT mice (n=3) received 2,000,000 CMFDA-labeled siControl and 2,000,000 CMTMR-labeled siCCL22 DC with 10 ng LPS in the right hind footpad. 18 hours later the right popliteal lymph node (draining LN) and the left popliteal lymph node (non-draining LN) were analyzed by flow cytometry. Error bars indicate SEM. P values were calculated relative to the siControl condition (*** $p < 0.001$). ns, not significant; nd, not detected.

3.2.3 CCL22 expression by dendritic cells influences interactions with regulatory T cells

To study the *in vivo* interaction of DCs and T_{Reg} as well as the influence of CCL22 in an antigen-depending setting, siControl- and siCCL22-treated OVA 323-339 peptide pulsed BMDC were labeled with CMTMR or CMFDA and coinjected into the right hind footpad of OT-II-Foxp3-GFP mice. After 18 hours, the animals were prepared for intravital imaging: the mice were held under constant anesthesia by an initial intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), the skin in the popliteal region was exposed and the popliteal lymph node was dissected open and covered in a heating chamber at 37°C. Subsequently, intravital imaging was performed using a two-photon microscope for the duration of 1 hour (Fig. 3.5a). CMFDA-labeled siControl-treated DC were assigned the blue channel, CMTMR-labeled siCCL22-treated DC the red channel, and the GFP fluorescence of resident T_{Reg} the green channel. A representative *in vivo* image of the T cell zone of the popliteal lymph node is shown in Figure 3.5b.

The acquired 4-dimensional image data were analyzed regarding instantaneous velocity of each cell group as well as contact time and frequency between either DC group and T_{Reg}. The instantaneous velocities of siControl- and siCCL22-treated DCs were calculated with automated software and were not significantly different but considerably slower than the velocity of T_{Reg} (Fig. 3.5c), a finding in line with previously published *in vivo* imaging studies (Matheu, Othy et al. 2015). No potentially confounding velocity difference was observed between the DC groups.

The contact time and the contact frequency for T_{Reg} and siCCL22-treated DC compared to siControl-treated DC were manually and independently assessed by two blinded readers. The mean contact time for siCCL22-treated DC was significantly shorter (Fig. 3.5d) and the mean contact frequency significantly lower (Fig. 3.5e). Taken together with the results from chapter 3.1, these data suggest an important role of the chemokine CCL22 in *in vitro* and *in vivo* cellular interactions between DCs and T_{Reg} during homeostasis as well as during antigen recognition. The results shown in Figure 3.5 represent three independent experiments.

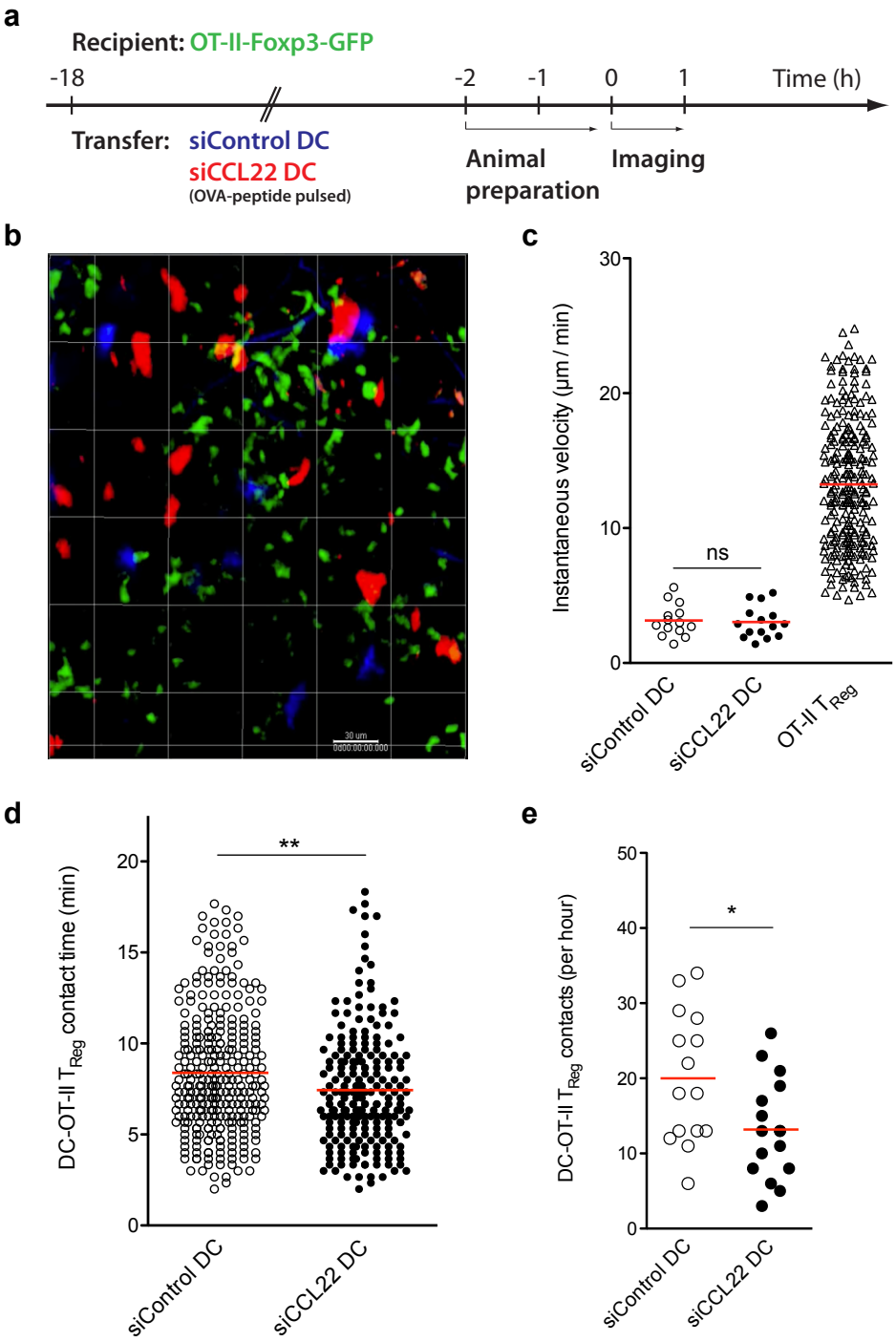

Figure 3.5: Intravital microscopy of cotransferred OVA 323-339 peptide-pulsed siControl and siCCL22 DC in the popliteal lymph node of OT-II-Foxp3-GFP mice

(a) 2,000,000 CMFDA-labeled siControl and 2,000,000 CMTMR-labeled siCCL22 BMDC were pulsed with OVA 323-339 (1 μg/ml) for 1 hour and thereafter coinjected with 10 ng LPS in the right hind footpad of OT-II-Foxp3-GFP mice. The right popliteal lymph node was prepared and intravital microscopy was performed for 1 hour. (b) Representative image of the lymph node demonstrating the Foxp3-GFP signal (green) and the cotransferred siControl (blue) and siCCL22 (red) DCs. (c) Instantaneous velocities of either population were measured using Bitplane Imaris software. (d) DC-OT-II T_{Reg} contact times were measured manually for siControl and siCCL22 DCs. (e) Absolute numbers of OT-II T_{Reg} contacts with either DC population were measured over 1 hour of imaging. P values were calculated relative to the siControl condition. ns, not significant.

3.3 Influence of the innate immune system on CCR4 expression

Innate immunity plays a key role in host defense and the initiation of adaptive immunity. Since we established that the CCL22-CCR4 axis influences DC-T_{Reg} interactions *in vitro* and *in vivo*, we next wanted to examine how this chemokine-chemokine receptor axis responds during various stimulations of the innate immune system. Primarily we aimed to study the regulation of the chemokine receptor CCR4 expression, as previous work of our research group focused on the regulation of the chemokine CCL22.

3.3.1 Regulation of CCR4 expression of regulatory T cells following stimulation of the innate immune system *in vitro*

To investigate the influence of innate immune stimulation *in vitro*, we incubated 500,000 WT splenocytes for 48 hours in the presence or absence of a variety of Toll-like receptor (TLR), RIG-I-like receptor (RLR) and Dectin-1 ligands, that are known as strong stimulators of innate immunity. Subsequently, we analyzed the CCR4 expression on T_{Reg} using flow cytometry (Fig. 3.6a, represents three independent experiments). The vast majority of stimuli led to a significant decrease of CCR4 expression on T_{Reg}, with the exception of the extracellularly applied TLR3 ligand poly (I:C) high molecular weight (HMW) and the TLR8 ligand poly U. On the other hand, transfection of the cells with poly (I:C) HMW, which is known to mainly interact with the intracellular receptor MDA5 of the RLR family, also led to a significant decrease of CCR4 expression. All in all, the data suggest that a multitude of innate immune stimuli lead to a downregulation of CCR4 expression of T_{Reg} *in vitro*.

3.3.2 Regulation of CCR4 expression of regulatory T cells following stimulation of the innate immune system *in vivo*

Moreover, we studied the regulation of CCR4 expression *in vivo* by subcutaneous injection of 100 μg of the TLR9 ligand CpG (or PBS as control) in WT C57BL/6 mice. After 48 hours, spleen and lymph nodes were harvested and subsequently analyzed by flow cytometry for CCR4 expression of T_{Reg}. The mice treated with CpG showed a significantly decreased CCR4 expression on T_{Reg} compared to untreated mice (Fig. 3.6b, represents two independent experiments). In addition to stimulation with a purified TLR ligand we performed an oral infection assay with WT C57BL/6 mice using *Salmonella typhimurium* with subsequent flow cytometric analysis 24 hours later. Mice orally infected

with *Salmonella typhimurium* showed a significant decrease of CCR4 expression on T_{Reg} compared to untreated mice (Fig. 3.6c, represents two independent experiments). Altogether, these results suggest that CCR4 expression is downregulated following innate immune activation.

