Dissertation

Submitted to the

Combined Faculty of Natural Sciences and Mathematics

of the Ruperto Carola University Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

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Born in: Bilbao, Spain

Oral examination: April 12th 2019

Characterization and modulation of CCR6/CCL20-mediated immunosurveillance in malignant melanoma

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INDEX

ABREVIATIONS

ACKR – Atypical Chemokine Receptor

ACD – Allergic Contact Dermatitis

AD – Atopic Dermatitis

APC – Antigen Presenting Cell

B-reg – B regulatory cell

BCC – Basal Cell Carcinoma

BD – Beta-Defensin

CD – Cluster of Differentiation

cDC – Conventional Dendritic Cell

CDKN – Cyclin Dependent Kinase Inhibitor

CHS – Contact Hypersensitivity

CIU – Chronic Idiopathic Urticaria

CKR – Chemokine Receptor

CSC – Cancer Stem Cell

CTLL – Cutaneous T-cell Lymphoma

DC – Dendritic cell

DMEM – Dubebco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

ECM – Extracellular Matrix

ELISA – Enzyme-Linked Immunoabsorbent Assay

FACS – Fluorescent Activated Cell Sorting

FBS – Fetal Bovine Serum

FCM – Flow Cytometry

GAG – Glycosaminoglycan

GC – Germinal Center

GPCR – G-protein Coupled Receptor

HBD – Human Beta-Defensin

HBSS – Hank's Balanced Salt Solution

IFN - Interferon

KDa – Kilo-Dalton

LN – Lymph Node

mDCs – Myeloid Dendritic Cells

MAPK – Mitogen Activated Protein Kinase

MDSC – Myeloid Derived Suppressor Cells

MHC – Major Histocompability Complex

NB-UVB – Narrow Band Ultraviolet B-light

NK – Natural Killer

NMSC – Non Melanoma Skin Cancer

OS – Overall Survival

PBS – Phosphate Buffered Saline

pDC – Plasmacytoid Dendritic Cell

PDL – Programmed Death Ligand

PMBC – Peripheral Blood Mononuclear Cell

SCC – Squamous Cell Carcinoma

SMG – Significantly Mutated Gene

RT – Room Temperature

TAM – Tumor Associated Macrophage

TGF – Transforming Growth Factor

TKI – Tyrosin Kinase Inhibitors

TLR – Toll Like Receptor

TME – Tumor Microenvironment

TNF – Tumor Necrosis Factor

TI – Tumor Infiltrating

TIL – Tumor infiltrating Leucocyte

T-reg – T Regulatory cell

TWT – Triple Wild-type

UV – UltraViolet

WT – Wild-type

Abstract

1. ABSTRACT

Chemokine ligand 20 (CCL20) expressed in the epidermis is a potent impetus for the recruitment of subsets of DCs, B-cells and memory T-cells expressing its exclusive receptor chemokine receptor 6 (CCR6) into the skin. CCL20 and a corresponding CCR6-expressing immune cell infiltrate have been detected in chronic inflammatory skin diseases and several malignancies, including melanoma. Yet, the functional contribution of the CCR6/CCL20 axis for the immune control of melanoma remains controversial. The characterization of CCR6-guided immune cell subsets and their functional contribution for the immune control of melanoma comprises the main focus of this project. We evaluated the homeostatic and inducible secretion of CCL20 by different murine and human melanoma cell lines by ELISA. Both murine (B16, Ret) and human (A375, C32) melanoma cell lines are capable of up-regulating CCL20 secretion upon stimulation with pro-inflammatory cytokines in vitro . In order to determine the functional relevance of CCR6 on local tumor growth, B16/F1 melanoma cells retrovirally transduced with a vector that constantly overexpresses CCL20 (B16-CCL20) were injected subcutaneously in C57BL/6 wt mice and congenic CCR6-knockout ($CCR6^{-/-}$) mice. While animals in both groups developed local tumors, we observed a significantly reduced tumor growth in $CCR6^{-/-}$ mice. By contrast, Wt and $CCR6^{-/-}$ control groups did not display differences in tumor growth rate. Our results suggest that CCL20 interactions in the microenvironment of cutaneous melanoma may be an essential factor for local tumor growth, although the precise mechanisms require further investigation.

1.- ZUSAMMENFASSUNG

Der Chemokin ligand 20 (CCL20) wird in der Epidermis exprimiert und ist ein wichtiges Signal für die Rekrutierung von Chemokin Rezeptor 6 (CCR6) - exprimierenden dendritische Zellen, B-Zellen und T-Gedächtniszellen in die Haut. CCL20 und das damit verbundene CCR6- exprimierende Immunzellinfiltrat konnten in chronisch entzündlichen Hauterkrankungen und verschiedenen Krebserkrankungen, wie Melanom, nachgewiesen werden. Die funktionelle Rolle der CCR6/CCL20 Moleküle in der Immunabwehr im Melanom ist noch nicht geklärt. Die Charakterisierung der CCR6-positiven Immunzellen und deren funktionellen Beitrag in der Immunabwehr gegen Melanom, stellt den Hauptfokus dieser Arbeit dar. Wir untersuchten verschiedene murine und humane Melanom-Zelllinien auf die basale und induzierbare Sekretion von CCL20 mit Hilfe von ELISA. Sowohl die murinen (B16, Ret), wie auch die humanen (A375,C32) Melanomzelllinien sind in der Lage, CCL20 als Antwort auf pro-inflammatorisch Stimuli in vitro hoch zu regulieren. Um die funktionelle Relevanz von CCR6 auf das Tumorwachstum zu beurteilen, wurden CCL20 überexprimierende B16/F1 Melanomzellen (B16-CCL20) in C57BL/6 Wildtyp (WT) Mäuse und C57BL/6 CCR6-Knockout Mäuse (CCR6 -/-) subkutan injiziert. Während Tiere beider Gruppen lokale Tumore entwickelten, konnte ein signifikant vermindertes Tumorwachstum in CCR6 -/- Mäusen beobachtet werden. Im Unterschied dazu zeigen WT und CCR6 -/- Kontrollgruppen keine sichtbaren Unterscheidungen im Tumorwachstum. Unsere Daten deuten darauf hin, dass Interaktionen von CCL20 im Tumormikromilieu beim kutanem Melanom möglicherweise einen essentiellen Faktor im lokalen Tumorwachstum besitzen, auch wenn die genauen Mechanismen dieser Interaktion noch weiter untersucht werden müssen.

Introduction

2. INTRODUCTION

2.1. Chemokines and their receptors, an historical overview

Chemokines are small cytokines of low molecular weight (7-12 KDa) whose main function, together with their Chemokine receptors (CKRs), is to drive different immune cell subsets to specific anatomical locations. However, besides this classical function, chemokines and their receptors have emerged as main players in an array of biological functions in both health and disease such as infectious diseases, hematopoiesis, cancer metastasis angiogenesis and neural development.

Although the study of chemoattractants started in late 1970s with the isolation of platelet factor 4 (PGF4, later CXCL4), it was not till 1987 that the field of chemokine research officially started after the successful cloning of the CXCL8 gene and its migratory effect on neutrophils [1]. Initially CXCL8 was misclassified as an interleukin (IL-8) and it was not till the analysis of its 3-dimension structure that could be correctly classified as a *bona fide* chemokine. In fact, unrevealing the 3-dimension structure of previously described proteins contributed the identification of other chemokines, like CXCL10 [2], CCL1 [3] and CCL3 [4]. The discovery of the first chemokines was the starting point of a race towards the identification of their receptors.

In the 1990s research in the field of chemokines rapidly expanded and it soon became clear that besides neutrophils, chemokines could target monocytes, innate immune cells, T- and B-cells. In addition to chemoattraction, new roles for chemokines were described in infections and inflammatory diseases, as well as in homeostatic processes and embryonic development [5]. Interestingly some homeostatic chemokines display lethal phenotypes in gene-deficient mice, such as CXCL12 [6].

In the year 2000 a new official nomenclature system was adopted in order to simplify the complexity of the previous naming system [7]. By then, the field of chemokine research had expanded to a great array of research areas, including studies on malaria [8] or on the human immunodeficiency virus (HIV) [9]. Chemokine ligand/receptor interactions soon proved to be more complex than initially speculated: Chemokine receptors need to sense a chemokine gradient to be expressed [10] and during inflammation processes, the ligands are able to modify the inflammatory milieu through binding, uptake and intracellular degradation of chemokines [11].

The fact that genes of many virus types target the chemokine system by expressing either inhibitors or chemokine-neutralizing proteins highlights the importance of this field of study [12].

Chemokine research is still an expanding field today. The many different and sometimes conflicting roles and functions of chemokine ligands/receptors make them a very attractive and interesting field of study. Major drug companies are investing enormous amounts of funds in chemokine research as a primary target in translational research. Up to date some FDA-approved compounds have emerged from these studies. Prominent examples are Maraviroc (Celsentri®, Pfizer), a CCR5 antagonist used as an entry inhibitor in the treatment of HIV-infected patients [13]; AMD3100 (Perixafor injection, Mozobil), a non-peptide chemokine receptor inhibitor targeting CXCR4 used in hematopoietic stem cell mobilization [14]; and Mogamulizumab (Poteligeo®, Kowa Hakko Kirin, Prizer) a humanized monoclonal anti-CCR4 antibody which was FDA-approved in August 2018 for the treatment of refractory or relapsed mycosis fungoides and Sézary syndrome [15].

The area of chemokine research has progressed continuously within the last two decades. Although the time of the molecular discoveries might be about to end, the need for a more comprehensive understanding of the precise roles, mechanisms and biological mediated by chemokines and their receptors is unchanged.

2.2. Chemokines

2.2.1. Structure

Most chemokines are low molecular weight proteins (7-12 kDa) that are synthesized in sequences of 20-25 amino acids. The characteristic basic tertiary architecture of all chemokine ligands consists on an N-terminal (signaling domain) followed by an N-loop, a three-stranded β-sheet and a C-terminal helix [16]. Biological activity of these proteins can be modified to various levels after being processed at the N-terminus. In fact, cleavage of chemokine N-terminus is understood as a natural mechanism for regulating chemokine function [17]. Although there have been some reports of chemokines that were processed at the C-terminus, the cleavage of up to six C-terminal amino acids has not shown to affect chemotactic activity [18].

2.2.2. Classification

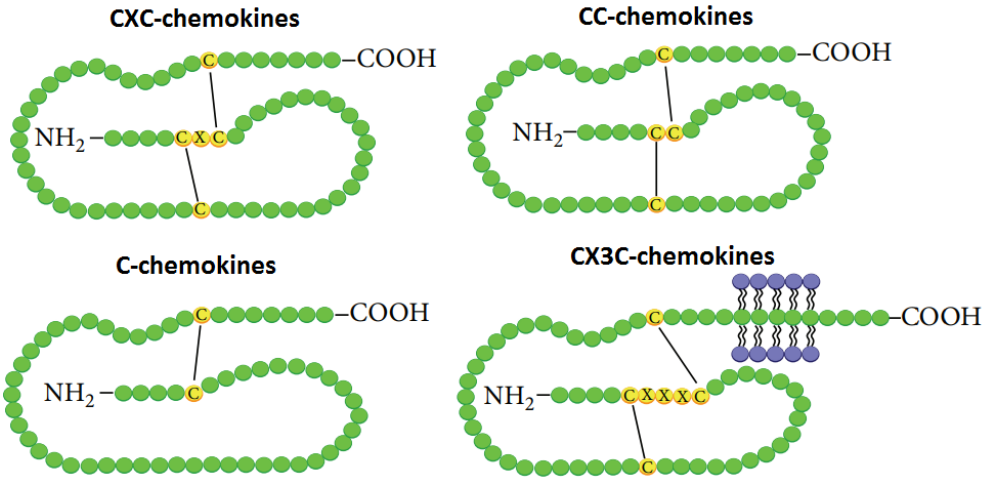
Chemokines are grouped into four subfamilies: CXC-, CC-, CX_3C- and XC- chemokines based on the spacing between their first two cysteine residues **(Intr. Fig. 1)** [19].

One of the two major subfamilies are the CXC-chemokines (also referred to as α-chemokines), which show one non-conserved amino acid between the two cysteines. They are subdivided into two categories based on the amino acid sequence located immediately before the first cysteine of the CXC motif. “ELR-positive” chemokines have a sequence of *glutamic acid-leucine-arginine* (ELR for short), while chemokines grouped as “ELR-negative” do not show this motif. This subdivision also reflects different roles of CXC chemokines as ELR-positive CXC chemokines typically induce the migration of neutrophils by interacting with CXCR1 and CXCR2 on their surface while CXC chemokines that lack the ELR motif tend to be more chemoattractant for lymphocytes [20,21]. To date 17 different CXC chemokines have been described and are named as CXCL1-17.

CC-chemokines are the second major subfamily (also called β-chemokines) and are characterized by the lack of an amino acid between the first two cysteines. Most of the members of this group have four cysteines before the CC motif (C4-CC) but a few CC-chemokines show six cysteines (6C-CC) [22]. Up to 27 chemokines have been identified in this group and are named as CCL1-27.

C-chemokines only have one cysteine at the N-terminal binding to another close to the C-terminal. Unlike the rest of the chemokine subfamilies only possesses one cysteine-cysteine bond. This group is only comprised of two members: XCL1 (also named lymphotactin-α) and XCL2 (or lymphotactin-β).

The last group is known as CX_3C-chemokines due to the presence of three amino acids between the first two cysteines. The sole CX_3C-chemokine that has been described is named fractalkine (CX_3CL1). Fractalkine is unique among chemokines due to its large size (373 amino acids) and the fact that is synthesized as a transmembrane protein whit its N-terminal domain facing the extracellular medium [23].



Intr. Fig. 1. Chemokines are divided into four subfamilies based on the spacing of amino acids between the first two cysteines. From up left, structure of CXC-, CC-, C- and CX_3C-chemokines (also known as α-, β-, γ- and Δ-chemokines). Adapted from Held & Feindt, 2015 [24].

Aside from the structural characteristics, chemokines can be categorized based on their function [25]. In this classification four subgroups can be distinguished: Homeostatic, Inflammatory, Dual-function and Plasma chemokines. These categories are not strict and not mutually exclusive [26].