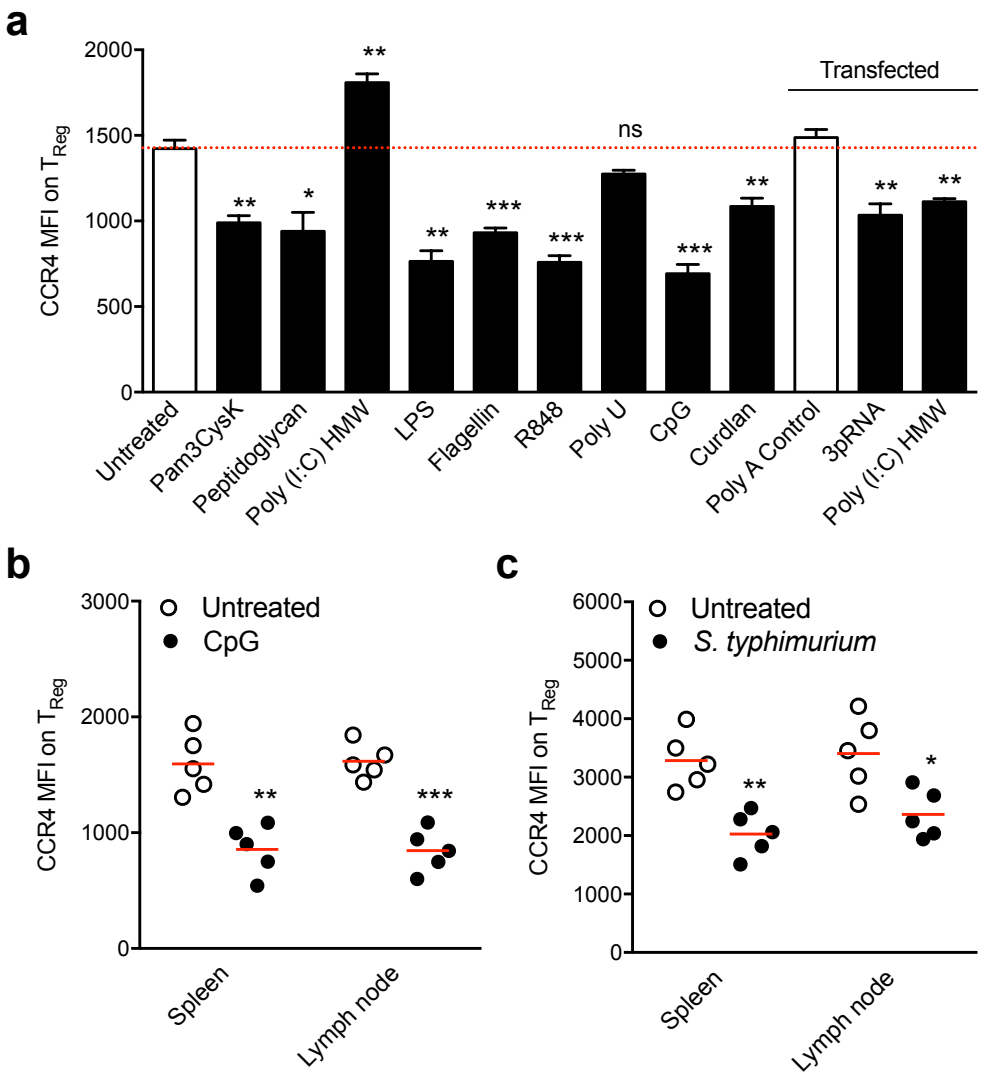

Figure 3.6: CCR4 expression on T_{Reg} following pathogen-recognition receptor stimulation *in vitro* and *in vivo* and following *S. typhimurium* infection *in vivo*

(a) 500,000 splenocytes of WT mice were incubated for 48 hours with TLR and RLR ligands and analyzed by flow cytometry for CCR4 expression on $CD4^+$-$Foxp3^+$ cells (T_{Reg}). Poly A control, 3pRNA and poly (I:C) HMW on the far right were transfected. Error bars indicate SEM. P values were calculated relative to the untreated or in case of transfection to the poly A Control condition (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). ns, not significant. (b) C57BL/6 mice were injected subcutaneously with PBS (n=5) or 100 μg CpG (n=5). (c) C57BL/6 mice were infected orally with 10^3 CFU of *S. typhimurium* (n=5) or untreated (n=5). 24 hours later splenocytes and lymph node cells were analyzed by flow cytometry for CCR4 expression on $CD4^+$-$Foxp3^+$ cells (T_{Reg}). P values were calculated relative to the untreated condition (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.4 Influence of the adaptive immune system on CCL22 and CCR4 expression

The DC exerts the pivotal role of presenting antigens to T and B cells, which it encounters in secondary lymphoid organs such as the lymph node. To promote these encounters, the DC makes use of chemokine-mediated migration involving proteins of the chemokine-chemokine-receptor family to attract its responder cells (Castellino, Huang et al. 2006, Semmling, Lukacs-Kornek et al. 2010). DCs interact with T_{Eff} and T_{Reg} and thereby stand at the crossroads of initiating or inhibiting an adaptive immune response. After studying the role of the innate immune system on CCL22 and CCR4 expression, we went on to investigate their regulation during an adaptive immune response.

3.4.1 Increased CCL22 production by dendritic cells following T cell activation

3.4.1.1 CCL22 production of splenocytes following T cell activation

Since CCL22 is a chemokine produced by DCs, we wanted to study the dynamic effect of an antigen-specific immune response on its production. To answer this question, we incubated 500,000 splenocytes of OT-II TCR transgenic mice with the specific antigen OVA 323-339 at a concentration of 1 μg/ml for 12, 18, 24, 48 and 96 hours. Subsequently, the supernatants were collected and the amount of CCL22 was measured using ELISA. The OVA 323-339-treated conditions showed significantly higher levels of CCL22, even after only 12 hours of incubation (Fig. 3.7, represents three independent experiments). The strongest relative increase was observed after 24 hours with the OVA 323-339-treated condition exceeding the untreated condition by more than 3-fold. A similar response was observed for the second CCR4 chemokine CCL17 (data not shown), whilst CCL17 concentrations were overall considerably lower. These results show that an adaptive immune response is associated with a strong and rapid induction of CCL22 production.

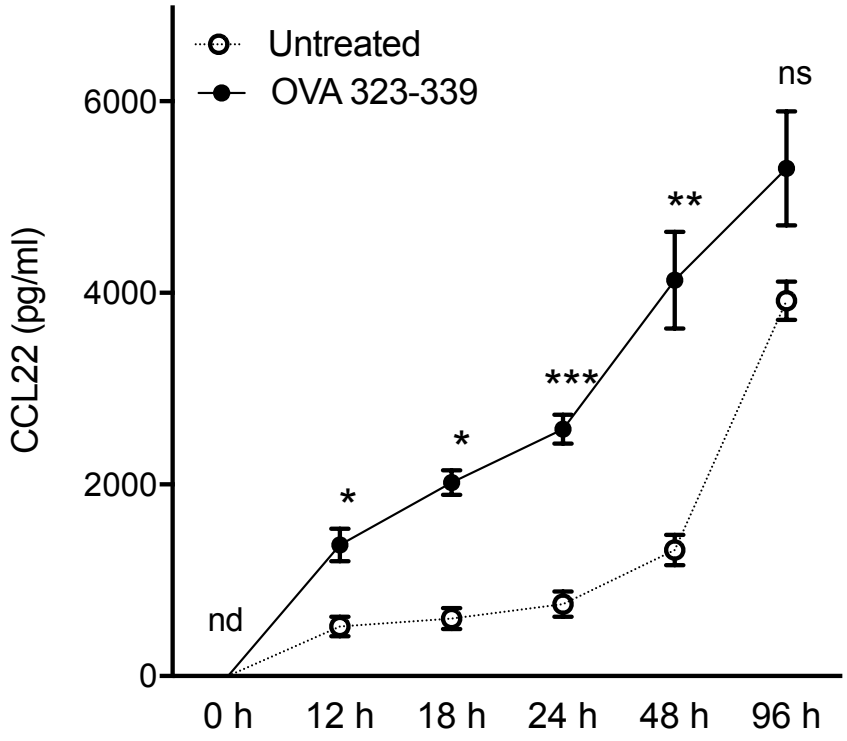

Figure 3.7: Kinetics of CCL22 production by OT-II splenocytes in the presence or absence of OVA 323-339

500,000 OT-II splenocytes were incubated for 12, 18, 24, 48 and 96 hours in the presence or absence of the OT-II TCR specific antigen OVA 323-339 (1 μg/ml). CCL22 levels were measured by ELISA and are depicted as absolute values. Errors indicate SEM. P values were calculated relative to the untreated condition (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). ns, not significant; nd, not detected.

3.4.1.2 CCL22 induction by T cell activation requires dendritic cells and T cells

Since we identified that T cell activation increased CCL22 production in splenocytes, subsequently we wanted to verify if this effect takes place during interaction of DCs and T_{Eff} alone. Thus, we coincubated 25,000 MACS-sorted DCs and 50,000 $CD4^{+}$-$CD25^{neg}$ T cells (T_{Eff}) of WT or OT-II transgenic mice for 48 hours with or without OVA 323-339 at a concentration of 1 μg/ml. Following coincubation, the supernatants were collected and CCL22 levels were measured using ELISA. The condition of DC and OT-II T_{Eff} showed a substantial increase in CCL22 production in the presence of cognate antigen (Fig. 3.8, represents four independent experiments). On the other hand, no significant difference was observed in the control condition with WT T_{Eff}. Previous results from our study group have shown that CCL22 *in vitro* is predominantly produced by DCs, but only if DCs are coincubated with T cells. Taken together, these results suggest that T cell activation through cognate antigen presented by DCs induces strong and rapid production of CCL22 by the antigen-presenting DC.

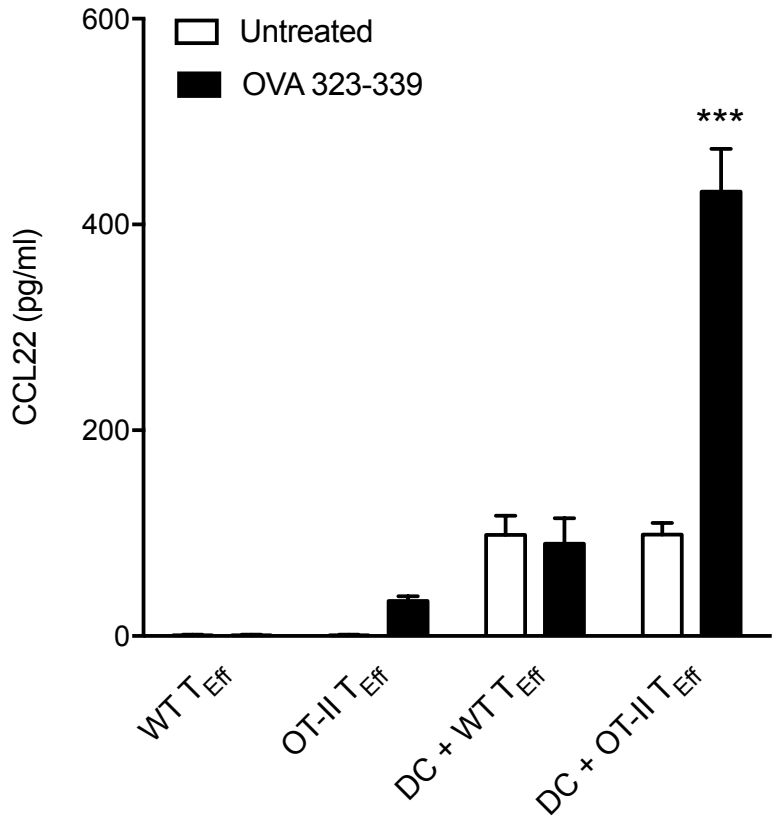

Figure 3.8: CCL22 levels of DCs and WT or OT-II T_{Eff} cells in the presence of OVA 323-339

25,000 $CD11c^+$-sorted DCs and 50,000 $CD4^+$-$CD25^{neg}$-sorted WT or OT-II T_{Eff} cells were incubated for 48 hours in the presence or absence of the OT-II TCR specific antigen OVA 323-339 (1 μg/ml). CCL22 levels were measured by ELISA. Error bars indicate SEM. P value was calculated relative to the untreated condition (*** $p < 0.001$).

3.4.2 Increased CCR4 expression of regulatory T cells following T cell activation

With the established induction of CCL22 production following T cell activation, we next wanted to study the effect of an antigen-specific immune response on CCR4 expression of T_{Reg}. To answer this question, we again tested murine splenocytes of OT-II TCR transgenic mice by initiating an adaptive T cell response with the specific antigen OVA 323-339 in an experimental setup identical to the one in chapter 3.4.1.1. The cells were analyzed by flow cytometry after incubation for 48 hours. In OT-II splenocytes incubated with the cognate antigen OVA 323-339 showed a significant increase in CCR4 expression on T_{Reg} but not T_{Eff} (Fig. 3.9, represents three independent experiments). No significant differences were observed for WT splenocytes. In addition, the results show that CCR4 expression is higher on naive T_{Reg} as compared to naive T_{Eff}. Taken together, these results show that antigen recognition by T_{Reg} is followed by an induction of their CCR4 expression.

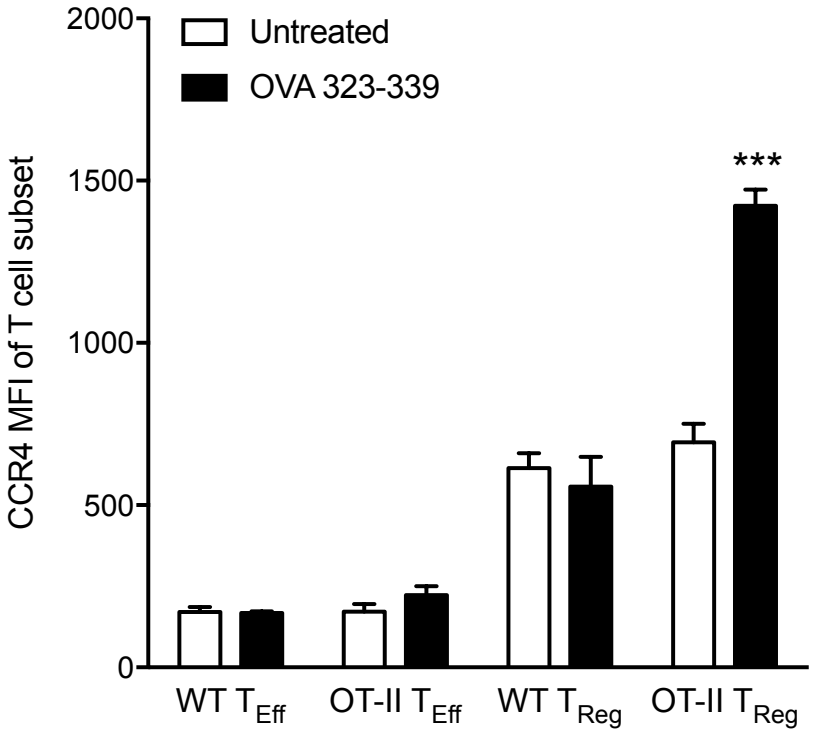

Figure 3.9: CCR4 expression of WT or OT-II T_{Eff} and T_{Reg} cells in the presence of OVA323-339

500,000 splenocytes of WT or OT-II mice were incubated for 48 hours in the presence or absence of the OT-II TCR specific antigen OVA 323-339 (1 μg/ml) and analyzed by flow cytometry. The CCR4 MFI was measured for $CD4^+$-$Foxp3^{neg}$ cells (T_{Eff}) and $CD4^+$-$Foxp3^+$ cells (T_{Reg}). Error bars indicate SEM. P value was calculated relative to the untreated condition (*** $p < 0.001$).

3.4.3 Differential regulation of CCL22 and CCR4 expression during combined activation of the innate and adaptive immune system

Previous experiments by our research group and new experiments in this thesis showed that CCL22 and its receptor CCR4 are both downregulated by a variety of stimuli of the innate immune system, like e.g. the TLR9 ligand CpG, and upregulated during T cell activation. The next point we wanted to study was the combined influence of an innate and adaptive immune response on the expression of CCL22 and CCR4. Therefore, we incubated 500,000 OT-II splenocytes with combinations of the TLR9 ligand CpG (5 μg/ml) and / or the cognate antigen OVA 323-339 (1 μg/ml). The condition stimulated with CpG and OVA 323-339 still showed a decrease in CCL22 production compared to the untreated condition (Fig. 3.10a, represents three independent experiments). On the other hand, the CCR4 expression on T_{Reg} showed a moderate decrease but was still higher than in the untreated condition (Fig. 3.10b, represents three independent experiments). Therefore, the opposing effects of innate and adaptive immune activation are possibly different on CCL22 production and CCR4 expression. Nevertheless, we

conclude that the downregulation by the innate immune stimulation is partly abolished by the adaptive immune response.

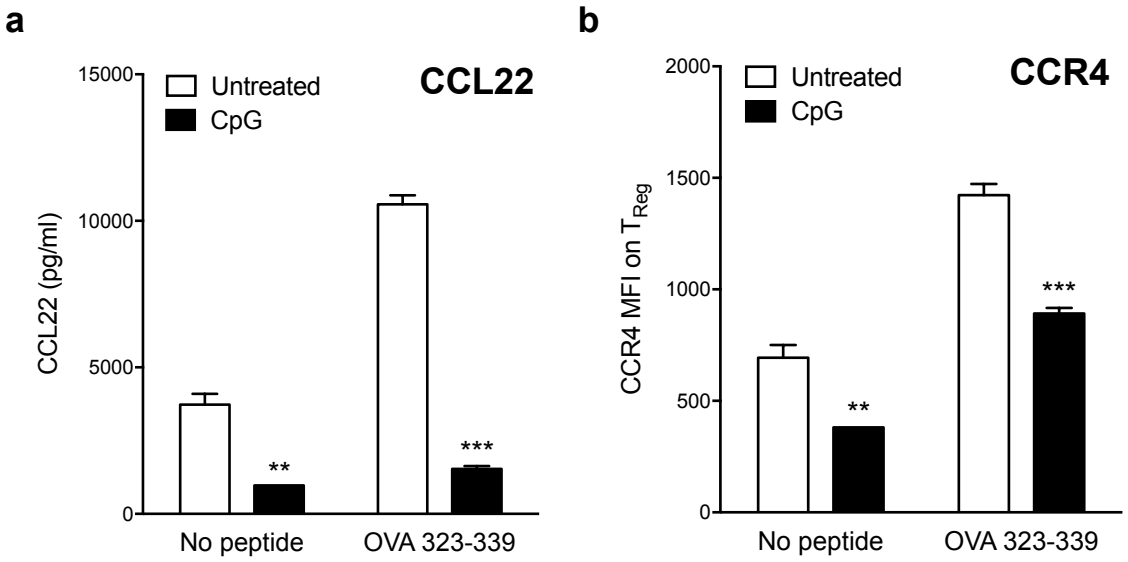

Figure 3.10: CCL22 production by OT-II splenocytes and CCR4 expression on OT-II T_{Reg} following TLR9 stimulation in the presence or absence of OVA 323-339
500,000 OT-II splenocytes were incubated for 48 hours with the TLR9 ligand CpG (5 μg/ml) in the presence or absence of OVA 323-339 (1 μg/ml). Thereafter supernatants were analyzed for CCL22 levels (a) by ELISA and splenocytes for CCR4 expression (b) on $CD4^+$-$Foxp3^+$ cells (T_{Reg}) by flow cytometry.

3.5 CCL22 expression by major dendritic cell subsets

Different DC subsets are known to exert specific functions in innate and adaptive immunity, e.g. to induce innate antiviral immune responses or to present antigen on MHC I and II to corresponding T and B cells for adaptive immune responses. Therefore, we were interested to elaborate which subsets are responsible for the production of the chemokine CCL22 to further understand its role in immunity. We studied CCL22 expression on the intracellular level by measuring the CCL22 mRNA levels in MACS-sorted DC subsets. As demonstrated in Figure 3.11, $CD8\alpha^+$ together with $CD4^+$ myeloid DCs are expressing higher levels of CCL22 mRNA compared to $B220^+$ plasmacytoid DCs. We could demonstrate the same differences on protein level by coincubation of these DC subsets with DC-depleted splenocytes by ELISA (data not shown). The $CD8\alpha^+$ and $CD4^+$ myeloid DC subsets are known to be involved in T cell interaction, whereas $B220^+$ plasmacytoid DC mainly function in innate antiviral immune responses. These data show that CCL22 is predominantly produced by DCs involved in T cell immunity.

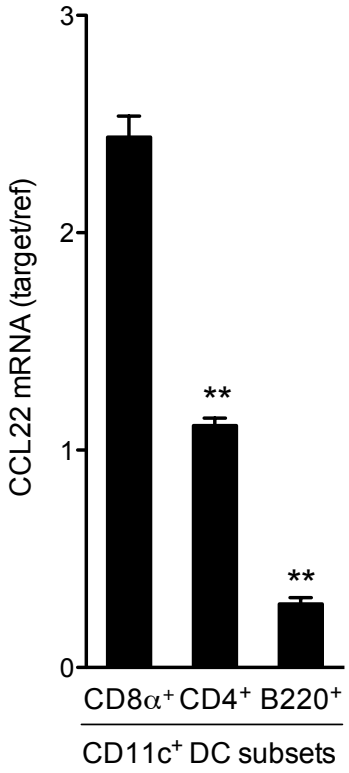

Figure 3.11: CCL22 mRNA expression of DC subsets
DC subsets were MACS-sorted from splenocytes of WT mice and their CCL22 mRNA expression was measured using RT-PCR. Errors indicate SEM. P values were calculated relative to the $CD8\alpha^+$ condition (** $p < 0.01$).

3.6 Influence of CCL22 on suppression mechanisms of regulatory T cells *in vitro*

T_{Reg} are known to make use of multiple mechanisms of immunosuppression, like e.g. inhibitory cytokines, suppression of DC function, metabolic disruption and cytolysis of T_{Eff} (Shevach 2009). In the previously described experiments, we investigated the impact of the CCL22-CCR4 axis on interactions of DCs and T_{Reg} *in vitro* and *in vivo* as well as its regulation during innate and adaptive immunity. The aim of the following experiments was to analyze the influence of CCL22 on T_{Reg} function *in vitro*.

3.6.1 Regulatory T cell suppression of dendritic cell function *in vitro*

One of the mechanisms of T_{Reg}-mediated immunosuppression on DCs is the downregulation of the costimulatory molecules CD80 and CD86 through trans-endocytosis of CTLA-4 (Wing, Onishi et al. 2008, Qureshi, Zheng et al. 2011). The

decreased expression of costimulatory molecules counteracts T cell activation and, in turn, can render T_{Eff} anergic. In order to study this *in vitro,* we set up a coculture experiment of 25,000 $CD11c^+$-sorted DCs with or without 50,000 $CD4^+$-$CD25^{neg}$-sorted OT-II T_{Eff} or 50,000 $CD4^+$-$CD25^+$-sorted OT-II T_{Reg} in the presence or absence of OVA 323-339 (1 μg/ml) for 48 hours. To investigate the influence of CCL22, we added anti-CCL22 blocking antibody (2 μg/ml) or recombinant CCL22 (1 μg/ml). After 48 hours of coincubation the cells were analyzed by flow cytometry for CD80 and CD86 expression on DCs. The addition of OT-II T_{Reg} to the coculture significantly decreased CD80 and CD86 expression by DCs. Changing the concentration of CCL22 by anti-CCL22 blocking antibody or recombinant CCL22 did not affect CD80 or CD86 expression (Fig. 3.12a-b, represents three independent experiments). The results do not support a role of CCL22 in *in vitro* suppression of costimulatory molecules.