Homeostatic chemokines are constitutively expressed in lymphoid organs and are related to homeostatic trafficking of various lymphocytes and other cells. A good example within this group is CXCL12, which is involved in the regulation and migration of bone marrow and thymic progenitor cells during immune cell development [27]. On the other hand, inflammatory chemokines are expressed under inflammatory conditions and are directly involved in the recruitment of immune cells to inflamed

tissues [28]. This group can be further sub-divided into two different categories based on the presence of an ELR (Glu-Leu-Arg) motif located before the first cysteine. ELR chemokines are mainly angiogenic (like CXCL8) while non-ELR chemokines have angiostatic properties (like CXCL4 or CXCL9) [29].

Some chemokines are involved in both homeostatic and inflammatory processes and are named "dual-function chemokines" (or dual-chemokines). CCL17 and CCL22 (both ligands of CCR4) constitute a good example of this category. This chemokine axis is involved in a series of homeostatic functions, like skin-homing T-cells, but is also involved in a series of conditions such as asthma and allergic diseases [30]. Finally, there is a subgroup of inflammatory chemokines known as "plasma chemokines" which are present at high concentrations in serum (like CCL14 and CCL15) [31].

2.3. Chemokine receptors

2.3.1. G-protein coupled receptors

CKRs are members of the seven-transmembrane G-protein-Coupled Receptor family (GPCR). They are mostly expressed on migrating cells and bind chemokines in the extracellular medium, triggering signaling pathways inside of the cell that usually lead to cell motility [16]. Like all GPCRs, CKRs are integrated in the membrane conformed as seven transmembrane helices with the N-terminus facing the external part of the cells and the C terminus facing the cytoplasm [32]. After binding to the chemokines, CKRs interact with G-proteins.

Heterotrimeric G proteins consist of three subunits α, β and γ. Although the GPCR family comprises almost 1000 different proteins, there is a relatively small number of G proteins to trigger a high number of intracellular signaling cascades [33]. 35 different genes encoding for G proteins can be found in the human genome, 16 of which corresponding to α-subunits, 14 to γ and 5 to β. [34]

The GPCR transduces the signal to a heterotrimeric G protein, which interchanges the bound GDP or the Gα subunit for GTP. This leads to the dissociation of the Gαβγ complex into Gα-GTP and Gβγ and to the activation of downstream effector by both subunits. The transduction of the signal ends when the intrinsic GTPase activity of the G protein hydrolyzes the GTP to GDP, resulting in the end of the cycle [35]. The Gβγ dimer activates a series of signaling cascades that lead eventually to chemotaxis and cell adhesion. It interacts with phospholipase C (PLCβ) [36], which leads to the hydrolysis of phosphatidylinositol bisphosphate, resulting in the generation of inositol trisphospate (IP3) and

diacylglycerol (DAG) [37]. The Gβγ dimer has proven to be critical for chemotaxis, while the Gα subunit seems not to be related to this process.

2.3.2. Electrostatic interaction and binding: Glucosamynoglycans (GAGs)

GAGs are carbohydrate structures that appear bound to protein cores of proteoglycans or attached to the extracellular matrix (ECM) of the cells. GAGs contribute to the immobilization of chemokines and the formation of gradients that adequately direct the migration of CKR-expressing cells, providing directional signals [38].

Chemokine-GAG interactions typically occur through electrostatic interactions of positively charged residues with highly sulphated and acidic GAGs [39]. Nevertheless, these interactions appear to be complex and recent evidence points to specificity of some chemokines for certain type of GAGs. Additionally, other factors like the state of oligomerization, the microenvironment or the monomer/dimer configuration of the chemokines can affect these interactions as well [40].

The immobilization of chemokines by GAGs allows them to act locally rather than in paracrine ways, preventing inadequate activation and desensitization of receptors on cells outside of the region of interest for a specific physiological situation [41]. GAGs have also been described to promote secretion of chemokine ligands from tumor cells and T-cells [42]

2.3.3. Chemokine /Chemokine Receptor binding

Chemokines are structured in a way that allows maintaining interactions with both GRK and GAGs, and the affinities for one or another have to be modulated in a way that enables the transfer of the ligand from the GAG to the receptor in the precise moment [39].

Once in contact with the CKR, the N-terminus from the receptor interacts with the chemokine core domain (formally named “chemokine recognition site 1”, CRS1) [43], at the same time that the N-terminus of the chemokine interacts with the ligand-binding pocket of the CKR (Chemokine recognition site 2, or CRS2). This interaction model is referred to as the “two-site model” of receptor activation, and CRS1 and CRS2 interactions can be decoupled, at least partially [44,45].

Once the chemokine and the CKR are bound together, the complex is rapidly internalized, hampering additional interactions of the CKR with other chemokines. This process is named “desensitization” and is a key mechanism for regulating the role of CKRs as it inhibits cell migration once leukocytes arrive at specific anatomical locations in response to a chemotactic gradient, like for example in the case of inflammation. Internalization of certain CKRs can be followed by the expression of different types of CKRs. This is called “CKR switching” and is associated with leukocyte function [46,47,48].

2.3.4. Chemokine receptor nomenclature

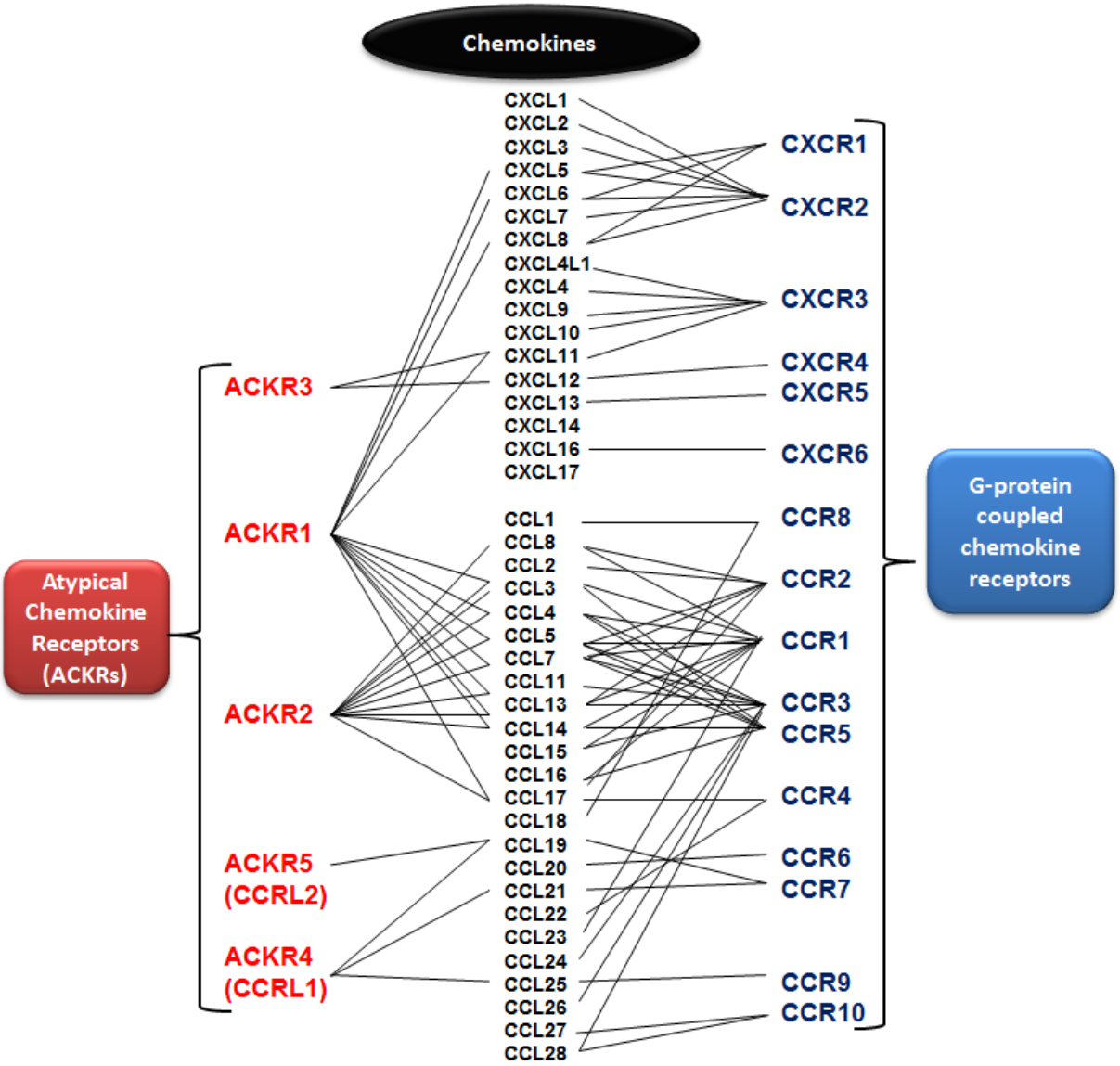
CKRs are named and classified based on their chemokine selectivity. Hence, CXC-chemokines bind to CXC-receptors. In humans there are up to 19 different CKRs, named CXCR1-6, CCL1-10, XCR1, XCR2 and CX3CR1 **(Intr. Fig. 2)**.

2.3.5. Atypical Chemokine Receptors (ACKRs)

Besides the classical CKRs, a series of so-called “atypical chemokine receptors” (or ACKRs) have been described in recent years. The ACKR family comprises four members (named ACKR1-4) and a fifth candidate named CCRL2 (CC-receptor like 2) **(Intr. Fig. 2)**. These are seven transmembrane proteins that do not signal through G proteins but are able to internalize the chemokine, promote its degradation or transport it across the cell [49]. For these reasons ACKRs can influence immune responses *in vivo* due to their ability to arrest chemokines from the local environment [50].

2.3.6. Exclusive receptors

The majority of the chemokines are able to bind to more than one CKR, and some CKRs have multiple ligands. This feature is common among chemokine/CKR pairs involved in inflammation processes. Promiscuity is restricted to chemokine class; a CXC receptor will always bind to CXC ligands but never to a CC ligand. The specific pathways that a CKR is able to activate depend on which ligand is binding the receptor, as well as on the cellular context. This functional selectivity is emerging as a key feature of CKRs and up to six CKRs have been described to show this characteristic [51]. On the other hand, some CKRs are also able to bind non-chemokine ligands. To quote an example, CXCR2 and CXCR4 have been observed to trigger cell migration after binding to macrophage migration inhibitory factor (MIF) [52].



Intr. Fig. 2. Human CXC- and CC-chemokines and their binding receptors. Right, GPCR CKRs. Left, ACKRs. Adapted from Bachelerie *et al.* 2014 [25]

On the other hand, CKRs involved in homeostatic processes bind usually to only one or two ligands that only interact with one specific CKR [[53]]. These "exclusive" chemokine/CKR pairs have been of great interest for research. Exclusivity simplifies research efforts since it lacks background effects caused by other ligands and eliminates the problem of redundancy in the chemokine system.

2.4. Biological functions of Chemokine/CKR interactions

Shortly after their discovery, chemokines were believed to be merely chemoattractant molecules (and were named based on this property). However, over the years it has become increasingly clear that chemokines are involved in a wide range or roles and functions. Today, it is assumed that any cell type can express chemokines and/or chemokine receptors [54]

2.4.1. Chemoattraction

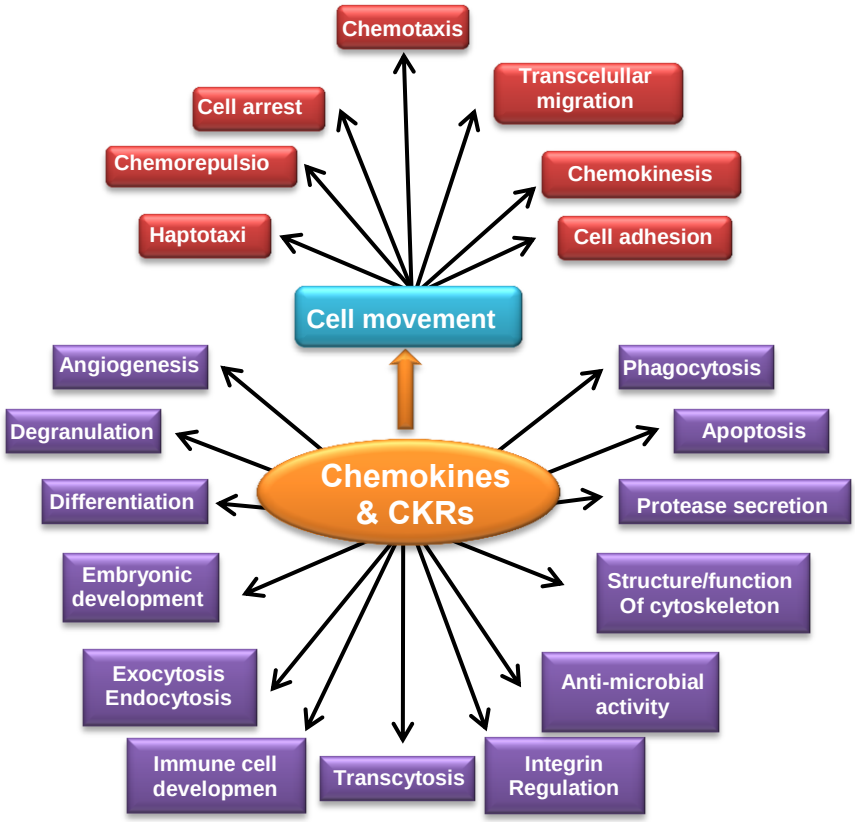
Chemokines, alongside with other chemoattractants, drive leucocyte subsets to specific anatomical locations under homeostatic conditions as well as in response to inflammation or tissue damage. Upon contact with chemoattractants, leucocytes undergo cytoskeleton rearrangement, cell shape changes and polarization, initiating cell motility along a concentration gradient [55]. Chemoattractants cooperate temporally and spatially. A good example of this phenomenon is the so-called "lipid-cytokine-chemokine cascade", were lipid mediators initiate leucocyte recruitment cascades that induce cytokine production, that trigger chemokine expression and release, thus amplifying the signal to other immune cells [56]. In this context, sequential chemokine engagement cascades have also been described, such as in neutrophil recruitment, for example [57]. In fact, the first observations of a chemokine gradient *in vivo* were performed using a neutrophil migrating model for the study of liver injury [58].

2.4.2. Other functions of chemokines

Apart from chemoattraction a wide variety of biological functions and processes may be induced by CKR activation on leucocytes, such as proliferation, differentiation, cytokine production, degranulation and respiratory burst **(Intr. Fig. 3)** [59]. Additionally, numerous chemokines have direct antimicrobial activity [60]. Besides immune cells, many cell types are able to express CKRs and respond in different ways to chemokines, including epithelial cells [61], endothelial cells [62], mesenchymal cells [63] and neurons [64]. Various chemokines are reportedly involved in angiogenesis regulation [65] and cancer cells of non-leukocytic origin can express CKRs and respond to chemokine ligands, triggering invasiveness, spread to draining lymph nodes (DLN) and metastasis seeding in distant tissues [66].