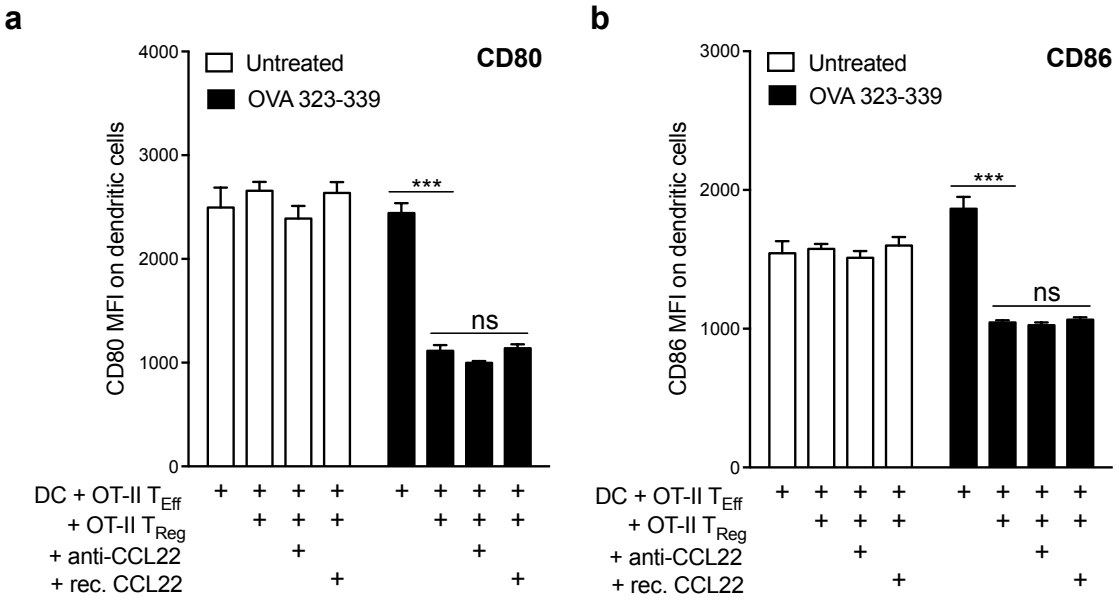

Figure 3.12: Antigen-dependent DC suppression assay by OT-II T_{Reg} at variable conditions of CCL22

25,000 $CD11c^+$-sorted DCs were coincubated for 48 hours as indicated with 50,000 $CD4^+$-$CD25^{neg}$-sorted OT-II T_{Eff}, 50,000 $CD4^+$-$CD25^+$-sorted OT-II T_{Reg}, anti-CCL22 blocking antibody (2 μg/ml) or recombinant CCL22 (1 μg/ml) in the presence or absence of OVA 323-339 (1 μg/ml) and subsequently analyzed by flow cytometry for CD80 (a) and CD86 (b) expression on dendritic cells. Error bars indicate SEM. P values were calculated relative to the DC + OT-II T_{Eff} condition (*** $p < 0.001$). ns, not significant.

3.6.2 Regulatory T cell suppression of effector T cell function *in vitro*

Aside from inhibiting the costimulatory capacity of DCs, the subsequent effect of T_{Reg}-mediated immunosuppression on T_{Eff} activation can be studied by assessing T_{Eff} proliferation and the expression of T cell activation markers. To study cell proliferation,

we used the same setup as in chapter 3.6.1 and added 5-bromo-2′-deoxyuridine (BrdU) to the coculture. BrdU is incorporated in dividing cells and was measured using flow cytometry. The addition of OT-II T_{Reg} significantly decreased OT-II T_{Eff} cell proliferation, but neither anti-CCL22 blocking antibody nor recombinant CCL22 altered the proliferation rate (Fig. 3.13a, represents three independent experiments). Furthermore, we analyzed the expression of the activation marker CD69 on OT-II T_{Eff}, which showed similar results (Fig. 3.13b, represents three independent experiments). These data do not support a role of CCL22 in *in vitro* suppression of T_{Eff} proliferation by T_{Reg}.

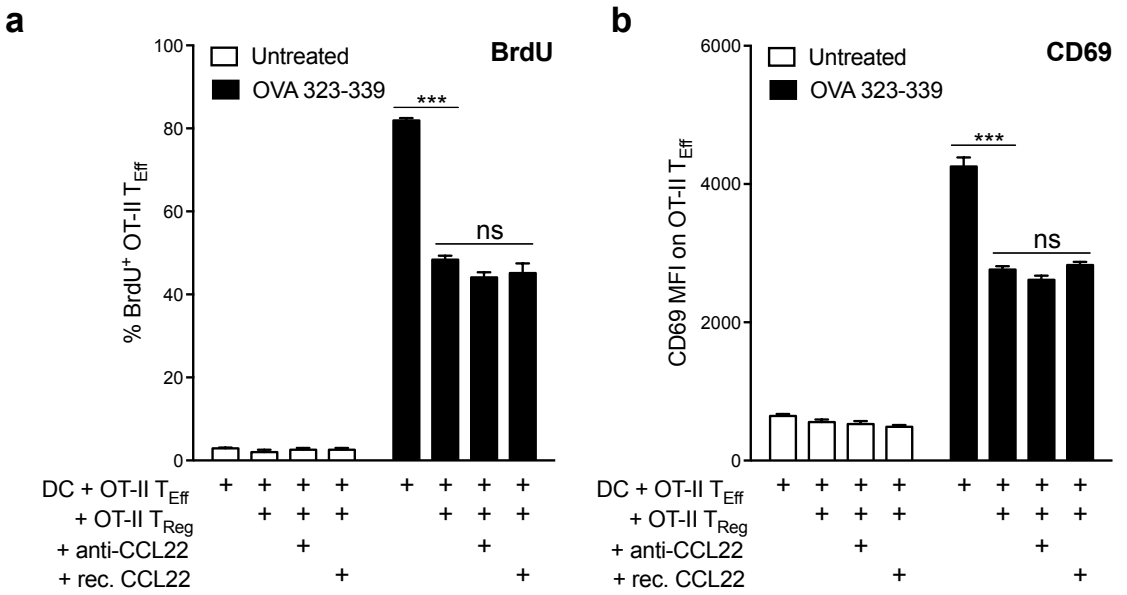

Figure 3.13: OT-II T_{Reg} suppression in an antigen-dependent proliferation assay at variable conditions of CCL22

25,000 $CD11c^+$-sorted DCs were coincubated for 48 hours as indicated with 50,000 $CD4^+$-$CD25^{neg}$-sorted OT-II T_{Eff}, 50,000 $CD4^+$-$CD25^+$-sorted OT-II T_{Reg}, anti-CCL22 blocking antibody (2 μg/ml) or recombinant CCL22 (1 μg/ml) in the presence or absence of OVA 323-339 (1 μg/ml). BrdU was supplied in the cell culture medium. Cells were subsequently analyzed by flow cytometry for BrdU uptake in OT-II T_{Eff} (a) and CD69 expression on OT-II T_{Eff} (b). Error bars indicate SEM. P values were calculated relative to the DC + OT-II T_{Eff} condition (*** $p < 0.001$). ns, not significant.

3.6.3 Regulatory T cell cytokine-mediated suppression *in vitro*

Further mechanisms of T_{Reg} include IL-2 inhibition and production of inhibitory cytokines such as IL-10. The influence of CCL22 on these mechanisms was studied *in vitro* in the same experimental setup as in chapter 3.6.1 and 3.6.2, and protein levels were quantified using ELISA. In the presence of cognate antigen, OT-II T_{Eff} produced large amounts of IL-2. OT-II T_{Reg} significantly decreased IL-2 levels in the coculture and

produced IL-10 in the presence of the cognate antigen OVA 323-339. Neither adding anti-CCL22 blocking antibody nor recombinant CCL22 altered IL-2 production by OT-II T_{Eff} (Fig. 3.14a, represents three independent experiments) or IL-10 production by OT-II T_{Reg} (Fig. 3.14b, represents two independent experiments).

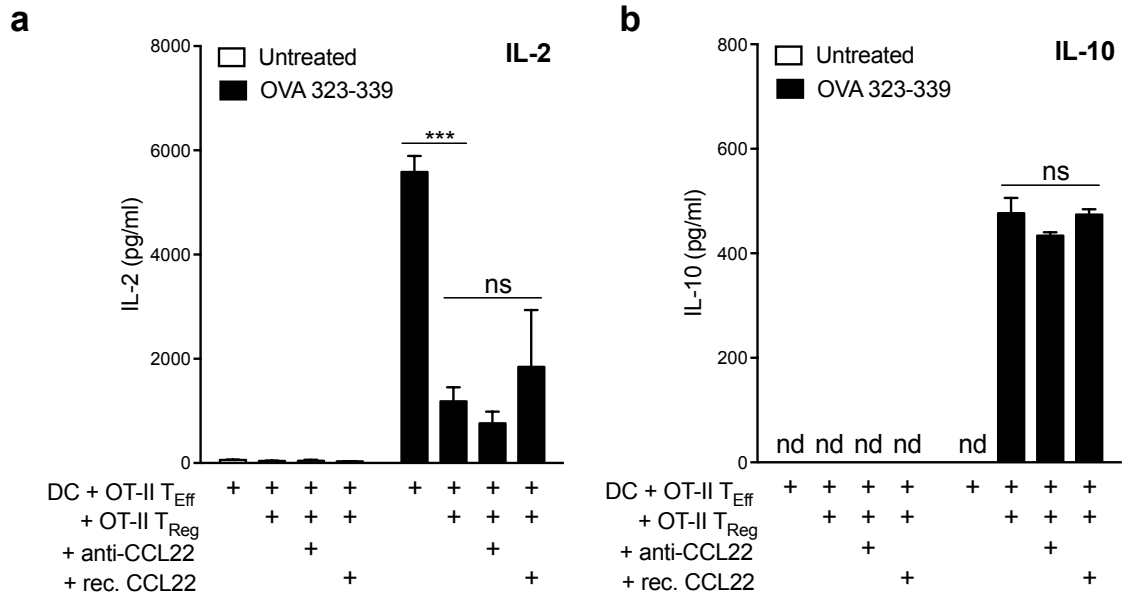

Figure 3.14: IL-2 and IL-10 cytokine production in antigen-dependent suppression assays at variable conditions of CCL22

25,000 $CD11c^+$-sorted DCs were coincubated for 48 hours as indicated with 50,000 $CD4^+$-$CD25^{neg}$-sorted OT-II T_{Eff}, 50,000 $CD4^+$-$CD25^+$-sorted OT-II T_{Reg}, anti-CCL22 blocking antibody (2 μg/ml) or recombinant CCL22 (1 μg/ml) in the presence or absence of OVA 323-339 (1 μg/ml). Supernatants were analyzed for IL-2 (a) and IL-10 (b) levels by ELISA. Error bars indicate SEM. P value was calculated relative to the DC + OT-II T_{Eff} condition (*** $p < 0.001$). ns, not significant; nd, not detected.

Taken together, no evidence supporting a function of CCL22 in *in vitro* T_{Reg} suppression could be found. However, these data can neither confirm nor exclude the function of CCL22 in *in vivo* adaptive immunity as the complexity of *in vivo* migration in interstitial tissues and cell interactions in secondary lymphoid organs cannot be adequately simulated in *in vitro* experiments. Therefore, *in vivo* studies are needed to investigate the role of CCL22 in immune tolerance.

4 DISCUSSION

Our knowledge on immune tolerance has been largely expanded during the last five decades. Pioneering work was performed by Sir Frank Macfarlane Burnet, who introduced the scientific community to the distinction between self and non-self and received the Nobel Prize in Physiology or Medicine in the year 1960. Since then, immune tolerance has been further dissected into central, peripheral and dominant tolerance mechanisms, with the latter being investigated for only about two decades.

Following the discovery of regulatory T cells (T_{Reg}), their important role in the pathogenesis of autoimmune diseases, in the management of organ transplantation as well as in immune evasion of malignant neoplasms soon became evident. These circumstances quickly made them a new promising target of immunotherapy. Efforts to increase or decrease T_{Reg} function depending on the pathological implication are successful, but then possibly accompanied by adverse effects like the onset of new autoimmune diseases. Therefore, understanding the exact mechanisms that balance the immune system are necessary. In this regard, targeting cell migration rather than cell function can be considered promising.

Based on the present thesis, I will discuss the fundamental migratory mechanisms involved in interstitial interaction of dendritic cells (DCs) and T cells and review the relevance of specific chemokines and chemokine receptors for T_{Reg} function. Then I will summarize and integrate the new insights from the reported results of this thesis and provide an overview of already established and currently envisioned clinical applications in the context of cancer and autoimmune diseases.

4.1 Interstitial migration in the interaction of dendritic cells and T cells within lymphoid and non-lymphoid tissues

The interstitial migration of leukocytes in lymphoid tissues, in particular the lymph node, is a highly organized process. Among the many immune cell types that circulate the lymph nodes, DCs seek to interact with different subpopulations of T lymphocytes, which are all in constant search for their cognate antigen. The adaptive T cell response involves

not only $CD4^+$ effector T cells (T_{Eff}) but also $CD8^+$ cytotoxic T cells (CTL), which also need to find their cognate antigen first. Therefore, a tricellular encounter is required, which would at first sight be expected as unusual if not virtually impossible. To facilitate the interaction of all involved cells, a sophisticated mechanism has evolved. The cognate interaction of DCs and T_{Eff} induces the production of chemokines CCL3, CCL4 and CCL5 in DCs, which subsequently attract CTLs to this specific DC (Castellino, Huang et al. 2006). This whole mechanism takes place within a lymph node, and can therefore be attributed to interstitial migration. Interestingly, the aforementioned mechanism to facilitate tricellular interactions is not exclusive as a similar program was identified for DC, T_{Eff} and NK cells involving other chemokines (Semmling, Lukacs-Kornek et al. 2010). We hypothesized such a mechanism for the interstitial tricellular encounter of DC, T_{Eff} and T_{Reg} in lymph nodes.

In *in vitro* experiments, we observed that the chemokine CCL22, produced by a modified immortalized dendritic cell line, attracted naive T_{Reg} and facilitated cell encounters in a dose-dependent manner. Furthermore, we used an experimental setup of splenic DCs, OT-II T_{Eff} and OT-II T_{Reg}, simulating the situation of tricellular encounters in the presence of cognate antigen. We could show that blocking CCL22 in the supernatant using neutralizing antibodies shifted the proportion of T_{Eff} to T_{Reg} in DC-T cell aggregates in the favor of T_{Eff}. Moreover, using an *in vivo* experiment based on intravital microscopy, we demonstrated a higher interaction frequency and longer interaction time of OT-II T_{Reg} with CCL22-producing DCs compared to CCL22-knock-down DCs in the setting of cognate antigen. Altogether, our results suggest a role of CCL22 in the interstitial interaction of DCs and T_{Reg} in homeostatic as well as inflammatory conditions.

Our results provide further evidence that DC-T_{Reg} interactions are observed *in vivo* within lymph nodes. The very first study using intravital microscopy showed prolonged DC-T_{Reg} interactions, whereas stable contacts between T_{Eff} and T_{Reg} were not observed (Tang, Adams et al. 2006), suggesting necessity of DC-T_{Reg} interactions for their *in vivo* function. Further insight was provided by an intravital microscopy study showing that the presence of T_{Reg} leads to an inhibition of stable DC-T_{Eff} interactions (Tadokoro, Shakhar et al. 2006). DC-T_{Reg} interactions have also been shown *in vivo* in the tumor tissue (Bauer, Kim et al. 2014) as well as in adjacent tertiary lymphoid structures (Joshi, Akama-Garren et al. 2015). Recent data could show the importance of T_{Reg} in lymph nodes during

immune homeostasis, in which they continuously suppress populations of auto-reactive T_{Eff} in certain clusters, again in the proximity of DCs (Liu, Gerner et al. 2015).

The impact of CCL22 to favor DC-T_{Reg} interactions observed in my experiments has to be put into context with previous studies. CCL22 has been shown to be upregulated in skin-resident DCs, also termed Langerhans cells, upon the initiation of DC maturation and subsequent lymph node homing (Tang and Cyster 1999). These CCL22-producing DC did not attract naive $CD4^+$ T cells to the lymph node but rather already cognate antigen-exposed activated $CD4^+$ T cells used in these experiments. This study, however, studied the homing of activated $CD4^+$ T cells and did not investigate interstitial migration or positioning. A second study on this topic showed that CCL22 is specifically expressed by DCs in the T cell zones and showed *in vitro* chemotactic properties for activated T cells (Kanazawa, Nakamura et al. 1999). Both studies, however, did not distinguish the $CD4^+$ T cell subpopulation in their experiments. In human peripheral blood mononuclear cells (PBMC), strong *in vitro* migration towards CCL22 was observed by activated T_H2-$CD4^+$ cells compared to T_H1-$CD4^+$ cells (Imai, Nagira et al. 1999). An *ex vivo* immunohistochemistry study in humans using inflamed skin and lymph nodes demonstrated that DC-T cell clusters showed CCL22 expression in DC and CCR4 expression in T cells, not further specifying the T cell subpopulations involved (Katou, Ohtani et al. 2001).

The only study so far to investigate the role of CCL22 in interstitial interactions of DC and T_{Reg} was performed in mesenteric lymph nodes (MLN) of mice (Onodera, Jang et al. 2009). MLN-DCs were shown to constitutively produce indoleamine-2-3-dioxygenase (IDO), an enzyme generating an immunosuppressive environment due to the degradation and subsequent depletion of the essential amino acid tryptophan. Furthermore, certain subsets of MLN-DCs produced CCL22 as a response to apoptotic cells. The IDO production by MLN-DCs was shown to be significantly reduced in the presence of CTLA-4-deficient T_{Reg} as well as in $CCR4^{-/-}$ mice, suggesting a role of CCL22 in DC-T_{Reg} interactions. Using static *ex vivo* immunohistochemistry of MLNs, however, no change in the frequency of colocalization of DC and T_{Reg} was reported when comparing wild type (WT) mice to $CCR4^{-/-}$ mice. The influence of CCR4 on the colocalization of DCs with T_{Reg} in the spleen or peripheral lymph nodes was not reported. Also, no studies of *in vitro* or *in vivo* interstitial migration were performed. Altogether, despite some previous evidence that CCL22 and CCR4 may interact to modulate T_{Reg}

function, our results are the first to show the impact of CCL22 on the interstitial interaction of DCs and T_{Reg}.

4.2 Chemokines and chemokine receptors in regulatory T cell function

Chemokines can affect cell function by controlling systemic migration in terms of homing to organs in which the cell needs to operate or in interstitial migration within lymphoid tissues to guide encounters with its functional cellular counterpart. In the following section, I will first discuss the known evidence regarding these differential influences for the chemokine CCL22 and its receptor CCR4. Based on that foundation, I will put our current and prior investigations of the regulation of CCL22 and its receptor CCR4 in different states of immune activation into context of the literature and the established CCL22-dependent interstitial interaction of DCs and T_{Reg} outlined in chapter 4.1.

The chemokine CCL22 has been demonstrated to be involved in the homing of T_{Reg} in autoimmune disease models as well as in transplant tolerance. In an adoptive T_{Eff} cell transfer model of inflammatory bowel disease the co-transfer of T_{Reg} can prevent the resulting colitis. When using $CCR4^{-/-}$ T_{Reg} for co-transfer the inflammatory bowel disease developed similar to transfer of naive T_{Eff} alone (Yuan, Bromley et al. 2007). It was shown that $CCR4^{-/-}$ T_{Reg} failed to locate to mesenteric lymph nodes (MLN) in the early stages, whereas their numbers were increased relative to WT T_{Reg} in MLNs during the later stages of active and fully developed disease. Aside from homing, the authors also suggested that the decreased frequencies in MLNs could be caused due to ineffective retention of $CCR4^{-/-}$ T_{Reg} in MLNs, supposedly due to their inability to create contacts to MLN-DCs. This, however, was not further investigated by *ex vivo* immunohistochemistry or *in vivo* imaging.

Concerning autoimmune disease models of type 1 diabetes using non-obese diabetic (NOD) mice, genetically engineered increased CCL22 production by endogenous islet cells as well as islet allografts conferred immune tolerance by increased homing of T_{Reg} to the pancreatic islets as well as lymph nodes of NOD mice (Montane, Bischoff et al. 2011, Montane, Obach et al. 2015). Other studies also demonstrated a role of CCL22-producing myeloid DCs in T_{Reg} homing to pancreatic lymph nodes in the same model of type 1 diabetes (Layseca-Espinosa, Korniotis et al. 2013). Opposed to these

data, one study found CCR4^{+} T_{Eff} to be responsible for the clinical course of type 1 diabetes in NOD mice (Kim, Cleary et al. 2002). Using adoptively transferred T cells from NOD mice into immunodeficient NOD-scid mice, *in vivo* neutralization of CCL22 resulted in decreased levels of insulitis as well as delayed onset of diabetes. These conflicting effects might probably be attributed to differential effects on T_{Reg} and T_{Eff} in a timely manner during the development of autoimmunity.

Beyond the forecited models of induced autoimmune disease, the selective absence of CCR4 on T_{Reg} in steady state conditions also results in spontaneous autoimmune reactions in the skin and the lungs as well as peripheral lymphadenopathy in mice (Sather, Treuting et al. 2007). Concerning the dysfunction of CCR4$^{-/-}$ T_{Reg} in the skin and the lungs, the impaired homing of these cells could clearly be held responsible. The peripheral lymphadenopathy, however, again raised the hypothesis regarding efficient interactions with target cells facilitated by interstitial migration, as CCR4 is not essential for lymph node homing. Regarding skin tolerance, these findings were further supported by a skin disease model of the autoimmune disorder vitiligo, which depended significantly on CCL22 (Eby, Kang et al. 2015). For the complete picture it should be kept in mind, however, that CCR4 has been implied in memory T cell homing to the skin (Campbell, Haraldsen et al. 1999) and to the lung (Mikhak, Strassner et al. 2013) as well.

With respect to transplant tolerance, the presence of CCR4 in T_{Reg} was essential for their homing to allografts in mouse models (Lee, Wang et al. 2005). In contrast, the further migration from allografts into draining lymph nodes was not affected by the absence of CCR4 on T_{Reg} (Zhang, Schroppel et al. 2009), but rather required the expression of CCR2, CCR5 and CCR7. These studies further support that CCR4 is dispensable for peripheral lymph node homing of T_{Reg}. The manifold implications of T_{Reg} migration and function, CCL22 and CCR4 in the setting of cancer will be discussed in further detail in chapter 4.3.

In the next section, I will recapitulate the role of CCL22 integrating the above-mentioned evidence, prior investigations on differential CCL22 regulation from our research group and the new insights provided by the experiments of this thesis.