Back to cell motility, chemokines may also control different types of cell movement in addition to classical chemotaxis. Examples include haptotaxis, trans-cellular migration and cell adhesion (hence stopping cell movement) [67,68]. Furthermore, chemokine gradients can work in a reverse way, causing

certain cells to move away from the concentration gradient. This process is called “chemo-repulsion” or “chemo-fugetaxis” [69].



Intr. Fig. 3. Functions of Chemokines and CKRs. Chemokines and their receptors participate in a vast array of functions besides cell movement. Scheme adapted from Hughes *et al.* 2018 [53]

2.4.3. Chemokines in disease

A well-coordinated function of the immune response is of vital importance. Specific leucocyte subsets have to be in the right location at the right moment for their immunological functions to be effective. Upon injuries or infection, innate immunity offers a rapid response to kill pathogens, start inflammation processes and repairing tissue damage. Adaptive responses and the generation of immune memory require precise coordination of leukocyte migratory processes. Without chemokines, this response is unsuccessful, immune tolerance is compromised and immunosurveillance fails. Nevertheless, chemokine-regulated leucocyte migration may contribute to tissue damage caused by immune malfunction or inflammatory processes such as allergies, chronic inflammatory disease, autoimmunity or cancer [53]. Moreover, many virus-related diseases involve chemokines. Several viruses carry genes that encode inhibitors that alter the function of CKRs of host immune cells as well as chemokine-neutralizing proteins of the host [53].

There is a long list of diseases reportedly regulated, mediated or caused by chemokines. Through the history of chemokine research there has been a series of groundbreaking discoveries implying chemokines in human disease. For example the malaria parasite *Plasmodium vivax* expresses a CKR that acts as a transcellular transporter or human chemokines [8]. Chemokines have also been related to HIV disease. CCR5 has been widely studied since it was described as a co-receptor for HIV entry on immune cells [70]. Moreover, studies found that some chemokines can act as HIV-suppressor factors and are implied in person-to-person transmission of this virus [71]. Implication of chemokines and their receptors in cancer is well documented in the study of several types of human cancer [72,73,74].

2.4.4. Chemokines and CKRs as therapeutic targets

Chemokines and their receptors are attractive targets in translational research since they are involved in a great array of pathways and key biological processes, especially immune-related functions. Techniques like genetic deletion or antibody neutralization have appeared as efficacious in treating diverse diseases in murine models [75]. There is a long list of therapeutics targeting CKRs in late clinical phases of development and few have been approved by the FDA [14,76]. Examples of the diseases and malignancies targeted include: HIV infection [77], T-cell lymphoma [78], Asthma [79], Multiple sclerosis [80], diabetes [81], arthritis [82] and various cancers [83,84].

Nevertheless, many therapeutic have failed during development despite of the strenuous effort of chemical and pharmacological industries for various reasons [85]. For instance, one major problem is that animal models of inflammatory diseases do not always predict accurately the efficacy in humans [86]. Further, the chemokine system is intricate and very redundant. Cells may express several receptors with high plasticity over time and most CKR may bind to several ligands and vice versa. Hence blocking one CKR may not have the desired effect since other chemokines may fulfill the physiological functions assigned to the inhibited receptor [87]. Another consideration is the potential toxicity derived from chemokine antagonists. Clinical trials with various CKR antagonists had to be stopped due to side effects [88].

2.5. An exclusive pair: The CCR6/CCL20 axis

One of the few exclusive chemokine/CKR pairs in the chemokine system is conformed by CC-chemokine receptor 6 (CCR6) and CC-chemokine ligand 20 (CCL20), referred to as the CCL20/CCR6 axis. CCL20 reportedly interacts exclusively with its receptor CCR6 in both humans and mice [89]. This chemokine axis has been the focus of many studies due to interesting features that make it different from most other chemokine axes.

First, supported by unusual genetic and structural characteristics, evidence points to an earlier phylogenetic origin CCL20/CCR6 axis in vertebrates than most of the other chemokines [90]. Second, unlike the majority of the chemokines, CCL20 displays a dual mode of expression since it is both expressed under homeostatic conditions and is inducible upon pro-inflammatory stimuli [91]. These facts, together with the wide array of leukocyte subsets that have been reported to express CCR6+ (i.e. immature dendritic cells, Th17, T-regulatory and γδ T-cells, B-cells, Macrophages) [108], involve the CCR6/CCL20 axis in a great array of different roles and functions.

2.5.1. CCL20

CCL20 was first described in 1997 by three independent research groups using bioinformatics techniques [92]**.** Due to these simultaneous discoveries, CCL20 was originally named in different ways (Macrophage inflammatory protein-3α (MIP-3α) [93], Liver-Activation regulated chemokine (LARC) [94] and Exodus-1 [95]) until the unified chemokine nomenclature in the year 2000. CCL20 is encoded by the SCYA20 [small inducible cytokine family A (Cys–Cys), member 20] gene, which in humans is located in the chromosome 2q33. The full length human CCL20 gene has 11.826 base pairs that comprise 4 exons and 3 introns encoding for a 70 AA protein in its mature form [94]. The CCL20 gene has two variants that differ only in three base pairs (Ala-27, GCA) close to the N-terminus. These two isoforms have been named the Ala-27 and Ser-27 forms of CCL20. Despite of the absence of one aminoacid, studies have revealed that the differences between both isoforms in terms of biological activity are minimal [96].

CCL20 is mainly expressed in organ-associated lymphoid tissues (lungs, lymph nodes, appendix), in the liver and by epithelial cells such as keratinocytes in the skin. CCL20 RNA has been found in several organs of the digestive system such as the tonsils, esophagus, stomach, small intestine and the appendix [97] Presence of CCL20 in these tissues points to its importance in inflammation and immune activation.

Additionally, neutrophils, natural killer (NK) cells, Th17 cells, B cells, Dendritic cells, macrophages and a variety of other immune cells have been reported to secrete or express CCL20 [98,99,100].

2.5.1.1. Homeostatic expression of CCL20 in the skin

Under healthy conditions CCL20 can be found in human epidermis at low levels, but can be rapidly upregulated upon a bacterial infection or wound generation. In the dermis, CCL20 is rarely expressed and infection or injuries do not trigger CCL20 mRNA expression [101].

Shortly after its discovery it was described that CCL20 showed modest anti-microbial activity. Like many other chemokines, the accumulation of positively charged residues on one specific point of its structure confers the molecule antimicrobial properties [102]. CCL20 has been reported to have anti-microbial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Candida albicans*, among other species of bacteria [103].

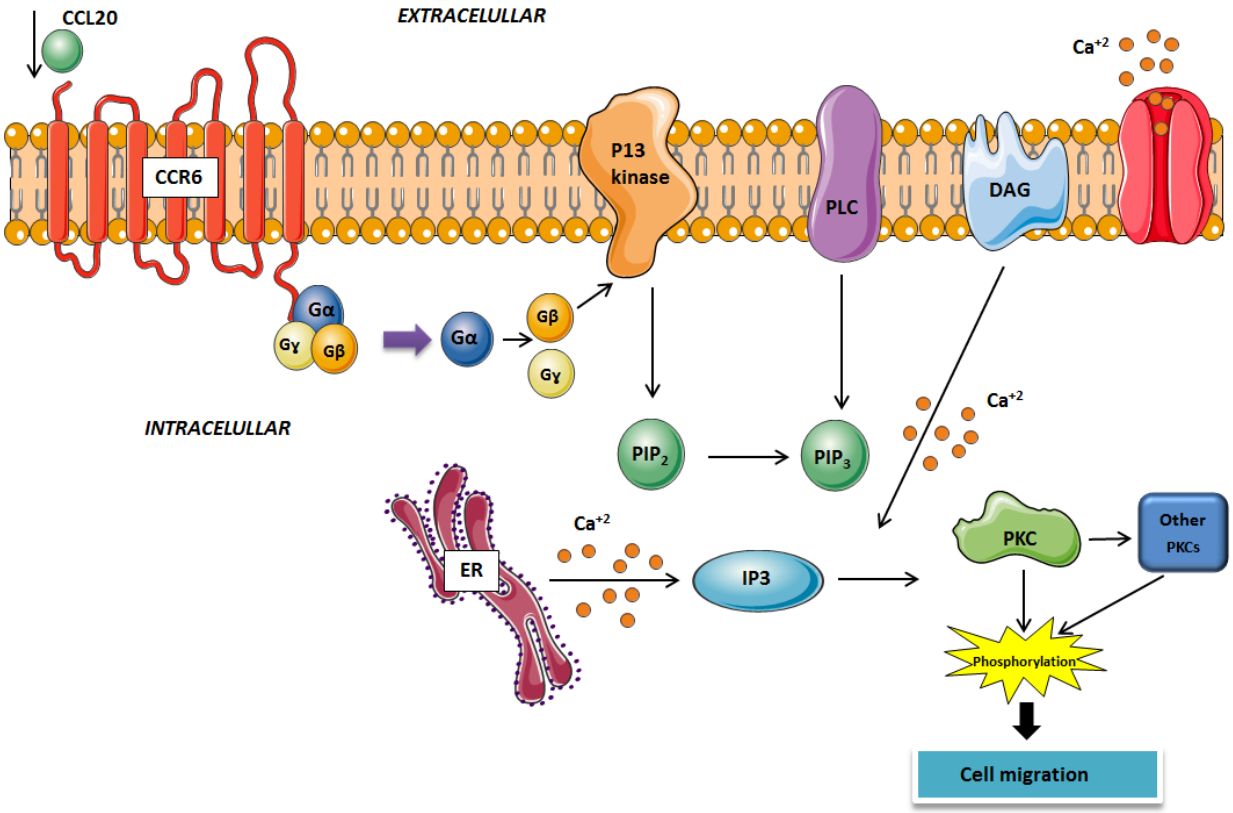
2.5.2. CCR6

CCR6 is a member of the CC-chemokine receptor family, also known as CD196 (Cluster of differentiation 196). The human CCR6 gene is located in the chromosome 6p27. Full length of CCR6 gene contains 27.890 bps encoding for a 374 AA protein in its mature form [104].

CCR6 is mainly expressed by leukocytes, therefore can be detected in immune-related tissues and organs such as the spleen, bone marrow, tonsils or lymph nodes. However, minor amounts of CCR6 mRNA have been detected in other tissues and organs such as kidney, lungs, liver, the gastrointestinal tract or the skin [105].

2.5.2.1. CCR6 ligands

The interaction of CCL20 with CCR6 was confirmed by CCL20 mediated calcium mobilization in cancer cells transfected with CCR6 [106]. Upon binding to CCL20, CCR6 activates G-proteins and signals through subunits of the Gαi family, triggering a signaling cascade that ends up activating calcium mobilization **(Intr. Fig. 4)** [107].



Intr. Fig. 4. CCR6 signaling pathway after CCL20-binding activation. After CCL20 binding, CCR6 activates the release of the Gβ and Gɣ G-protein subunits that further activate PLC and PI3 kinase. PI3 triggers the conversion of PIP_2 to PIP_3, that activates Ca^{+2} mobilization across IP_3. Together with DAG, IP3 activates PKC alongside with additional PKCs that end up triggering cell migration. PI3: Phosphoinositide 3 kinase, PLC: Phospholipase Cβ2, DAG: diacylglycerol, PIP_2: Phosphatidlinositol 4,5-biphosphates, PIP_3: Phosphatidlinositol 4,5-bisphosphates 3, IP_3: Inositol triphosphate, PKC: Protein kinase C, ER: Endoplasmic reticulum. Adapted from Ranasinghe & Eri, 2018 [108]

To date CCL20 is the only known chemokine that can both bind and signal through CCR6. However other molecules are capable of binding and activating CCR6, such as anti-microbial peptides named β-defensins (Human Beta-defensins, HBDs) have been identified. Further additional chemokines such as CCL5 or CCL14 have been shown to be able to partially bind CCR6 without being able to trigger any signal [113].

2.5.2.2. Additional CCR6 binding chemokines

Shortly after the discovery of CCR6, except for CCL20 most chemokines were excluded as possible binding partners for this CC-receptor. One of this early studies showed that the two most similar CC-ligands to CCL20 (in terms of AA sequence), CCL19 and CCL21 were not able to activate CCR6-transfected cells [109]. These first studies contributed to the notion of CCL20 as the unique partner for CCR6.

However, some studies have reported that other chemokines could bind to CCR6. For example, CCL5 and CCL14 were found to be able to partially desensitize CCR6 in vitro reducing the capability of CCL20 to mobilize intracellular calcium [110]. Another chemokine, CCL18 was found to mediate chemotaxis and calcium mobilization through CCR6 on human lung fibroblasts [111]. However, CCL18 did not have a strong influence in the mobilization of lung-infiltrating CCR6+ T-cells [112]. Taken together, although CCL18 can signal through this CKR under certain circumstances, evidence shows that CCR6 is not a dominant receptor for CCL18.

Some authors have pointed out the fact that some newly discovered chemokines have not yet been tested for their ability to activate CCR6 (such as CCL24 or CCL27), and that most chemokine binding studies have been solely based on calcium mobilization assays, which is not always an appropriate method to study specific receptor binding and activation processes [113].

On the other hand, although CCR6 expression has been correlated to CCL20, the expression levels of this CKR do not necessarily reflect CCL20 activity. For instance, CCR6+ human peripheral blood B-cells have been reported to fail to respond to CCL20, as well as to respond to CCL20 without changes in CCR6 protein or mRNA expression [114]. In another study, T-cells reportedly did not increase their responsiveness to CCL20 upon upregulation of CCR6 [115].

2.5.2.3. β-Defensins

Defensins are a family of antimicrobial peptides that were first isolated from leucocytes during the 1960s and comprise more than 10 different members. They play key roles in innate immunity and are divided into two main subfamilies in humans: α- and β-defensins [116]. BDs are small molecules (2-6 kDa) that can be classified as well within the broader group of host defense peptides. They are expressed under homeostatic conditions but its production can be induced in response to pathogens or pro-inflammatory cytokines by epithelial cells of the epidermis of the skin (mainly by keratinocytes) and mucosal tissue of the gastrointestinal and respiratory tract [117]. Therefore HBDs are important elements of immunity of surface-organs [118] by linking adaptive and immune responses through the modulation of cytokine expression and induction of chemotactic migration of various immune cells.