The chemokine CCL22 is constitutively expressed in high amounts by immature DCs in lymph nodes (Kanazawa, Nakamura et al. 1999), but not by immature DCs in tissues like

the skin (Tang and Cyster 1999). Upon PAMP-induced maturation, DCs in the skin *in vivo* as well as isolated DCs *in vitro* show rapid upregulation of CCL22. In strong contrast to the skin, lymph-node resident DCs, however, show a rapid and strong downregulation of CCL22 *in vivo* in response to various PAMPs (unpublished observations). When culturing complete cells isolated from lymph nodes, this downregulation can also be observed *in vitro*, and was shown to be mediated by cytokines and to depend on the presence of T cells by our research group (unpublished observations). In this thesis, I further investigated the regulation of CCL22 production by DCs in cognate DC-T_{Eff} interactions *in vitro* and found a strong upregulation of CCL22 as early as 12 hours after antigen exposure and lasting for a period of 2 to 4 days. A similar regulation could be detected for its corresponding receptor CCR4 on T_{Reg} *in vitro* and *in vivo*. Using a multitude of *in vitro* experimental assays to assess the immunosuppressive function of T_{Reg}, I could not establish an impact of modulating CCL22 levels by recombinant CCL22 or anti-CCL22 blocking antibodies. Studying chemokines *in vitro*, however, has obvious limitations, as the complex *in vivo* environment cannot be simulated appropriately.

In the chain of reasoning, the experiment that would clarify the physiologic *in vivo* function of CCL22 and its receptor CCR4 in immune tolerance is the analysis of knock-out mice and their phenotype. Here, it should be considered that CCR4 also recognizes CCL17, so that only the knock-out of CCL22 would lead to unequivocal conclusions regarding the function of CCL22. The phenotype of $CCL22^{-/-}$ mice has not been previously studied. Therefore, our research group generated the $CCL22^{-/-}$ mouse. During homeostatic conditions, spontaneous immune dysregulations or autoimmune diseases were not detected in these mice (unpublished observations). However, following vaccination with Ovalbumine significantly stronger T and B cell responses were observed in $CCL22^{-/-}$ mice compared to WT mice. Moreover, the $CCL22^{-/-}$ mice were more severely affected in models of autoimmune disease like dextran sulfate sodium (DSS)-induced colitis compared to WT mice (unpublished observations).

The proposed model for the function of CCL22 in immune tolerance during the steady state and during the inflammatory state, generated using data from this doctoral thesis, prior data from our research group and existing literature, is visualized in Figure 4.1.

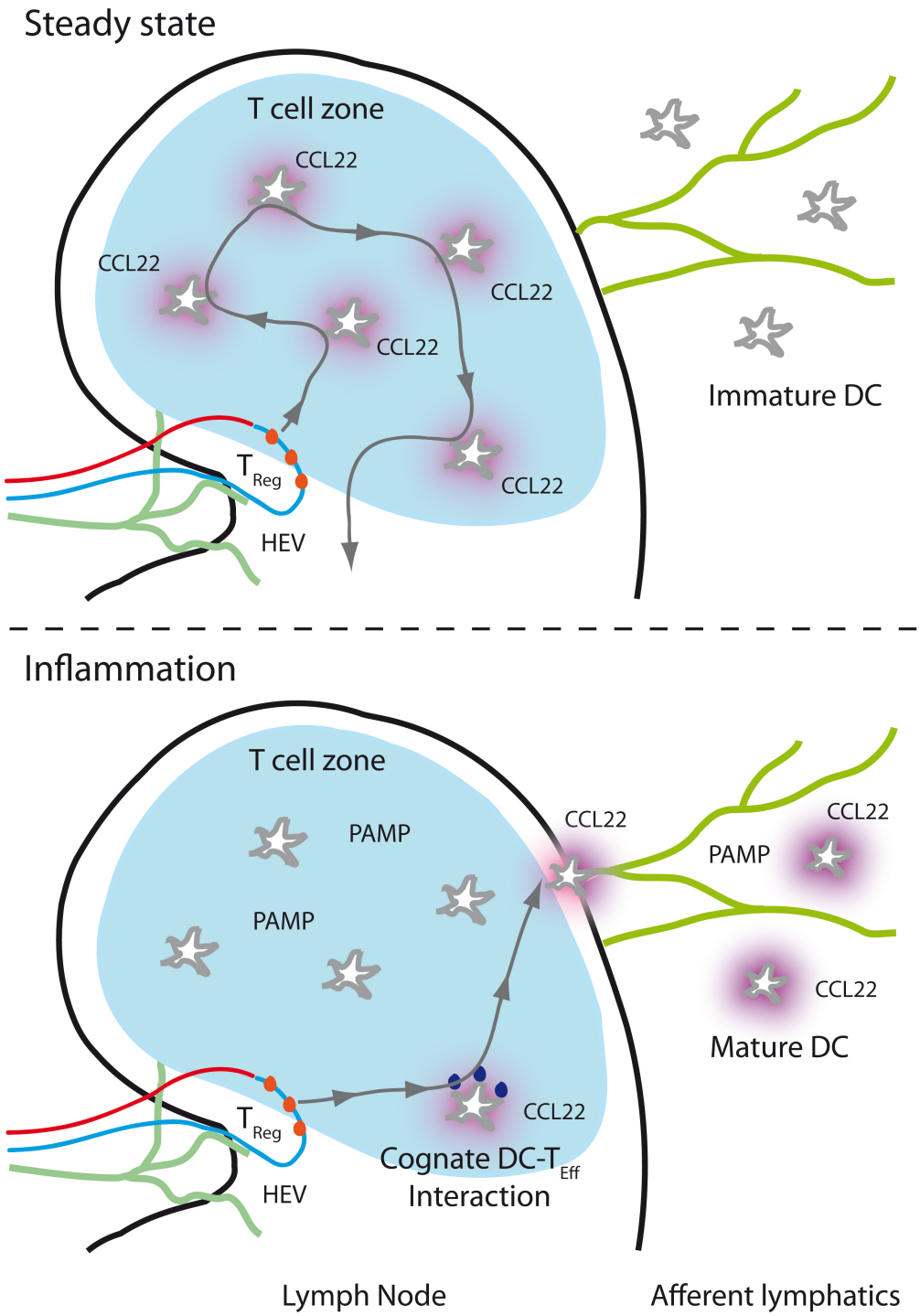

Figure 4.1: Proposed model of CCL22-guided interstitial migration of regulatory T cells for immune tolerance in the steady state and in inflammatory conditions

In the steady state, the constitutive CCL22 production by immature lymph node-resident DCs facilitates DC-T_{Reg} encounters to allow continuous screening for self-antigen in secondary lymphoid organs. Acute inflammation, however, almost completely reverses this setup: (1) immigrating DCs from peripheral tissues carrying antigens feature strongly induced CCL22 production, (2) lymph node-resident DCs exhibit strong reduction of

CCL22 production, whereas (3) lymph node-resident DCs in cognate antigen interactions with T_{Eff} preserve some CCL22 production despite inflammation. Collectively, this model suggests that T_{Reg} in this setting could easily identify, interact and if necessary control immigrating DCs as well as lymph node-resident DCs that are on the verge of T cell priming – both of which should be tightly observed to maintain immune tolerance in case of self-antigen presentation.

4.3 Regulatory T cells and chemokines as targets of immunotherapy in cancer

Cancer is one of the leading causes of mortality in the ageing societies of developed countries. The pathogenesis of malignant tumors is still a subject of intensive studies, and the search for targeted therapies to fight these devastating diseases ongoing. The vast majority of cancer therapies were and are still based on cytotoxic effects of chemotherapeutic agents. Tumor immunotherapy is an approach to modulate the patient's immune system in order to fight the cancer cells. William Bradley Coley is considered the pioneer of tumor immunotherapy. In the late 19th century, he made the observation that patients with infections after cancer surgery had a better prognosis, which motivated him to develop a mixture of killed bacteria termed Coley's toxin. Almost one century later, the Bacillus Calmette-Guérin (BCG) vaccine, an attenuated strain of *Mycobacterium bovis*, is used as the first-line therapy for non-invasive bladder cancer (Lamm, Blumenstein et al. 1991). Since then, many new ways of immunotherapy were discovered. In the recent decade, T_{Reg} have drawn attention as they accumulate in various types of cancer and are linked to poor clinical outcomes.

The antitumor immune response is directed against multiple antigens of the tumor. These tumor-associated antigens have been shown to share properties with self-antigens rather than foreign antigens, and therefore antitumor immunity can in part be seen as a kind of autoimmunity (Nishikawa and Sakaguchi 2010). Accordingly, T_{Reg} could have a negative influence on antitumor immunity. In fact, this role for T_{Reg} was first verified by experiments using anti-CD25 antibody-mediated depletion of T_{Reg}, which resulted in the rejection of established tumors across a broad variety of tumor entities (Onizuka, Tawara et al. 1999, Shimizu, Yamazaki et al. 1999). In addition, T_{Reg} depletion can prevent the development of cancer in murine carcinogenesis models (Teng, Ngiow et al. 2010). Therefore, T_{Reg} display an interesting target in cancer therapy.

The very first study in the setting of human cancer reported a negative impact of increasing amounts of T_{Reg} in the tumor tissues on the survival of human ovarian cancer patients (Curiel, Coukos et al. 2004). The accumulation within the tumor was shown to be driven by CCL22. In the same tumor entity, another migratory mechanism involving CCL28 and CCR10-dependent migration of T_{Reg} could be identified (Facciabene, Peng et al. 2011), again with predictive value regarding the clinical course of the disease. In common cancer types with considerable socioeconomic influence like human colorectal cancer or human breast cancer, T_{Reg} also represent a serious risk factor for poor clinical outcome (Gobert, Treilleux et al. 2009, Deng, Zhang et al. 2010, Faget, Biota et al. 2011, Menetrier-Caux, Faget et al. 2012, Saito, Nishikawa et al. 2016).

From a mechanistic point of view, the function of T_{Reg} in suppressing antitumor immunity was shown to be dependent on CTLA-4 expression (Wing, Onishi et al. 2008) and *in vivo* imaging of T_{Reg} function within tumors demonstrated local intratumoral DCs to be the cellular interaction counterpart (Bauer, Kim et al. 2014). Further evidence for the interaction between DCs and T_{Reg} in the context of cancer is provided by the functional involvement of TGFß (Chen, Pittet et al. 2005), IDO (Sharma, Baban et al. 2007), OX40 (Piconese, Valzasina et al. 2008) and by perforin-dependent DC death exerted by T_{Reg} in tumor-draining lymph nodes (Boissonnas, Scholer-Dahirel et al. 2010), all of which were shown to depend on cognate antigen recognition.

From a clinical point of view, the metastatic tumor burden is in general the life-limiting aspect of the disease. In this regard, T_{Reg} have been shown to favor lung metastases in breast cancer by homing mechanisms (Olkhanud, Baatar et al. 2009) and by T_{Reg}-produced receptor activator of nuclear factor-kB (RANK) ligand (RANKL), which was necessary for breast cancer cells to metastasize to the lung (Tan, Zhang et al. 2011).

A medical breakthrough in the field of human tumor immunotherapy was the randomized clinical trial with ipilimumab, an antibody that targets CTLA-4. It showed improved survival in patients with metastatic melanoma (Hodi, O'Day et al. 2010). The increase in the median overall survival from 6.4 months to 10.0 months made ipilimumab the first drug to effectively improve the clinical course of metastatic melanoma and therefore ipilimumab was quickly established as the first-line therapy. Severe immune-related adverse events occurred in about 10% of patients, mainly including dermatologic,

gastrointestinal and endocrine adverse events. These were, however, reversible in most cases with immunosuppressive treatment. An elaborate analysis of the implication of CTLA-4 on T_{Eff} and T_{Reg} using compartmentalized expression of human CTLA-4 in mice suggested that the effect of anti-CTLA-4 treatment depends on blockade of CTLA-4 on T_{Eff} as well as on T_{Reg} (Peggs, Quezada et al. 2009). However, a later study identified the antibody-mediated Fc-dependent depletion of intratumoral T_{Reg} as the key mechanism underlying the clinical success of anti-CTLA-4 antibody immunotherapy (Simpson, Li et al. 2013). Newly developed antibodies such as nivolumab target other T cell molecules like the immune checkpoint inhibitor programmed cell death protein 1 (PD-1), which however is mainly expressed on activated T_{Eff} rather than T_{Reg}.