In this context BDs have been observed to recognize, bind and activate CCR6. Concretely, HBD-1, 2 and -3 were shown to be capable of competing with CCL20 and even displacing it from CCR6, inducing

chemotactic-mobilization of CCR6-expressing HEK293 cells, immature DCs and memory T-cells [119]. Other studies have confirmed the fact that HBDs are biologically active ligands for CCR6. HBDs were able to recruit DC precursors to neovasculature in tumors through CCR6 [120]. HBD-2 was shown to arrest Th17 cells in inflammatory tissues with the same affinity as CCL20 [121].

However, under certain conditions HBDs do not show as much affinity for CCR6 as CCL20. HBD-2 was proven to be 35 times less effective than CCL20 in displacing radio-iodinated CCL20 from CCR6, and chemotactic response of CCR6 expressing human spermatozoa was shown to be more efficient in response to CCL20 than to HBDs [122]. Additionally, evidence discards implication of HBDs in CCR6 mediated T and B-cell biology [113]. In fact, involvement of HBDs in CCR6-mediated immuno-modulatory activities has been surrounded by controversy, with reports showing contradicting results [123,124]. On the other hand HBDs have also been shown to bind and signal through other CKRs such as CXCR4 [125] and CCR2 [126]. Despite controversies, it has been demonstrated that HBDs can compete with CCL20 as a ligand under proper conditions and are able to induce chemotaxis through CCR6 [113].

2.6. CCR6/CCL20 axis in health and disease

Under healthy conditions, the CCR6/CCL20 axis plays a key role in the homing of immune cells to the intestinal epithelium [91]. Studies using CCR6 knock-out ($CCR6^{-/-}$) mice have found that in the absence of this CKR T-cells accumulate in the intestinal mucosa, T-regulatory and B-cell levels within the Peyer's Patches decrease and development of M cells is inhibited [89,127].

The main functions of the CCR6/CCL20 axis in the peripheral steady-state immune system are not completely clear. It has been suggested that in the absence of CCR6, other chemokine or chemokine-independent systems are able to compensate the effects of the lack of this CKR in the basal trafficking events of the peripheral immune system [**Fehler! Textmarke nicht definiert.**].

2.6.1. CCR6/CCL20 in the immune system

Numerous leucocyte types have been observed to possess CCR6+ subpopulations. Subsets of lymph node resident naïve CD4+ T-cells that start to differentiate into Th1, Th2 and Th17 upon antigen presentation upregulate CCR6. While not so widely expressed by Th1/2 cells, a great fraction of Th17 is CCR6+. This implies that CCR6 has a key role in organizing adaptive immunity [**Fehler! Textmarke nicht definiert.**].

Different cytokines such as TGF-β, IL-6, IL-17 or IL-21 can induce CCR6 expression on Th17 cells [128]. CCL20 expression can be induced in a series of different T-cell subpopulations by lipopolysaccharide (LPS) as well as by some cytokines like IL-1α, TNF-α, IFN-ɣ, IL-1β, IL-17 or IL-21 [129]. IL-23 mediated Th17 differentiation can induce inflammation and is able to induce the release of CCL20. This CCL20 can create a feedback loop (acting in an autocrine and/or paracrine way) that would create a self-perpetuating cycle at inflammatory locations [130].

Naïve T-cells can also give raise to Foxp3+ T-regulatory cells that can express CCR6. These immune cells are mainly disease suppressive and are able to downregulate inflammatory T-cell proliferation [131]. Once T-regs have infiltrated inflammatory sites, these cells can suppress effector T-cell responses by releasing TGF-β and IL-10, which downregulate CCL20 expression [132].

CCR6 has also been related to the biology of γδ-T Cells. Defects in the downregulation of CCR6 in γδ-T-Cell precursors can lead to abnormal accumulation of these cells in the fetal thymus as well as a decrease in the epidermal tissues [133]. On the other hand, CCR6 is required for the trafficking of mature

γδ-T-Cells in the skin [134]. Several B-cell subsets are able to express CCR6. The CCR6/CCL20 axis regulates certain B-cell responses [135] and is involved in the basic kinetics of germinal centers (GC) and B-cell differentiation [136]. CCR6 can be expressed by naïve and activated B-cells and is necessary for the production of high-affinity antibodies [137]. Memory B-Cell precursors have also been shown to express CCR6 and it is believed that this can be a hallmark in the differentiation between Memory B-cells and Plasma cells (PCs) [138].

Interestingly, the CCR6/CCL20 axis seems to play a role in the coordination of T and B-cells interactions during humoral immune response. It has been observed that CCR6+ follicular T-helper cells (Tfh) that express CCL20 can retain this chemokine into the cell surface in order to enhance the generation of T-B cell conjugates with CCR6+ B-cells [139].

CCR6 is also expressed in DCs, which play a key role in the initiation of primary immune responses and are essential in both innate an adaptive immunity. Recruitment of immature and mature DCs in mucosal immunity is CCR6-dependant [104]. CD11b+ and CD11b- DC subtypes found in Peyer's Patches regulate the development of Th2 and Th1 cells respectively and have been found to have CCR6+ subpopulations [140].

The CCR6/CCL20 axis has been related to macrophages as well. Tumor associated macrophages (TAMs) have been reported to attract CCR6+CD4+ cells (mostly T-regs) through the secretion of CCL20 in some malignancies like colorectal cancer [122] or skin cancer [141].

2.6.2. CCR6/CCL20 as a key player in human diseases

CCR6 and CCL20 have been reported to be involved in several aspects of human disease, especially in auto-inflammatory disorders. The CCR6/CCL20 axis may regulate tolerance and auto-inflammation by engaging CCR6-expressing T-regulatory cells and immature DCs. Upon inflammatory conditions however, the axis may switch its role and mediate immune defense through activation of CCR6-expressing Th1/Th17 cells [142]. When the CCR6/CCL20 axis is disrupted, malfunctions in these systems can occur and could lead to adverse immunologic function of several systems that culminate in a number of diseases [111].

In the recent years the CCR6/CCL20 axis has been linked to various disorders of very different origins. Implication of CCR6+ immune cells has been reported for pulmonary sarcoidosis, where CCL20 expressing lung interstitial tissue attracted CCR6+ T-cells, DCs and macrophages [142]. Looking at renal disorders, CCR6 deficiency in nephritic mice caused an imbalance of T-regs/Th17 cells and caused renal damage and high mortality compared to controls [143]. Furthermore, CCR6 has also been studied in autoimmune renal disease and the prevention of allograft rejection in kidney transplantation [144]. In the liver, intrahepatic increase of CCR6 and CCL20 was correlated with chronic liver disease [145] and it has been reported that CCR6-deficient mice are not able to recruit IL-17-expressing γδ T-cells towards inflammation sites in the liver, resulting in acute liver fibrosis [146]. CCR6 and CCL20 have also been related to brain disorders. Transfer of CCR6- Th17 cells significantly inhibited experimental autoimmune encephalomyelitis [147].

Another study found a correlation between the amount of brain-infiltrating CCR6+ γδ T-cells and cerebral ischemia related tissue damage [148]. Neutralization of CCL20 resulted in improved clinical signs on an experimental model for dry eye disease, by reducing the amount of Th17 infiltrating cells into the ocular surface [149]. Some studies have also focused on the role of this chemokine axis in clinical reproduction and contraception. Capacitated sperm exhibits a directional movement towards CCL20, and under homeostatic conditions sperm motility is known to be related with CCR6/CCL20 interactions in the reproductive tracts of both sexes [150]. In the gut the disruption of the CCR6/CCL20 axis is a contributing factor responsible for inflammatory disorders in the gastrointestinal tract [151]. CCR6 and CCL20 have been also involved in obesity and diabetes. Inflammation of the adipose tissue driven by this axis has been related to obesity. In this study, CCL20 overexpression in adipocytes had a positive correlation with the body mass index (BMI) [152]. The disruption of the CCR6/CCL20 axis in the pancreas has been suggested as a therapeutic mechanism to hamper inflammation-triggered dysfunction observed in both obese and diabetic individuals [153]. Implications of this chemokine axis have also been observed in HIV studies. CCR6 has been shown to act as a receptor for HIV invading T-helper cell subsets alongside with CXCR4 and CCR5, although with lower affinity [154].

2.6.2.1. CCR6/CCL20 in inflammatory skin diseases

Since CCR6 is expressed by leukocyte subsets that drive inflammation in the skin, a large series of studies have focused on the contribution of the CCR6/CCL20 axis in various acute and chronic inflammatory skin diseases.

For instance, reduced CCL20 production caused by keratinocyte deficiency in atopic dermatitis (AD) results in a decrease of CCR6+ leucocytes and exposes patients to bacterial and viral infections that can lead to the aggravation of eczemas. For instance, keratinocyte deficiency caused by a skin disorder named atopic dermatitis (AD) results in reduced CCL20 production, which leads to CCR6 downregulation that exposes patients to viral infections that may lead to eczemas. In fact, AD patients are more likely to have small mutations in the CCL20 gene, displaying the same phenotype [155]. Another study found that CCR6- subpopulations of T-regs cells with high expression of CD25 would acquire Th2-like properties in AD lesions [156].

CCR6+ immune cells have also been reported to play key roles in contact dermatitis. CCR6+ Th17 and Th22 cells were found to infiltrate inflamed skin regions in a study model for allergic contact dermatitis (ACD) [157]. In another study, researchers found that $CCR6^{-/-}$ mice showed altered responses in contact hypersensitivity (CHS) and delayed-type hypersensitivity (DTH) suggesting a defect in the activation and migration of CD4+CCR6+ T-cells [**Fehler! Textmarke nicht definiert.**]. Subsets of NK cells expressing a specific set of chemokine receptors including CCR6 have also been found to contribute to accumulate in ACD [158]. Additionally, increased amounts of CCL20 have been found in skin samples from patients with AD when compared to healthy controls [159].

CCR6 has been linked to urticarial as well. In a study about chronic idiopathic urticarial (CIU), peripheral blood mononuclear cells (PBMCs) from patients with CIU had increased levels of CCR6 mRNA [160]. However, the precise role of CCR6 in this disease still needs further research.

In psoriasis, a common chronic inflammatory autoimmune skin disease, CCR6 and CCL20 are highly expressed in skin lesions and immunological activation is mainly driven by CCR6+ Th17 cells [161]. Injection of IL-23 in the skin of mice results in psoriasiform dermatitis, resembling human psoriasis. It has been reported that CCR6-deficient mice fail to develop psoriasiform lesions in the skin after IL-23 intradermal injections, which promotes accumulation and proliferation of Th17 cells. Furthermore, keratinocyte derived CCL20 contributed to recruit additional CCR6+ expressing T-cells towards psoriatic-

like lesions [162,163]. Therefore inhibition of CCR6 has been suggested as a possible therapeutic treatment, in particular in addition to modern antibody therapies effectively targeting Th17-mediated skin inflammation in psoriasis [164].

In recent years there have been some approaches in this field. Using narrow band ultraviolet B-light (NB-UVB) phototherapy researchers were able to decrease CCR6 expression in PBMCs from psoriatic patients [165]. More recently, an approach using a modified CCL20 variant that could bind CCR6 but did not trigger a strong chemotactic response could prevent psoriatic inflammation in an IL-23 dependent mouse model for psoriasis [163]. Several additional research groups are also experimenting with different compounds that downregulate CCL20 expression, alone or together with other cytokines [166,167,168].

Besides Th17 cells, additional CCR6-expressing immune cell subsets have also been linked to psoriasis. These include γδ T cells [161]; Th22 cells, which secrete cytokines (IL-22, IL-13 and TNF-α) that contribute to the pathogenesis of psoriatic lesions [169]; and neutrophils. Neutrophils have been recently suggested to be the cause of CCR6+ T-cell migration towards psoriatic lesions. According to this, epidermis infiltrating neutrophils secrete IL-17A, which triggers IL-23 and CCL20 expression by keratinocytes, thus attracting CCR6+ Th17 cells and enhancing proliferation of this leucocyte subset [170].

2.6.3. CCR6/CCL20 in cancer

Cancer initiation, growth and metastasis are part of a sequential and selective process regulated by local tissue conditions, growth factors, cell-adhesion molecules and chemokines. In this context, chemokines can control the progression and outcome of cancer through different mechanisms.

For instance, chemokines drive the migration of immune cells towards tumors, which may result in anti-tumor immune responses or enhanced tumor growth [171]. Chemokines are also able to regulate angiogenesis through the interaction with endothelial cells expressing certain CKRs [172]. Through direct interaction with tumor cells, proliferation and apoptosis can be directly controlled by chemokines as well [173].

Cancer cells may use chemokines during metastatic processes to directly invade the lymphatic system and spread through the blood, as well as to settle and develop in a new location [174]. The fact that tumor cells could use CKRs to direct organ-specific tumor metastasis has attracted the attention of the

scientific community. Tumor cell surface signals, together with chemotactic gradients could allow cancer cells to migrate towards specific metastatic destinations [175].

In this context, CCR6 and CCL20 have been reported to be involved at different stages of tumor progression in cancer. In pancreatic carcinoma, stimulation of CCR6+ tumor cells with CCL20 triggered tumor cell proliferation, migration and invasion of tissues, pointing to a role of mechanisms used by autocrine and paracrine secreted CCL20 [176]. In colorectal cancer, CCL20 mediated macrophage recruitment into the intestine was reported to cause spontaneous intestinal tumorigenesis [177]. CCR6 and CCL20 can also affect tumor progression in coordination with other chemokines, like in ovarian cancer, where changes in the chemokine network involving CCL20- and CXCR2- related pathways result in enhanced tumor development [178].

2.6.3.1. CCR6/CCL20 in skin cancer

In skin cancer, CCR6 has been suggested to be involved in the regulation of metastasis alongside with other CKRs. In squamous cell carcinoma (SCC), downregulation of CCR6 followed by upregulation of CCR7 and CXCR4 promoted tumor migration to regional lymph nodes [179]. Downregulation of CCR6 followed by expression of CCR2 in γδ T-cells was shown to be of high importance in the trafficking of these cells towards the dermis in human papillomavirus-associated uterine cervical SCC [180]. Similar mechanisms have been observed for metastasis of basal cell carcinoma (BCC) tumors [181].

A few studies have focused on the involvement of CCR6 and CCL20 in the progression of cutaneous T-cell lymphoma (CTCL). Advanced CTCL showed high expression of IL-22, CCR6 and CCL20, but not of IL-17 [182]. In fact CTCL produced IL-22 was found trigger CCL20 by keratinocytes, which attracted CCR6+ dendritic cells that initiated inflammatory responses in the skin [183]. In addition, upregulation of CCR6 and CCL20 enhanced metastatic processes in CTCL. Considering this data, the inhibition of the CCR6/CCL20 axis has been suggested as a therapeutic strategy for advanced CTCL [184].

Several groups have studied the implication of the CCR6/CCL20 axis in melanoma but the precise functional contribution to this malignancy remains controversial (See **2.9**)

2.7. Skin Cancer

Skin cancer is the most common type of neoplasia worldwide, with a major preponderance among the Caucasian population and higher incidence in regions with intense solar exposure. 35-45% of all cancers among the Caucasian population are a type of skin cancer [185]. This value is lower among Hispanic (4-5%), Asian (2-4%) and African populations (1-2%) [186]. It is also related to professions with longer exposure to ultraviolet radiations (UV). The incidence among fair-skinned populations has keep increasing during the last 30 years, while in darker pigmented populations has remained more or less stable, partially due to UV-photo-protection provided by higher amounts of melanin and different structures of the melanosomes [187, 188].

Skin cancer is commonly divided into melanomas and non-melanoma skin cancer (NMSC). Every year two million new cases of NMSC are diagnosed in Europe and the United States [189,190]. These carcinomas can be divided into two main groups: Basal Cell Carcinoma (BCC) and SCC. There are other types of cutaneous tumors (although much less frequent that BCCs and SCCs) such as adnexal tumors, Merkel cell carcinomas, Darier-Ferrand tumors, and B and T cutaneous lymphomas [192].

The major environmental risk factor for BCC and SCC is UV radiation. Therefore it appears predominantly in photo-exposed areas like the head and neck (90% of the cases). Additional risk factors include radio-dermatitis, burn scars, chronic ulcers, immunosuppression and hereditary disorders such as occulocutaneous albinism and xeroderma pigmentosum [186].

BCC and SCC are the most common types of cutaneous tumors. BCC has an incidence of approximately 150-744 cases for every 100.000 individuals in Caucasian populations, whereas SCC appears with a frequency of 42-132 cases for every 100.000 Caucasians. SCC is the cause of death in 75% of all the individuals with non-melanoma cutaneous tumors, although it only represents 20% of non-melanoma cutaneous tumors [191].

Since not all types of skin cancers, in particular (NMSCs) such as SCC and BCC, are routinely registered in national tumor registries and in most of the cases are not a cause of death, the real incidence and mortality rates are difficult to acknowledge. Nevertheless these cases should lead to an underestimation of skin cancer incidences worldwide and as skin cancer constitutes a major economic and health burden in terms of diagnosis, therapies and follow-ups in the field of oncological pathology [192].

2.7.1. Melanoma

Melanoma represents the sixth most frequent neoplasia among men and the seventh among women [186]. The incidence of melanoma in Europe and the US has been increasing between 3-7% annually for the past years and is one of the tumor types with the highest mortality rates [193].

Nevertheless the increase in the incidence seems to be partially related to more awareness and prevention among the population and early diagnosis, resulting in higher detection rates of melanoma without a parallel increase in the mortality rate [194]. Improvements in clinical diagnosis and surgical techniques, together with more accurate classification guidelines of melanoma have contributed to these advances as well, and melanoma patients with high risk of relapse are identified with better precision [189].

While early tumors may be cured by surgical excision alone, metastatic and locally advanced disease is a major concern in melanoma patients and has been associated with poor prognosis and low survival rates.

2.7.1.1. Epidemiology, risk factors and prognosis

Melanoma is a malignancy that develops from the pigment containing cells known as melanocytes that start to proliferate uncontrollably for various reasons [192]. Melanoma can be categorized into two different subgroups based on etiopathogenetic differences: On one hand most common melanomas show a superficial diffusion arising *de novo* or from a pre-existing nevus. Most melanomas grow primarily in an horizontal manner that may turn into a vertical growth pattern over time, gaining metastatic potential. On the other hand some melanomas arise *de novo* alongside with a fast vertical growth, aggressive biological behavior and high metastatic potential shortly after its appearance. The pathogenic mechanisms of this less frequent form of melanoma are yet to be elucidated [195].

Slow evolution melanomas appear to be related with the exposure to UV radiation, with risk factors including accumulated dose of radiation, type of exposure (intermittent or continuous) and sunburn. Sun exposure in genetically susceptible individuals can induce melanocytic naevi (or moles), high frequencies of which imply increased risk for melanoma development. Having ≥100 melanocytic naevi increases the risk for lifelong melanoma development in seven orders of magnitude. Asymmetric and irregular shapes of naevi are an additional risk factor [196].

Melanoma is more common among individuals from ages between 40 to 60 years but it also affects those in adolescence and at late stages of life (80 years or more). The average age at melanoma diagnosis is 57 years [197]. In adults between 20 to 29 years, melanoma is one of the most common types of cancer [198]. Melanoma appears more frequently in the back for male individuals and in the legs for females [199].

The process of metastasis is commonly believed to be a late event during tumor development. However, metastasis can initiate at any phase of tumor progression, even during primary tumor formation, and is influenced by many factors: The host immune response, the tumor microenvironment, and most importantly, the capacity of the metastatic cell to generate a new tumor in a new location [200,201,202]. The mechanisms that underlie these processes are still to be precisely elucidated. The genetic and epigenetic backgrounds of tumor cells could explain the differences in metastatic potential, and this should be reflected when comparing primary and metastatic tumors of the same patient. Evidence points to considerable functional heterogeneity within melanoma tumors, since it seems that certain tumor cell subpopulations have higher potential to repopulate the tumor mass and resist certain stress factors (like hypoxia or drug toxicity) [203,204].

Metastasis of melanoma is associated with poor prognosis. Nevertheless this depends on the specific location of distant metastases. The metastases located in the skin, subcutaneous tissues and lymph nodes are associated with longer survival than visceral metastases [205].

Superficial spreading melanoma comprises almost 70% of all melanoma-diagnosed cases. There are other types of melanoma that differ from superficial spreading melanoma in terms of morphology, radial growth, metastatic potential and anatomical locations [206]. These melanomas represent the remaining 30% of cases and include: Lentigo melanoma, Acral lentiginous melanoma, Nodular melanoma, Amelanotic melanoma and uveal melanoma [192].

2.7.1.2. Melanoma pathogenesis

2.7.1.2.1. Molecular pathogenesis

A complex interaction between exogenous and endogenous events is required for the transformation of melanocytes into melanoma. The genetic basis of melanoma tumors has been widely investigated in recent years [207].

The first genetic data from melanoma tumors was obtained from patients who had relatives also affected by melanoma. Familiar background is reported in close to 10% of all melanoma patients. From this cases, 40% carry high-risk germline mutations in the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene [208,209]. CDKN2A encodes two tumor suppressor proteins, $p16^{INK4A}$ and $p14^{ARF}$, through alternative promoters and alternative splicing. Protein $p16^{INK4A}$ plays a maintenance role in cell-cycle control by the activation of a signal cascade that inactivates the retinoblastoma-associated protein (RB), while $p14^{ARF}$ controls de degradation of cellular tumor antigen p53 (encoded by the retinoblastoma gene TP53) through a series of intermediaries [210]. Therefore, mutations in CDKN2A induce G1-S cell-cycle transition due to the loss of two key regulators of cellular homeostasis, RB and p53 [211]. Germline mutations in the gene of the $p16^{INK4A}$ downstream effector cyclin-dependent kinase 4 (CDK4) have also been found in melanoma-prone families [212]. Other less common germline mutations have been reported (for example, in BAP1 [213] or POT1 [214] genes) but overall, more than half of all familial melanomas have an unknown genetic basis [215].

Sporadic melanomas constitute around 90% of all melanomas and are usually driven by low/moderate risk alleles that possess high prevalence and low penetrance, indicating that environmental factors are likely to be the main cause of malignant transformation [216,217]. Various population studies have related the melanocortin 1 receptor gene (MC1R), which has more than 100 allelic variants, with red hair, low tanning capability and higher melanoma risk [218]. The MCR1 gene controls the regulator of pigmentation encoded by the gene MITF (micropthalmia-associated transcription factor), and therefore the production of melanine. Mutations in MCR1 can induce different levels of UV light highly protective eumelanine or less-protective pheomelanin [219]. Germline polymorphisms have also been reported in other pigmentation-related genes such as ASIP, OCA2, PAX3 or SOX10 [220].

Various reports cast doubt the hypothesis that melanoma is only caused by UV directed mutations. A good example of this is that primary melanomas can arise in non-sun exposed skin or in internal organs

[215]. The signaling cascade with the highest oncogenic and therapeutic relevance in melanoma is the one controlled by the mitogen-activated protein kinase (MAPK), and most of the mutations found in this gene are not related to direct UV damage [221]. Other common mutations that are not caused by UV-light are $NRAS^{Q61L}$ and $NRAS^{Q61R}$ (detectable in approximately 15-20% of melanomas) [207]. Nevertheless the causal role of UV light in these mutations cannot be completely excluded, since indirect effects like the generation of free radicals resulting from the interaction of UV light with melanin can act as mutagens that indirectly cause genetic mutations [222].

However mutations in single genes are generally not sufficient to result in melanoma development. The most accepted hypothesis is that mutations in these genes constitute the first steps of the transformation of melanocytes, which require further mutations in additional genes that would eventually lead to the development of melanoma [223]. In fact more than 80% of nevi carry BRAF and NRAS mutations [224].

2.7.1.2.2. Molecular classification of melanoma

The recent development of targeted therapies to treat advanced-melanoma with activating driving mutations has been significantly successful [225]. Therefore the scientific community has been trying to generate a framework to be used for personalized therapies in patients with melanoma [226]. In this context, melanomas can be classified into 4 different genomic groups (or subtypes) based on significantly mutated genes (SMGs) that are directly related to the MAPK pathway.

The main genomic subtype is the one characterized by hot-spot mutations in the BRAF gene. Approximately 50% of all advanced melanomas carry a mutation in this gene (with V600E being the most common one) [227]. Most melanomas from this category harbor UV-caused mutations and are more frequent among younger patients [226]. The RAS subtype is the second largest group (with a frequency of approx. 25%), and includes mutations in the 3 genes belonging to this superfamily (N-, K- and H-RAS). Certain hot-spot NRAS mutations are anti-correlated with some specific BRAF mutations [228]. The third most frequent group is the NF1, with close to 15% of all cases). Most of the melanomas with these mutations occur in sun-exposed skin and are more common in older individuals [229]. A fourth group is named as the Triple Wild-Type (TWT) subtype, which is more heterogeneous and is characterized by a lack of hot-spot BRAF, RAS or NF1 mutations. Most of these melanomas (around 70%) do not harbor UV-caused mutations [226].

2.7.1.3. Melanoma interactions with the microenvironment

During the development of melanoma, cancer cells interact with different cellular components within the tumor microenvironment. On one hand there are interactions between melanoma cells and different components from the stroma, such as fibroblasts, endothelial cells or infiltrating immune cells [230]; and on the other hand tumor cells interact with tumor cells from different sub-compartments within the tumor, that are characterized by the differences in the access to oxygen and nutrients [231].

Interactions between tumor cells and these cellular components can occur at different molecular levels, mainly via cell-cell contact or through the secretion of cytokines and growth factors [232]. These molecules can remodel the microenvironment by affecting tumor progression, growth and metastasis, as well as other processes such as angiogenesis or tumor cell apoptosis [233]. Differential secretion of certain chemokines can attract specific immune cell subsets, which in turn secrete more molecular signals that affect the final outcome of melanoma tumors [234].

2.7.1.3.1. Tumor infiltrating leucocytes (TILs)

Advanced melanoma tumors contain considerable amounts of immune cells. The contribution of the immune response to the final outcome of malignant melanoma tumors depends on various factors and the prognostic value of tumor infiltrating leucocytes (TILs) has been a matter of controversy in the field of immunotherapy [235]. It has been suggested that the reason behind the contradicting data on the prognostic impact of TILs in melanoma is that the effect of the immune infiltrate may be more pronounced in advance-stage melanoma compared to early stage melanoma [236].

Several immune cell subsets have been reported to infiltrate melanoma tumors. One of the first immune cell types that can be detected at early stages of tumor growth are dendritic cells (DCs). These antigen presenting cells (APCs) process tumor antigens into peptides that will be later presented trough the mayor histocompatibility complex (MHC) to effector cells [237]. Signals from the tumor microenvironment stimulate Toll-like receptors (TLRs) that trigger migration of the DCS towards regional lymph nodes and present the tumor antigens through the MHC to T-cells (T-cell priming) [238].

Once T-cells have been activated, they can migrate towards the tumor microenvironment. This process is referred to as “homing” of T-cells. This process is regulated through the expression of cell adhesion molecules by the T-cells as well as chemokines secreted by tumor cells. Once they have infiltrated, T-cells recognize melanoma cells and initiate a cytotoxic signal that can kill the tumor cells [239].

The presence of T-cells (CD4+ and CD8+) has been widely demonstrated to be a good prognostic factor in different types of cancer [240,241]. However, their role in melanoma is not completely clear. While some studies have found a positive correlation between the presence of CD4+ and CD8+ T-cells within the tumor and good tumor prognosis, others studies found no association between the overall infiltrating CD3+ T-cells and better melanoma outcome [236]. It has been suggested that these differences may be originated by methodological differences on the analysis of the TILS. For instance, some groups evaluated the infiltrate of the whole tumor [242], while others restricted the analysis of TILs exclusively to specific tumoral regions [243]. The state of activation of the T-cells has been another source of controversy, since not all the studies have reported the proportion of activated T-cells found in the samples. T-cells found within the tumor are frequently defective and show depressed proliferation capability or hampered cytotoxic activity [244]. Therefore it is recommended to characterize the activation state of T-cells in addition to a quantitative determination. In general, high peritumoral density of activated T-cells is associated with a good prognosis [245].

Among CD4+ T-cells, FOXP3+ T-regulatory cells (T-regs) can also be found within melanoma tumors and are implicated in the inhibition of antitumor immune response [246]. Nevertheless the presence of this immune cell type has also been a matter of controversy. While some studies reported a correlation between the amount of T-regs within the tumor and higher survival [247], most of the reports have found no association between the density of this immune cell subset and melanoma outcome [248].

The density of B-cells within melanoma tumors is relatively low and their contribution to melanoma outcome has been a matter of debate as well. On one hand B-cells can promote tumor growth through the secretion of certain antibodies and inhibitory factors, and on the other hand they can work as effective APCs activating effector T-cells [236]. Discrepancies regarding the pro- or anti-tumorigenic role of B-cells have been related to the high diversity of functions that these cells carry [249]. Despite these controversies, studies that analyzed the density of B-cells alongside with activated T-cells have been more consistent. High B-cell infiltration together with activated T-cells provides higher survival chances [250].