Aside from CTLA-4, other cell surface proteins of T_{Reg} have been extensively studied in preclinical settings in mice regarding their potential as targets of immunotherapy. OX40 is a member of the family of costimulatory molecules and is constitutively expressed in murine T_{Reg} cells and transiently expressed in activated T_{Eff}. OX40 regulates the differentiation and clonal expansion of $CD4^+$ T_{Eff} as well as activation of CTLs. In mice, OX40 agonists inhibit the development of tumors and are able to reject established tumors, both depending on the presence of T_{Reg} (Piconese, Valzasina et al. 2008). In humans, OX40 agonists have been tested in phase I clinical trials and represent a strong immune-stimulating agent with first positive clinical results in cancer patients (Curti, Kovacsovics-Bankowski et al. 2013).

Another example of a costimulatory molecule on the cell surface of T_{Reg}, but also other $CD4^+$ T cells, is the protein GITR (Glucocorticoid-Induced TNF-receptor family Related gene). Anti-GITR antibody treatment has been shown to inhibit T_{Reg}-mediated suppression, enhance T_{Eff} and CTL anti-tumor responses and lead to the eradication of established tumors (Ko, Yamazaki et al. 2005). The mechanism of GITR targeting plays an important role. Depleting antibodies likely lead to tumor rejection by T_{Reg} depletion (Kim, Shin et al. 2015). On the other hand, agonistic GITR antibodies were shown to modulate the differentiation of $CD4^+$ T cells. The generation of induced T_{Reg} (iT_{Reg}) was inhibited, whereas the generation of T_H9 cells was strongly enhanced, which contributed significantly to the anti-tumor immune response (Kim, Kim et al. 2015).

Taken together, various approaches in antibody-mediated targeting of T_{Reg} have proven to be effective in murine and human cancer. For further drug development, new

strategies of combining established treatments as well as the screening of new cell surface markers that allow specific T_{Reg} targeting have great potential to contribute to the developing field of cancer immunotherapy.

As the above-mentioned directly T_{Reg}-targeted immunotherapies are often accompanied by autoimmune side effects, altering the T_{Reg} migration to tumors through chemokines might be promising as an alternative target. Chemokines are a large family of proteins with multifaceted functions in innate and adaptive immunity. Here, I will focus on chemokines and chemokine receptors with T_{Reg}-related functions and their implications in cancer.

T_{Reg} are recruited by solid malignancies as a means of immune escape and subsequent tumor progression. In particular, the amount of chemokines CCL22 for the receptor CCR4 and CCL28 for the receptor CCR10 were found to have high correlations with the amount of intratumoral T_{Reg} and most notably with the clinical outcome in human ovarian cancer patients (Curiel, Coukos et al. 2004, Facciabene, Peng et al. 2011). Among chemokines associated with T_{Reg} accumulation, CCL22 is so far the most studied chemokine. CCL22 is present in a variety of solid human tumors like breast cancer (Gobert, Treilleux et al. 2009), Hodgkin lymphoma (Ishida, Ishii et al. 2006), gastric adenocarcinoma (Mizukami, Kono et al. 2008), esophageal cancer (Maruyama, Kono et al. 2010) and in the malignant pleural effusion of lung cancer (Qin, Shi et al. 2009). In the studies investigating clinical implications, the CCL22 levels within the tumor correlated with the clinical progression of the disease. In the majority of cases, the main producers of the intratumoral CCL22 are identified as myeloid cells, in particular DCs.

The suppression of intratumoral CCL22 production by Toll-like receptor (TLR) or RIG-I–like receptor (RLR) ligands leads to reduced T_{Reg} recruitment and lower numbers of intratumoral T_{Reg} (Anz, Rapp et al. 2015). In this activation of innate immunity, the main mediator of CCL22 suppression is interferon alpha (IFNα). Most notably, the well-established therapeutic effectiveness of TLR and RLR ligands was shown to depend on intratumoral CCL22 reduction. This could be proven by genetically modified CCL22-producing tumor cell lines, in which these therapies were ineffective.

Not only the chemokine CCL22 but also its receptor CCR4 on T_{Reg} represents an interesting target of immunotherapy. Targeting CCR4 by small molecule antagonists acts

as a strong adjuvant in vaccination through decreased function of T_{Reg} (Bayry, Tchilian et al. 2008). The *in vivo* mechanism of these antagonists, however, was not elucidated. The combination of these CCR4 antagonists with vaccination against tumor antigens resulted in strong anti-tumor CTL responses and significantly reduced tumor growth (Pere, Montier et al. 2011). In the vaccine draining lymph node, decreased numbers of T_{Reg} were observed. It was, however, not further assessed whether this is due to decreased homing or decreased retention of T_{Reg} in this lymph node, which itself could be a result of decreased interstitial DC-T_{Reg} interactions.

Opposed to CCR4 small molecule antagonists, an anti-CCR4 depleting antibody called mogamulizumab has been originally developed for treatment of adult T-cell leukemia. The reasoning was not based on direct targeting of T_{Reg} but rather depletion of leukemia cells, which were identified to strongly express CCR4. It soon emerged that CCR4 has actually two targets with the CCR4-expressing malignant cells as well as CCR4-expressing T_{Reg} and might exert a second effect in abolishing the T_{Reg}-mediated immune evasion (Ni, Jorgensen et al. 2015). Unfortunately, the associated T_{Reg} depletion also led to severe immune-related adverse events in some patients (Fuji, Inoue et al. 2016), as was observed in anti-CTLA-4 antibody treatment.

Interestingly, mogamulizumab also strongly amplified tumor antigen-specific immune responses in melanoma patients through the depletion of T_{Reg} (Sugiyama, Nishikawa et al. 2013), which suggested to expand the use of mogamulizumab on solid malignancies. In a recent phase I clinical trial of patients with lung cancer and esophageal cancer, Mogamulizumab also induced strong tumor antigen-specific T cell responses (Kurose, Ohue et al. 2015). The T_{Reg} depletion was highly efficient even at low doses and lasted for more than 6 months. These initial observations illustrate the potential of CCR4 as a target of immunotherapy and warrant further research in larger phase II clinical trials. In this context, our results represent an additional pathophysiologic explanation for the effectiveness of mogamulizumab and CCR4 small molecule antagonists.

Last but not least, the function of CCR4 as a chemokine receptor that promotes DC interactions can also be exploited in adoptive T cell therapy (Rapp, Grassmann et al. 2016). Modifying CTLs *ex vivo* to overexpress CCR4 proved to significantly enhance the efficacy of adoptive T cell therapy by directing CTLs to DCs, which resulted in increased CTL activation.

Taken together, T_{Reg} and the CCL22-CCR4 axis represent promising targets of immunotherapy in cancer. Initial feasibility in humans was already established for CCR4. CCL22 has so far not been studied in the human context in clinical trials. Yet in this setting, our data suggest that targeting the interaction of DCs and T_{Reg} by neutralizing CCL22 may be a promising alternative immunotherapy approach, as the severe immune-related adverse events resulting from T_{Reg} depletion in case of anti-CCR4 antibody therapy could potentially be circumvented.

4.4 Regulatory T cells and chemokines as targets of immunotherapy in autoimmune disease

Autoimmunity can cause a heterogeneous group of diseases that manifest across all organs of the body that are surveilled by immune cells. The common ground for this disease entity is the reaction of the body's own immune system against self-antigens. The pathogenesis of many autoimmune diseases is under continuous investigation and the search for targeted therapies ongoing. The very first application of an immunotherapy in human autoimmune disease used cortisone in patients with rheumatoid arthritis and was performed 1948 by Philip Showalter Hench, who later received the Nobel Prize in Physiology or Medicine in 1950. In the recent decade, the crucial involvement of T_{Reg} in the development of different autoimmune diseases was discovered, making them an interesting therapeutic target.

The dysfunction or the paucity of T_{Reg} within the affected organ represents one of many underlying pathophysiological mechanisms that disrupt the physiological immune balance (Buckner 2010). The various leukocytes that ultimately mediate the disease differ significantly between the specific autoimmune disorders and include innate as well as adaptive immune cells. Among the lymphocytes driving the disease are in particular the $CD4^+$ T_{Eff} subpopulation with T_H1 and T_H17 differentiation (T_H1 and T_H17 cells), CTLs and B cells.

In autoimmune diseases, the adoptive transfer of T_{Reg} can prevent disease onset in multiple murine models (Roncarolo and Battaglia 2007). However, the clinical goal in active autoimmune disease is to re-establish tolerance to the self-antigens involved in the specific autoimmune disorder (Sakaguchi, Powrie et al. 2012). Recent success was

achieved with the first phase I clinical trial of adoptive T_{Reg} transfer in patients with type 1 diabetes (Bluestone, Buckner et al. 2015). Nevertheless, the major obstacle of adoptive T_{Reg} therapies is to reach cost-effective manufacturing and broad availability to be a viable therapeutic option (Trzonkowski, Bacchetta et al. 2015).

As the presence of T_{Reg} in the specific organ can in general be considered favorable for the clinical outcome of the autoimmune disease, systemically altering the chemokines to direct migration of T_{Reg} does not represent a valuable therapeutic mechanism. However, localized application of chemokines like CCL22 is effective in skin autoimmune disorders such as vitiligo (Eby, Kang et al. 2015). Moreover, in the context of transplant tolerance as a cure for autoimmune diseases, reinforcing T_{Reg} migration to the allograft constitutes a reasonable approach. In the transplantation of islet cells in mouse models of type 1 diabetes, the genetic modification of induced chemokine CCL22 expression by transplanted islet cells induces tolerance towards foreign antigens and promotes allograft survival (Montane, Obach et al. 2015).

Besides adoptive T_{Reg} transfer, an approach to induce tolerance is to administer self-antigen-loaded DCs with tolerogenic properties to the patient, which is termed negative vaccination. My data show that modulating CCL22 secretion by DCs affects the interaction frequency with T_{Eff} and T_{Reg}. Furthermore, vaccination of $CCL22^{-/-}$ mice led to significantly stronger T cell responses compared to WT mice (unpublished observations). In accordance, increasing the chemokine receptor CCR4 expression of adoptively transferred T cells also resulted in stronger T cell responses through increased interaction with DCs (Rapp, Grassmann et al. 2016). These observations suggest that inducing CCL22 secretion of transferred DCs could potentially render them tolerogenic, whereas the inability to produce CCL22 leads to an increase in the DC's immunogenic potential. Taken together, increasing DC-T_{Reg} interactions through modulation of the CCL22-CCR4 axis may represent an alternative approach to balance the immune system towards a tolerogenic state in autoimmune diseases.