Macrophages can also be found at high numbers within melanoma tumors and play a key role in tumor-associated inflammation. TAMs can serve as APCs can stimulate T-cell responses as well as secrete toxic substances that contribute to antitumor cytotoxic activity [251]. Tumor infiltrating macrophages can also

secrete chemokines that attract additional immune cells towards the tumor site that further enhance antitumor immune responses [141]. Nevertheless, macrophages can also contribute to tumor progression through the stimulation of angiogenesis or enhancing tumor invasion and metastasis [252].

2.7.1.3.2. Immune escape in melanoma

Antitumor immune surveillance cannot always be effective. For instance, high numbers of incompletely matured DCs may lead to tolerance instead of generating a proper antitumor immune response [253]. Since tumor cells have high mutation rates, even in the event of proper T-cell activation, mutations in melanoma cells can lead to the loss of the old and the generation of new antigens that cannot be longer recognized by the T-cells [254].

Tumor progression can occur even in the presence of a strong anti-melanoma immune response. Once tumor growth has started, cancer cells are able to modulate the immune response through several different mechanisms [**Fehler! Textmarke nicht definiert.**]. For instance, even upon proper activation of T-cells, the absence of the proper chemoattractants at the tumor microenvironment can inhibit homing of these leucocytes to the tumor. Melanoma tumors are also able to hamper the immune response by secreting suppressor factors [244].

Another of these mechanisms is to encompass immune-checkpoint pathways that are responsible for self-tolerance. For example, tumor cells can limit T cell effector activity by the expression of programmed death ligands 1 (PDL1) and 2 (PDL2), which are the ligands of the surface receptor programmed cell dead protein 1 (PD-1). In addition, other T-cells, B-cells and NK cells are also affected since they also express PD-1. Another example of how tumor cells can hamper immune response is by the expression of the cytotoxic T lymphocyte protein 4 (CTLA4), which mitigates the magnitude of the initial T-cell activation by dendritic cells within the lymph nodes [255]. Other immunosuppressive mechanisms are the downregulation of tumor-associated antigens or the secretion of inhibitory factors like transforming growth factor-β (TGF-β) or IL-10 [256].

2.7.1.4. Cytokines and chemokines in the tumor microenvironment

Chemokines within the tumor microenvironment can profoundly influence tumor cells as well as other components of the stroma, thus having an important impact on tumor biology. Melanoma cells have

been reported to express several CKRs (including CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, CXCR7, CCR1, CCR2, CCR5, CCR6, CCR7, CCR9 and CCR10, among others) and to produce chemokines (such as CXCL1, CXCL2, CXCL6, CXCL7, CXCL8, CXCL10, CCL2, CCL5 and CCL20) that are able to act in paracrine and autocrine ways [257].

Together with growth factors, proteases and other cytokines, tumor cells can use chemokines to generate chronic inflammatory conditions that lead to the generation of an immunosuppressive environment [258]. Through this type of mechanism melanoma cells can influence fibroblast and macrophages to acquire pro-tumorigenic functions [259]. Additionally, while the tumor is growing, mobilization of myeloid-derived suppressor cells (MDSCs) and T-regs towards the tumor site can be enhanced by tumor-derived chemokines such as CXCL5 [260]. Melanoma derived chemokines can further induce T-regulatory cells to express immunosuppressive cytokines such as IL-10 and TGF-β that can hamper the function of anti-tumor T-cells [261].

The precise role of a specific chemokine CKR pair in the immune control of melanoma can be difficult to elucidate when it is not studied in a greater context. For instance, CXCL10 stimulates Th1 cells, which release interferon-ɣ (IFN-ɣ) that contributes to melanoma regression [262] while CXCL4 activates Th2 cells, which release certain interleukins (like IL-4 or IL-13) that facilitate tumor progression [263]. Both CXCL10 and CXCL4 signal through the same CKR, named CXCR3 [264]. On the other hand, it has been reported that inhibition of CCL27 contributes to immune evasion and tumor progression [265]. However, another study found that when B16 melanoma cells overexpress its receptor CCR10 they are more resistant and are more protected against Fas-mediated apoptosis [266].

Melanoma derived CXCL12 has also been observed to play dual roles in anti-tumoral immune responses. On one hand CXCL12 has been observed to reduce the immune response by attracting CXCR4+ DC precursors that alter antigen-presenting mechanisms of other DCs [267]. However, it has been reported that CXCL12-expressing B16 cells induce specific memory responses to the tumor [268] and that CXCL12 expression attracts CXCR4+ T-cells which facilitate tumor regression [269].

Nevertheless, there are numerous examples where consistent observations have allowed clarifying the precise role of these compounds. One of these examples is CCL21, one of the ligands for CCR7. Under normal conditions, CCL21 is produced in the lymph node and is able to attract both CCR7+ APCs and

CCR7+ melanoma cells. It has been observed that melanoma tumor cells are able to express CCL21 and CCL19 (also a ligand for CCR7) creating an autocrine effect referred to as “autologous chemotaxis”, which induces tumor cell migration towards the lymph node [270]. In another study, authors reported that expression of CCL21 by B16 melanoma cells caused larger tumors despite attracting greater amounts of leukocytes and APCs towards the tumor site. These observations could not be reproduced using immune-deficient mice, pointing to the immune system as the responsible for this outcome. Melanoma tumors expressing high amounts of CCL21 attract more T-reg cells that contribute to immune tolerance and therefore progression of the tumor, while low expression of this chemokine within the tumors results in increased migration of cytotoxic T-cells that contribute to tumor regression [271].

Chemokines can influence tumor growth by the modulation of other processes such as angiogenesis. A series of melanoma-produced chemokines can bind to CKRs expressed by endothelial cells triggering angiogenic processes. In general, ELR+ CXC chemokines (like CXCL1-3, CXCL5 or CXCL8) are more angiogenic while those chemokines lacking the ELR motif (like CXCL4, CXCL9 or CXCL10) promote angiostasis [20]. Knocking down angiogenic chemokines can lead to a reduction in tumor growth, migration and invasion [272]. CXCL6 and CXCL8 have been observed to promote angiogenesis through the recruitment of neutrophils that trigger the degradation of the matrix [273]. On the other hand, upregulation of angiostatic chemokines like CXCL4 can hamper the vascularization of growing tumors slowing down tumor growth [274]. Therefore, an imbalance between the proportion of angiogenic and angiostatic chemokines can determine the outcome of a melanoma tumor.

Chemokines and their receptors may also play a key role in metastatic spread of melanoma [275]. Melanoma cells are able to overexpress CXCR4, CCR7 and CCR10. Since CXCL12, the ligand for CXCR4, is mostly expressed in tissues like the lymph nodes, liver, lung and bone marrow, it has been suggested that CXCR4 be a factor of selective spread of metastatic cells towards this anatomical locations [276]. As a matter of fact, CXCR4 might contribute to tumor spread towards these tissues [277] and tumor cell subpopulations of various types of cancer express this receptor [278]. In another study, researchers observed a 10-fold greater capacity for metastasis of B16 murine melanoma cells to the lung when they were transfected with CXCR4 [198]. Additionally, CXCL12 has been reported to promote tumor growth by angiogenic properties [279].

In the case of CCR7, its ligands (CCL19 and CCL21) are mostly expressed in lymph nodes and lymph vessels, and interactions with their receptor have been associated in the development of lymph-node

metastasis in mice [280]. On the other hand, CCR10 is suspected to the pathogenesis of skin metastases, since its ligand (CCL27) is mainly expressed in the basal cells of the epidermis [276].

In addition to CKR expression, the secretion of chemokines by melanoma cells has been suggested to be part of a coordinated mechanism that generates circuits or autocrine loops in the tumor cells themselves. For example, some chemokine ligand/receptor pairs such as CXCL12/CXCR4 and CCL21/CCR7 can act in an autocrine way stimulating the proliferative and invasive capacity as well as the metastatic potential of melanoma tumor cells [281,282].

Finally, chemokines have also been linked to tumor initiation and drug resistance since they control key signaling pathways of cancer stem cells (CSCs). Although under normal conditions CSCs remain quiescent, they can start to proliferate upon certain changes at the microenvironment [283]. It has been reported that CXCL12 is able to trigger the migration of dormant CSCs into the blood stream [284]. Some CKRs have been linked to CSC chemotaxis such as CXCR1 [285] or CXCR4 [286].

2.7.2. Novel therapeutic options for advanced melanoma

In recent years the research of mutations responsible of tumorigenesis and immune-evasion mechanisms from melanoma tumors has contributed to the generation of effective treatments that have improved the overall survival (OS) in late stage melanoma patients [287].

One of the most success full therapies is the use of immune-checkpoint inhibitors. Among the current first-line immunotherapies, drugs such as Ipilimumab or Nivolumab are being widely used and can improve the OS of advanced melanoma patients [287]. These compounds are monoclonal antibodies that block CTLA-4 (Ipilimumab) or PD-1 (Nivolumab), altering T-cell activation and effector phase respectively [288,289]. Current studies have focus on the beneficial effects of therapies that combine two or more of these components in order to improve the efficiency of these treatments [290].

Another current strategy for the treatment of advanced melanoma is the use of targeted therapies. These consist in the blockade of signaling pathways from mutated genes such as BRAF or MAK. One example is the use of tyrosine kinase inhibitors (TKIs) of the BRAF kinase. Since $BRAF^{V600}$ mutations are the most common mutations in melanoma (approx. 50% of all cases), the use of BRAF inhibitors in these

cases has been shown to improve the outcome in patients with melanomas harboring this mutation [291].

While these novel therapeutic strategies have proven to significantly improve the OS and the general prognosis of patients with advanced melanoma, a considerable proportion of patients does not show a durable response to therapy and most of the compounds bring additional side effects like toxicity [292].

Nevertheless, of new combinations of immunotherapeutic agents and targeted therapies are currently under development and tested in numerous clinical trials worldwide. Alongside with the discovery of new check-point inhibitors, the exploration of new targeted therapies for a more effective individual treatment will likely increase the effectivity of the current treatment strategies [290].

2.8. CCR6/CCL20 axis in melanoma

The role of the CCR6/CCL20 axis in melanoma is still not completely understood and a matter of debate. The fact that this chemokine/CKR pair may control both the effectors (i.e. CCR6+ CD4+ T-cells, Th17 cells) and the regulators (i.e. immature CCR6+ DCs, CCR6+ T-regs) of an anti-melanoma immune response has contributed to this controversy [**Fehler! Textmarke nicht definiert.**]. Therefore targeting this axis in melanoma requires careful consideration and precise experimentation, and could only be of use under specific circumstances [**Fehler! Textmarke nicht definiert.**]. In fact, in order to obtain an effective therapeutic effect, inhibition of additional trafficking receptors could be necessary. For instance, it has been observed that the CCR1/CCL9 axis is capable of compensating the absence of CCR6/CCL20 in a series of conditions, such as the recruitment of DCs to Peyer's Patches [140].

2.8.1. CCR6+ Immune subsets in melanoma

A few studies have focused on the role of CCR6+ immune cells within melanoma tumors, mainly on DCs. It has been reported that melanoma patients show increased numbers of plasmacytoid DCs (PDCs) and myeloid DCs (MDCs) in the circulation. In this study, it was observed that PDCs expressed high levels of CCR6 and could migrate towards CCL20 expressing melanoma tumors, leading to an enhanced anti-tumor immune response [293]. Other CCR6+ immune cell subsets have anti-tumor properties as well. For instance, Th17-polarized CD4+ T-cells are able to mediate the eradication of advanced melanoma in mice [294].

However, other CCR6 expressing leucocytes may contribute to the generation a pro-tumorigenic environment. Accumulation of highly immunosuppressive leucocytes such as T-regs and memory T-cells in the tumor microenvironment contributes to tumor progression and enhanced metastasis in the Ret mouse model for melanoma [295]. In humans, CCR6+ is a marker for a subset of effector-memory T-cells that can hamper strong immune responses [296]. Nevertheless, the contribution of immunosuppressive CCR6+ leucocytes to melanoma tumor progression requires further research.

2.8.2. CCL20 in the melanoma microenvironment

It has been reported that some melanoma cells are able to produce and secrete CCL20 in the tumor microenvironment [297] and that the expression of this chemokine is highly upregulated in 3D cultures when compared with monolayer growing in vitro models [298]. Additionally, other cells within the

stroma such as tumor infiltrating macrophages can express CCL20. It has been shown that macrophage produced CCL20 can enhance the metastatic potential of melanoma tumors [141].

However the precise role of tumor CCL20 in melanoma progression has not been yet completely elucidated. A recent study using CCL20 DNA-vaccines in a B16 model for melanoma found that anti-tumor immune response was enhanced when CCL20 was tagged with the melanoma antigen gp100. Coupling of CCL20 to Gp100 facilitated the activation of APCs like CCR6+ dendritic cells that later could activate effector T-cells faster than CCL20 alone [299].

2.8.3. CCR6/CCL20 axis in melanoma progression

In addition to CCL20, melanoma cells are also able to express CCR6 [141]. Recently a series of research groups have focused their efforts in unraveling the contribution of the CCR6/CCL20 axis to melanoma progression with ambiguous outcomes. Comparing intradermal melanoma tumor growth between Wt and $CCR6^{-/-}$ mice, no differences were found in terms of tumor growth or immune cell interactions. In this same study, researchers found no differences in the relative amounts of T-regs, Th17 and CD8+ effector T-cells within the tumor tissue and the regional lymph nodes of B16-F10 injected Wt and $CCR6^{-/-}$ mice [300].

On the other hand, loss of CCR6 in T-cells was observed to correlate with secondary lymphoid organ metastases. A decrease of CCR6 expression was also associated with increased lung metastases and a drop in CCR6+ T-cells has been highlighted as a hallmark of metastatic dissemination into the lymph nodes [301].

Thus, decrypting the contribution of CCR6 and CCL20 to melanoma growth and progression requires a comprehensive insight into the role of the different CCR6+ immune cell subsets, the specific contribution of the various CCL20 expressing cells and the effect of CCL20/CCR6 expression by melanoma cells.

3. AIM OF THE THESIS

The general objective of this thesis was to evaluate the distinct functional contribution of CCR6 interactions with CCL20 in modulating anti-melanoma immune response as well as to study the contribution of this chemokine axis to melanoma tumor growth and progression.