5 SUMMARY

Immune tolerance by regulatory T cells (T_{Reg}) requires continuous interactions with dendritic cells (DCs) to screen the organism for potentially harmful self-antigen presentation. Ultimately, this interaction keeps effector T cells (T_{Eff}) with auto-reactive properties in check as the contact with DCs, T_{Reg} and cognate antigen induces anergy, a state of non-responsiveness. The efficient collaboration of these three immune cell types therefore dictates the delicate balance between immunity and tolerance.

The aim of this study was to investigate the role of the chemokine CCL22 as a mediator of DC-T_{Reg} interactions. Based on previous observations of CCL22-dependent migration of $CCR4^+$ T_{Reg} and CCL22 production by DCs, we hypothesized that CCL22 recruits T_{Reg} to DCs and increases their interaction frequency. Therefore, I investigated DC-T_{Reg} interactions *in vitro* and *in vivo*. In addition, I studied the influence of innate and adaptive immune stimulation on CCL22 and CCR4 expression.

I showed that DC-T_{Reg} interactions were significantly more frequent *in vitro* and *in vivo* for DCs that secreted CCL22 compared to DCs that did not secrete CCL22. *In vivo*, I studied dynamic interactions in peripheral lymph nodes by intravital microscopy and observed longer antigen-dependent DC-T_{Reg} contact times for DCs that secreted CCL22. These results suggest that the homeostatic production of CCL22 by DCs serves as a signal to recruit T_{Reg} to support continuous immune tolerance during the steady state.

Studying the influence of the innate immune system on the CCL22-CCR4 axis, I observed a decrease of CCR4 expression by T_{Reg} after stimulation with TLR and RLR ligands. In contrast, adaptive immune responses in the form of cognate-antigen DC-T_{Eff} interactions led to strong and rapid induction of CCR4 expression by T_{Reg} and further to CCL22 production by DCs. This strong induction following cognate-antigen interaction with T_{Eff} might represent a mechanism of immune surveillance, as more T_{Reg} will be recruited to the DCs that interact with T_{Eff}.

These new mechanistic insights advance our comprehension of T_{Reg} function and may help to make use of their enormous potential in clinical applications for autoimmune diseases, for cancer and beyond.

6 ZUSAMMENFASSUNG

Immunologische Toleranz durch regulatorische T-Zellen (T_{Reg}) beruht auf stetigen Interaktionen mit dendritischen Zellen (DCs), um die potenziell gefährliche Präsentation von Selbstantigenen im Organismus zu unterbinden. Diese Interaktion hält auto-reaktive T-Effektor-Zellen (T_{Eff}) unter Kontrolle, da der Kontakt mit DCs, T_{Reg} und dem passenden Antigen eine Anergie der DC und T_{Eff} erzeugt. Eine effiziente Zusammenarbeit dieser drei Immunzellen bestimmt somit die feine Balance zwischen Immunität und Toleranz.

Ziel dieser Studie war es, die Rolle des Chemokins CCL22 als Mediator der DC-T_{Reg}-Interaktion zu untersuchen. Basierend auf früheren Beobachtungen der CCL22-abhängigen Migration von $CCR4^+$ T_{Reg} und der CCL22-Produktion durch DCs, stellten wir die Hypothese auf, dass CCL22 T_{Reg} zu DCs migrieren lässt und die Häufigkeit der Interaktionen steigert. Darüber hinaus untersuchte ich den Einfluss der Stimulation des angeborenen und adaptiven Immunsystems auf die Expression von CCL22 und CCR4.

Ich zeigte, dass DC-T_{Reg}-Interaktionen *in vitro* und *in vivo* signifikant häufiger sind, wenn DCs CCL22 sezernieren verglichen mit DCs, die dies nicht taten. *In vivo* untersuchte ich dynamische Interaktionen in peripheren Lymphknoten mittels intravitaler Mikroskopie und beobachtete längere DC-T_{Reg} Kontaktzeiten für DCs, die CCL22 sezernierten. Diese Ergebnisse deuten darauf hin, dass die homöostatische Produktion von CCL22 durch DCs als Signal zur Rekrutierung von T_{Reg} fungiert, um eine stete immunologische Toleranz im Ruhezustand zu gewährleisten.

Bezüglich des Einflusses des angeborenen Immunsystems auf die CCL22-CCR4-Achse, beobachtete ich eine Abnahme der CCR4-Expression bei T_{Reg} nach Stimulation mit TLR- und RLR-Liganden. Demgegenüber führten adaptive Immunantworten durch antigen-spezifische DC-T_{Eff}-Interaktion zu einer starken und schnellen Induktion der CCR4-Expression bei T_{Reg} und der CCL22-Produktion durch DCs. Dieser starke Anstieg nach einer antigen-spezifischen DC-T_{Eff}-Interaktion könnte ein Mechanismus der immunologischen Toleranz sein, um mehr T_{Reg} zu diesen DCs zu leiten, welche gerade mit T_{Eff} interagieren.

Diese neuen mechanistischen Erkenntnisse verbessern unser Verständnis der Funktion von T_{Reg} und können dabei helfen, deren großes Potenzial in der klinisch-therapeutischen Anwendung bei Autoimmun- und Krebserkrankungen zu nutzen.

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8 APPENDIX

8.1 Abbreviations

A

α	Anti
APC	Allophycocyanin
APC	Antigen-presenting cell
ATP	Adenosine triphosphate

B

BSA	Bovine serum albumin
BMDC	Bone marrow-derived dendritic cells

C

CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CpG	Oligonucleotide with cytosine-(phosphate)-guanine motifs
cpm	Counts per minute
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleid acid
ds	Double-stranded

E

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

F

FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isocyanate
Foxp3	Forkhead box p3

FSC	Forward scatter

G

GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPF	High power field
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase

H

HMW	High molecular weight

I

IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunglobulin
IL	Interleukin
IVM	intravital microscopy

L

LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
LPS	Lipopolysaccharide

M

mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MDA5	Melanoma differentiation-associated gene 5
MDC	Macrophage-derived chemokine
mDC	Mature dendritic cell
MLN	Mesenteric lymph node
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
Min	Minute(s)
Mio	Million(s)
mRNA	Messenger RNA

MyD88	Myeloid differentiation factor 88
MP-IVM	Multi-photon intravital microscopy

N

nd	Not determined
NEAA	Non-essential amino acids
ns	Not significant

O

ODN	Oligodesoxynucleotide
OVA	Ovalbumine
OVA 323-339	Ovalbumine peptide recognized by the T cell receptor of OT-II transgenic T cells
OT-II	Derived from mice transgenic for a chicken OVA 323-339 specific T cell receptor

P

PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
poly (dA:dT)	Poly (deoxyadenylic-deoxythymidylic) acid sodium salt
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PFA	Paraformaldehyde
PGN	Peptoglycans
PHA	Phytohemagglutinine
PI	Propidium iodide
PLN	Peripheral lymph node
PRR	Pattern recognition receptor
PYD	Pyrin domain

R

RIG-I	Retinoic acid-inducible gene-I

RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction

S

SEM	Standard error of mean
SLO	Secondary lymphoid organs
ss	Single-stranded
SSC	Sideward scatter
siRNA	Small interfering ribonucleic acid

T

TARC	Thymus- and activation-regulated chemokine
TCR	T cell receptor
T_{Eff}	$CD4^+$-$CD25^{neg}$-T-helper cell, effector T cell
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
T_{Reg}	$CD4^+$-$CD25^+$-$Foxp3^+$-regulatory T cell

V

VLE	Very low endotoxin

W

WT	Wild type

8.2 Publications

8.2.1 Original publications

1. Anz D, Mueller W, Golic M, **Kunz WG**, Rapp M, Koelzer VH, Ellermeier J, Ellwart JW, Schnurr M, Bourquin C, Endres S. CD103 is a hallmark of tumor-infiltrating regulatory T cells. *International Journal of Cancer* 2011 Nov 15;129(10):2417-26.

2. Rapp M, Wintergerst MWM, **Kunz WG**, Vetter VK, Knott MML, Lisowski D, Haubner S, Moder S, Thaler R, Eiber, S, Layritz P, Kühnemuth B, Stutte S, Bourquin C, von Andrian UH, Endres S, Anz D. Constitutive expression of CCL22 maintains immune homeostatsis by recruiting regulatory T cells to dendritic cells, *Manuscript submitted 2017*.

8.2.2 Oral presentations

1. **Kunz WG.** Antigen-specific regulatory T cell function

Immunopharmacology Workshop, Munich, Germany, 2010

2. **Kunz WG.** Dendritic cell interaction with regulatory T cells – the influence of innate immune stimulation

Graduiertenkolleg 1202 Results Colloquium, Munich, Germany, 2011

3. **Kunz WG.** Chemokine-guided interactions of dendritic cells and regulatory T cells

Floor meeting, Immune Disease Institute, Boston, USA, 2011

4. **Kunz WG.** Chemokine-guided interactions of dendritic cells and regulatory T cells

Results colloquium, FöFoLe-Program, Herrsching, Germany, 2011

8.2.3 Poster presentations

1. **Kunz WG**, Rapp M, Endres S, Anz D. Influence of innate immune stimulation on regulatory T cell surveillance over dendritic cell function

The Third International Conference on Regulatory T Cells and Th Subsets and Clinical Application in Human Disease (China Treg 2012), Shanghai, China 2012

8.3 Curriculum vitae

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09/12	Shanghai East Hospital, Department of Radiology, Tongji University
08/12	Addenbrooke's Hospital, Department of Medicine, University of Cambridge

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07/17	Junior Researcher Fund, Medical Faculty Ludwig-Maximilians-Universität München (47,500 €)

Stipends & Awards

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12/16	ECR Invest in the Youth 2017
10/16	RSNA Trainee Research Prize Category: Health Service, Policy and Research Category: Nuclear Medicine
04/13	ERASMUS-Stipend (Université Paris 7 Diderot)
06/12	Students' Excellence Program for final year internships abroad by the Ludwig-Maximilians-Universität München
05/12	Else-Kröner-Fresenius-Foundation, Bilateral Exchange Program with Jimma University in Jimma, Ethiopia
03/10	Friedrich-Naumann-Foundation, Full study scholarship by the Federal Ministry of Education and Research
02/10	Research Training Group (Graduiertenkolleg) 1202 of the German Research Foundation
06/06	Prize of the German Physics Society

Memberships

Radiological Society of North America, German Society of Radiology, German Society of Neuroradiology, European Society of Radiology, European Society of Neuroradiology, European Society of Oncologic Imaging

8.4 Acknowledgements

First and foremost, I want to express my gratitude to Professor Stefan Endres, MD for the opportunity to work on my doctoral thesis in the Division of Clinical Pharmacology. The research environment that he creates is inspiring and as exceptional as his support for every member of the staff. The opportunity to witness a part of his "back to the bench" experience during his sabbatical was unique and memorable.

I am indebted to PD Dr. David Anz, MD for providing me with this interesting and exciting research project, for continuously finding time to brainstorm, develop and share new ideas and for his guidance through the complex topic of immunology and experimental research. Moreover, I want to thank Dr. Moritz Rapp, PhD for his steady support in experiments, data analysis and writing as well as for his assistance with graphic design.

I am much obliged to Professor Ulrich von Andrian, MD, PhD for the chance to conduct a part of my thesis in his laboratory. In this respect, I want to thank his former research fellow Dr. Susanne Stutte, PhD, for her exceptional assistance.

Last but not least, I thank all my fellow doctoral students and co-workers for the pleasant and welcoming atmosphere in the laboratory.

Eidesstattliche Versicherung

Kunz, Wolfgang Gerhard

Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

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Interaction of Dendritic Cells and Regulatory T Cells

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