To do so, we studied the kinetics and distribution of CCR6-guided immune cell subsets to the tumor site and draining lymph nodes (TDLN), as well as the effect of CCL20 on tumor initiation and progression in a CCR6 deficient environment. Furthermore, stimuli by which the expression of CCR6 ligands may be modulated at tumor site and/or the skin-draining LN and their potential relevance for anti-tumoral immune responses were part of our study.

3.1. Aims and experimental approach

1) In vitro characterization of CCL20 expression by human and murine melanoma cell lines under homeostatic conditions and in response to inflammatory stimuli
2) In vivo characterization of CCR6/CCL20 dependent immune responses in a transplantable B16 melanoma model using Wild-type (Wt, C57BL/6J) and congenic CCR6 knock-out ($CCR6^{-/-}$) mice by the analysis of
    a. Tumor growth kinetics
    b. Tumor microenvironment (TME)
        i) Cellular components: Tumor infiltrating Leucocytes (TILs)
        ii) Soluble components: cytokine/chemokine composition

3) Study of potential autocrine effects of CCR6/CCL20 interactions in melanoma

Materials & Methods

4. MATERIALS & METHODS

4.1. Buffers and Solutions

Complete Medium: Dubebcos Modified Eagle Medium without L-Glutamine (DMEM, Gibco) with

- 10% Fetal Bovine Serum (FBS, Sigma-Aldrich)
- 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma-Aldrich)
- 1% Penicillin/Streptomycin (Sigma-Aldrich)
- 1% L-Glutamine (Sigma-Aldrich)
- 1% MEM non-essential amino-acids (Ge-Healthcare)

Digest Solution: Hank's Balanced Salt Solution (HBSS, Biochrom-MERCK) with 1 µg/ml Collagenase IV (Sigma Aldrich) and 25 µg/ml DNAse I (Sigma Aldrich)

Erythrocyte Lysis Buffer: Ammonium-Chloride-Potassium (ACK) lysis buffer pH 7.3. dH_2O with:

- 155 mM NaH_4Cl
- 10 mM $KHCO_3$
- 130 mM EDTA (Disodium ethylenediaminetetraacetate dehydrate)

FACS Buffer (for FCM): 3% FBS in PBS

Freezing Medium

- 50% Complete Medium
- 30% FBS (Sigma-Aldrich)
- 20% Dimethyl sulfoxide (DMSO, Sigma-Aldrich)

Percoll gradient solutions: Percoll™ (GE-Healthcare)

- **40%:** 60% DMEM (with 1% L-Glutamine), 36% Percoll, 4% 10X PBS
- **80%:** 20% DMEM (with 1% L-Glutamine), 72% Percoll, 8% 10X PBS

Phosphate Buffered Saline (PBS): 1% PBS Dulbecco powder (Biochrom-MERCK) in dH_2O pH 7.4

Wash Buffer (for FCM): HBSS with 0,1% Bovine Serum Albumin (BSA, Sigma-Aldrich)

4.2. Cell lines and cell culture

Murine Ret and Human C32 and A375 melanoma cells were gently provided by Daniel Roth from the German Center for Cancer Research (DKFZ) in Heidelberg, Germany. Murine B16/F1 melanoma cells (referred to as B16) transduced with cDNA encoding firefly (*Photinus puralis*) luciferase (B16-luc) in the PMSCV retroviral vector (Clontech) [302], were the kind gift from Dr. Sam Hwang (Medical College of Wisconsin)

Human (C32, A375) and murine (B16, Ret) melanoma cells were cultivated in Complete Medium, and incubated at 37 º C in a 5% CO2 environment. $4x10^5$ cells were cultured in T75 flasks and passed after detection of 70-80% of visual confluence twice a week, washing them previously with room temperature (RT) PBS and detaching with trypsin (GE Healthcare). 10µg/ml puromycin (Pur) was added for PMSCV-vector (Luciferase) selection to culture medium of B16 melanoma cells after each passage.

4.3. CCL20 detection by Enzyme-Linked Immunoabsorbent Assay (ELISA)

$5x10^4$ human (C32, A375) and murine (B16, Ret) melanoma cells were seeded in 24-well plates (Falcon) and stimulated with increasing concentrations of human or murine TNF-α (50, 100 and 200 ng/ml) and human or murine IL-1α (10, 20 and 40 ng/ml) alone or combined (50ng/ml TNF-α + 10 ng/ml IL-1α) for 24, 48 and 72h. After these time-points supernatants were collected and centrifuged to eliminate cells in suspension. CCL20 was then measured by means of ELISA following the guidelines from human and murine CCL20 detection kits (R&D Systems) and analyzed with a Multiskan Ex ELISA plate reader (Thermo-Fischer). All incubation times were performed at 37 º C in a 5% CO^2 environment.

4.4. Generation of retrovirally transduced melanoma cells

B16-luc-PMSCV cells were cultured for up to 15 passages until stimulation with TNF-α was not capable to induce CCL20 expression anymore. This CCL20-non-expressing cell line (B16 parental), alongside with two pLNCX2 vectors (one carrying a mCCL20 gene) from (Clontech) were provided to Dr. Rainer Will from the Stable Isogenic Cell line Service at the German Center for Cancer Research (DKFZ, Heidelberg) in order to generate two retrovirally transduced B16 cell lines. Using “Sir” cell line technology Dr. Will provided us with two new cell lines, B16-Control (B16 cells transduced with the empty pLNCX2 vector) and B16-CCL20 (B16 cells transduced with the CCL20 bearing pLNCX2 vector). Both pLNCX2 vectors carried a selection gene resistant to G418-sulphate. Therefore in addition to 10µg/ml Pur, 400µg/ml of G418-sulphate was added to the medium of B16-Control and B16-CCL20 cells after each passage.

To measure CCL20 homeostatic expression, $5x10^4$ cells of each newly generated cell line were seeded into 24-well plates and supernatants were collected and analyzed by means of ELISA after 24, 48 or 72 h.

In order to confirm in vivo CCL20 expression, $4x10^5$ cells of B16-Control and B16-CCL20 cell lines were injected subcutaneously in the right flank of Wt and $CCR6^{-/-}$ that had been shaved locally 24 hours before. 15 days after inoculation, animals were sacrificed, tumors were dissected and blood samples were taken.

Tumor digestion was performed by manual fragmentation followed by 1,5 hours of incubation with Digest Solution at 37ºC and filtered through a 70 µm strainer (Falcon). The resulting suspension was centrifuged at 200g and CCL20 was analyzed from the remaining supernatant by means of ELISA in 96-well plates (Falcon). Blood was extracted by cardiac puncture, centrifuged at 7000 g and CCL20 was measured from the serum by means of ELISA in 96-well plates (Falcon)

4.5. XTT proliferation assay

$5x10^4$ B16-Control and B16-CCL20 cells were seeded in 96-well plates (Falcon) and cultured for 24 and 48h with different concentrations of mCCL20 (PeproTech) as indicated. XTT/PMS solution was prepared according to the XTT kit protocol of the manufacturer (Advance Targeting Systems). 50 ul of XTT/PMS were added to each well and plates were incubated for 2h at 37ºC. Absorbance was measured at 450 nm with a Multiskan Ex ELISA plate reader (Thermo-Fischer).

4.6. Wound Healing Assay

$1x10^5$ B16-Control and B16-CCL20 cells were seeded in 12-well plates (Falcon) and cultured at 37 º C in a 5% CO^2 environment to near confluence. After 6h of culture with complete medium without FBS, a linear wound was created gently by scraping a 100 ul sterile pipette tip through the complete diameter of the cell monolayer. Subsequently the well was washed twice with PBS to remove detached cells. The monolayers were further incubated at 37 º C in a 5% CO2 environment with complete medium without FBS and photographs (x20 magnification) of the wounds were taken at 0, 4, 8 and 24h after wounding. Percentage of wound-closure was calculated using ImageJ Software.

4.7. Flow-cytometry (FCM) of melanoma cells

$5x10^5$ B16-Control and B16-CCL20 melanoma cells were seeded in 24-well plates (Falcon) with complete medium. After 24h cells were washed with RT PBS and detached with trypsin (GE Healtcare). Cells were suspended in FACS buffer and incubated at 4 º C with anti-mouse CD16/32 (eBioscience, Cl 93) at a 1:100 concentration for 10 mins in order to block the Fc receptors. Cells were then washed and incubated for 30 min with APC-labeled anti-CCR6 (Biolegend, Cl 29-2L17) and APC-labeled Armenian Hamster IgG Isotype Ctrl Antibody (Biolegend, HTK888) at 37ºC to enhance CKR turnover. Cells were then washed and 7AAD (eBiosciences) was added as a death cell marker before measuring CCR6 signal by means of FCM. All samples were analyzed using a Galios 10 Colors, 3 Lasers (B15-R1-V2 Configuration) flow cytometer from Beckman Coulter.

4.8. Mice strains and stabling

Wild-type (Wt) C57BL/6J mice were purchased from Charles River Laboratories (Germany). Congenic C57BL/$CCR6^{-/-}$ ($CCR6^{-/-}$) mice, generated as previously described [162], were kindly provided by Joshua M. Farber (Laboratory of Molecular Immunology, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, Maryland, USA) and redeliver by embryonic transfer at the Interfacultary Biomedical Faculty (IBF) of Heidelberg University (Heidelberg, Germany). All mice were maintained in specific pathogen-free conditions at the IBF of Heidelberg University and used for experiments at 6-8 weeks of age. Animal care and procedures followed the regulations, specifications and obligations given by the German protection of Animals Act. All experimental procedures were approved by the institutional review boards and performed in accordance with the institutional guidelines for animal welfare.

4.9. Tumor growth experiments

The right flank of Wt and $CCR6^{-/-}$ mice was shaved 24 hours before tumor cell inoculation. $4x10^5$ B16, B16-Control or B16-CCL20 melanoma cells were subcutaneously injected in the right flank. Tumor growth was monitored every day after tumor inoculation using a caliper. Tumor area (TA) was calculated with the formula: TA = (major radius)*(minor radius)*π. Mice were routinely euthanized using CO_2 15 days after tumor inoculation followed by dissection and tumor and lymph node extraction. In accordance with the institutional guidelines for animal welfare, mice were euthanized earlier in case that the diameter of the tumor reached >20 mm or the animal showed signs of suffering or discomfort.

For the evaluation of the effect of recombinant mCCL20 (rCCL20) on B16-Control tumor progression, local injections of rCCL20 (PreproTech) were administrated every 2 days at the site of tumor injection from day 2 after inoculation. The amount of injected rCCL20 was modulated according to tumor size: 50 ng for tumors up to 75 mm^2, 100 ng for tumors between 75-125 mm^2 and 200ng for tumors bigger than 125 mm^2.

For the evaluation of the effect of anti-mCCL20 (aCCL20) antibodies on B16-CCL20 tumor progression, local injections of aCCL20 (Biotechne, Cl 114908) were administrated every 2 days at the site of tumor injection from day 2 after inoculation. The amount of injected aCCL20 was modulated according to tumor size: 100 ng for tumors up to 75 mm^2, 200 ng for tumors between 75-125 mm^2 and 400ng for tumors bigger than 125 mm^2

4.10. Cell isolation

For the isolation of TILs, tumors were dissected and DLN were separated before digestion. Digestion was performed by manual fragmentation followed by 1,5 hours of incubation with Digest Solution at 37ºC. The resulting suspension was centrifuged at 50g to remove undigested skin remnants, and the collected supernatant was then centrifuged at 200g. After this centrifugation the supernatant was collected for a posterior analysis by Legendplex (see **4.12**) and the pellet was washed twice with Wash Buffer and centrifuged again at 200 g. Pellets were then incubated for 3 minutes with Erythrocyte Lysis Buffer at RT. After another washing step, pellets were resuspended in 40% Percoll solution and gently placed in falcon tubes containing 80% Percoll solution. Falcon tubes were centrifuged at 325g for 23 min at RT, with low ascending and descending rates. Interface between 40% and 80% Percoll layers was collected and filtered through a 40µm cell strainer. Filtrated suspension was washed and centrifuged at 425g for 10 min at RT. The resulting cell pellet was resuspended in 100 µl FACS buffer and analyzed by means of FCM.

For the isolation of DLN immune cells, lymph nodes were manually disrupted over a 40µm cell strainer (Falcon) and the collected suspension was washed twice with FACS buffer before proceeding to FCM analysis.

4.11. Flow cytometry of TILS and DLN immune cells

Each sample of isolated TILs and DLN cell suspensions was split in half to be analyzed with two different panels of antibodies: Panel A and Panel B. All samples were analyzed using a Galios 10 Colors, 3 Lasers (B15-R1-V2 Configuration) flow cytometer from Beckman Coulter. For the Panel A, cells were suspended in FACS buffer and incubated with anti-mouse CD16/32 (eBioscience, Cl 93) for 10 mins in order to block the Fc receptors. Cells were then washed and incubated at 4 º C for 30 mins with **Panel A antibody combination**. After an additional washing step, 7AAD was added and cells were analyzed by means of FCM. All incubations were performed at 4ºC and all washing steps were done with cold FACS buffer.

Panel A antibody combination

Antigen	Fluorophore	Clone	Company	Working Concentration
CD45R	FITC	RA3-6B2	eBioscience	1:200
CD8	PE	53-6.7	BD Pharmigen	1:200
CCR6	APC	29-2L17	Biolegend	1:100
CD5	APC-alexa780	53-7.3	eBioscience	1:200
CD3	Pacific Blue	145-2C11	Biolegend	1:200
CD19	BlueViolet510	103	BD Pharmigen	1:200
Dead Cell Marker	7AAD	-	Invitrogen	1:100

For Panel B cells were suspended in FACS buffer and incubated with Zombie Violet at RT for 10 minutes. After a washing step, Fc block and **Panel B antibody combination** staining was performed as described for panel A, without adding PE anti-FoxP3. After antibody staining, cells were resuspended in fixation buffer from the FoxP3 fixation/staining kit from (Invitrogen) and FoxP3 staining was performed according to the guidelines from this kit. Once this was performed, fixed cells were resuspended in FoxP3 fixation/staining kit wash buffer and analyzed by means of FCM.

Panel B antibody combination

Antigen	Fluorophore	Clone	Company	Working Concentration
CD45R	FITC	RA3-6B2	eBioscience	1:200
Foxp3	PE	FJK-165	Invitrogen	1:100
CCR6	APC	29-2L17	Biolegend	1:100
CD11c	APC-alexa780	N418	eBiosciences	1:200
CD3	Pacific Blue	145-2C11	Biolegend	1:200
CD4	BlueViolet510	RM4-5	Biolegend	1:200
Dead Cell Marker	Zombie Violet	-	Biolegend	1:1000

4.12. LEGENDPLEX

Cytokine composition from cultured melanoma cells and fresh supernatants from dissected tumors were analyzed by means of LEGENPLEX™ technology (BioLEGEND). Samples were incubated with antibody-tagged beads in 96-well plates and analyzed by means of FCM following the guidelines from the product protocol. Data was analyzed by using the LEGENDPLEX™ Software (v7.1). A customized bead combination was designed to detect the concentration of IL-1α, IL-6, IL-9, IL-10, IL-12p70, IL-17A, IL-22, IL-23, IL-27, INF-ɣ, TNF-α, TSLP and CCL2.

4.13. STATISTICAL ANALYSIS

In vitro results are represented as the mean ± standard deviation (SD) of the indicated number of independent experiments and significance was calculated using Two-tailed Student T-test. In vivo results are represented as the mean ± standard error of the mean (SEM) of the indicated number of independent experiments and significance was calculated using Two way ANOVA, Bonferroni post-test. All statistical calculations were performed with GraphPad Prism software. P-value < 0.05 was considered as statistically significant.

Results

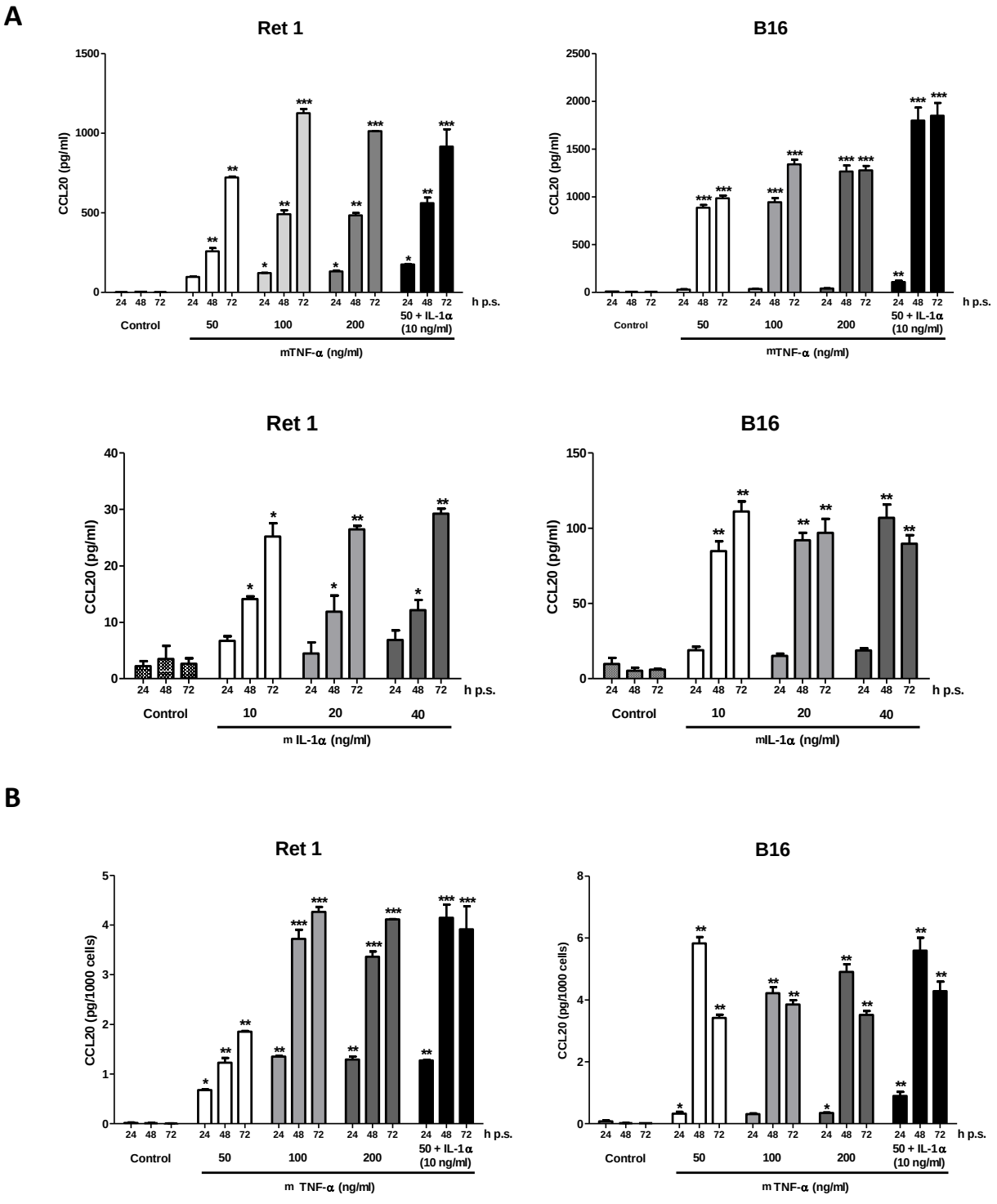
5. RESULTS

5.1. CHARACTERIZATION OF CCL20 EXPRESSION BY MELANOMA CELLS

5.1.1. mCCL20 expression by murine melanoma cells *in vitro* .

In order to test the capability of murine melanoma cell lines to produce CCL20, we cultured B16 and Ret mouse melanoma cells for 24, 48 and 72h in complete growth medium in presence or absence of pro-inflammatory cytokines mTNF-α and mIL-1α, which have been described to upregulate CCL20 in certain cell types [297] and are present in the tumor microenvironment [303,304]. Cell culture supernatant was collected after each time point and mCCL20 levels were measured by means of ELISA.

Under homeostatic conditions mCCL20 production was weak or non-existent. However, it could be triggered upon stimulation with pro-inflammatory cytokines. Stimulation with increasing concentrations of either mTNF-α or mIL-1α induced a significant increase of mCCL20 expression after 48h. In the case of stimulation with mTNF-α, significant increases of mCCL20 could be observed after 24h. Overall mTNFα was able to induce significantly higher levels of mCCL20 than mIL-1α (**Fig. 1**).


A
Ret 1
B16
CCL20 (pg/ml)
h p.s.
Control
50
100
200
50 + IL-1α (10 ng/ml)
mTNF-α (ng/ml)
Ret 1
B16
CCL20 (pg/ml)
h p.s.
Control
10
20
40
m IL-1α (ng/ml)
B
Ret 1
B16
CCL20 (pg/1000 cells)
h p.s.
Control
50
100
200
50 + IL-1α (10 ng/ml)
m TNF-α (ng/ml)

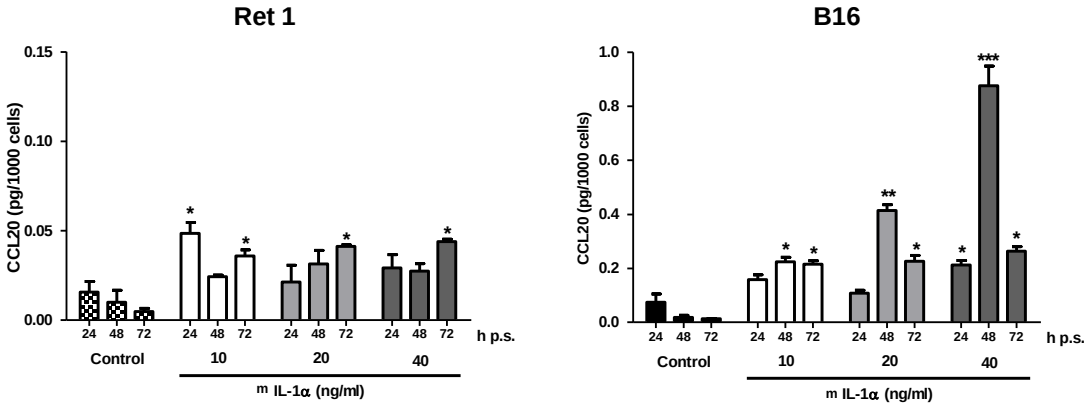


Figure 1. Murine melanoma cell lines express mCCL20 in vitro under stimulation with mTNF-α and mIL-1α. 5x10^4 Ret1 and B16 melanoma cells were seeded in 24 well plates, stimulated with increasing concentrations of TNF-α or co-stimulated with mTNF-α and mIL-1α. Medium was removed after 24, 48 and 72 hours respectively and mCCL20 was measured by means of ELISA. (*h.p.s.*, hours post stimulation) **A.** Total mCCL20 detected in the supernatants expressed in pg/ml. **B.** pg of mCCL20 produced per 1000 melanoma cells. Bar graphs represent mean ±SD from three independent experiments. Unpaired T-test *(* P<0.05, ** P<0.005, *** P<0.0005*)

5.1.2. hCCL20 expression by human melanoma cells in vitro.

We performed similar experiments described in the previous section using two human melanoma cell lines (C32 and A375) in order to verify hCCL20 production capability (**Fig. 2**).

Our results paralleled with those obtained using murine melanoma cell lines. Both human melanoma cell lines showed weak or non-detectable hCCL20 expression under homeostatic conditions, but expression could be triggered after being cultured in presence of hTNF-α or hIL-1α. However, especially in the case of C32 melanoma cells, the magnitude of the responses to these pro-inflammatory cytokines were significantly weaker compared to B16 or Ret murine melanoma cell lines. Additionally, co-stimulation with hTNF-α and hIL-1α resulted in a tremendous increase on hCCL20 production after 72h in A375 cells, while in C32 cells, addition of hTNF-α and hIL-1α caused the same effect as hTNF-α alone.

Altogether, these results showed that the constitutive expression of CCL20 in both murine and human melanoma is weak or non-detectable *in vitro* . However CCL20 expression can be easily triggered by pro-inflammatory cytokines. Additionally, the magnitude of CCL20 induction upon stimulation varies between different melanoma cell lines.

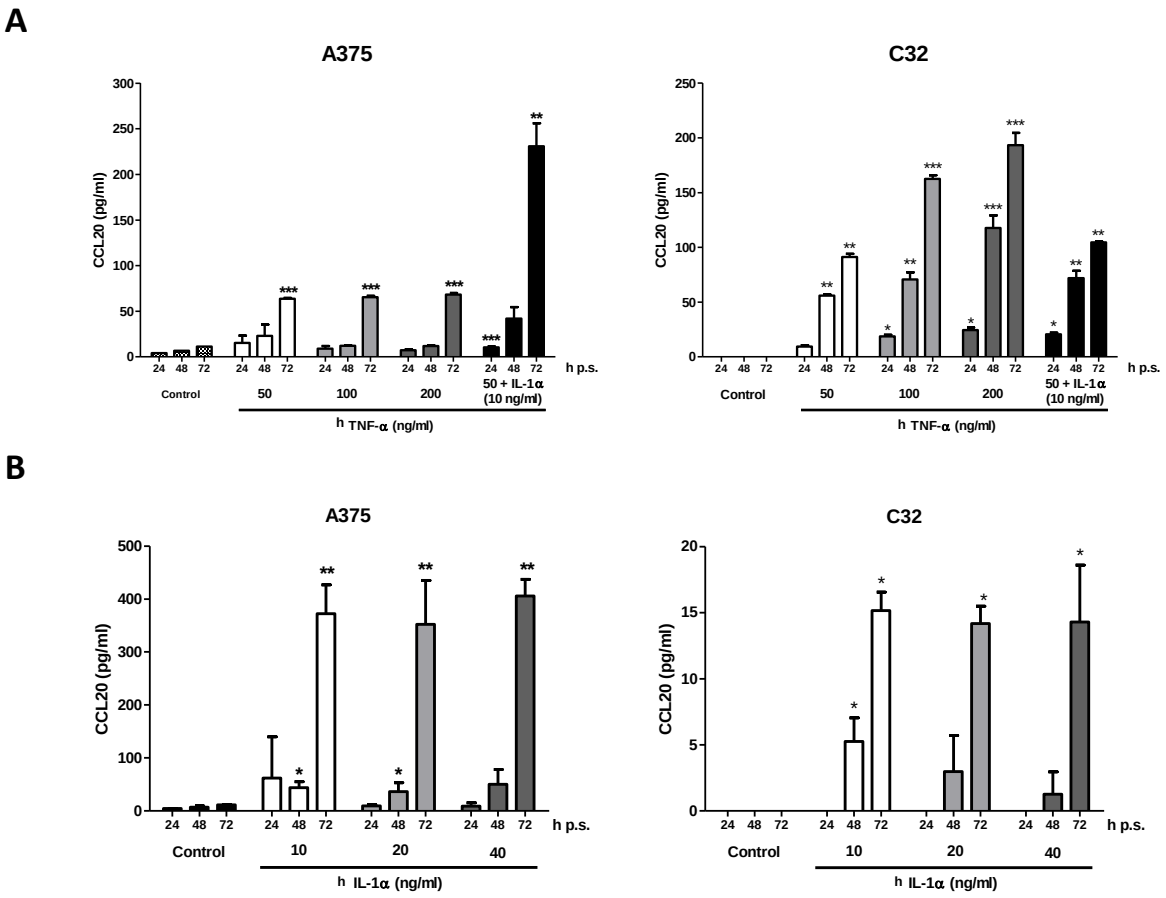


Figure 2. Human melanoma cell lines express hCCL20 in vitro under stimulation with hTNF-α and hIL-1α. 5x10^4 A375 and C32 human melanoma cells were seeded in 24 well plates and stimulated with increasing concentrations of hTNF-α or co-stimulated with hTNF-α and hIL-1α. Medium was removed after 24, 48 and 72 hours respectively and CCL20 was measured by means of ELISA (*h.p.s.*, hours post stimulation). **A.** Total hCCL20 detected in the supernatants expressed in pg/ml. **B.** pg of hCCL20 produced per 1000 melanoma cells. Bar graphs represent mean ±SD from three independent experiments. Unpaired T-test *(* P<0.05, ** P<0.005, *** P<0.0005*)

5.1.3. CCL20 expression decreases over in vitro passages in B16 melanoma cells

Our collaborator Prof. Fran Balkwill from Barts Cancer Institute in London communicated that she commonly observes a loss of chemokine expression after 10 or more passages in several cell lines cultured in vitro, and recommended to monitor carefully any loss of expression. Subsequent research of literature led to find similar phenomena in some publications [305,306]. Indeed CCL20 production progressively decreased after in vitro passages **(Fig. 3)**. We observed that this "passage effect" was taking place in other melanoma cell lines as well**.**

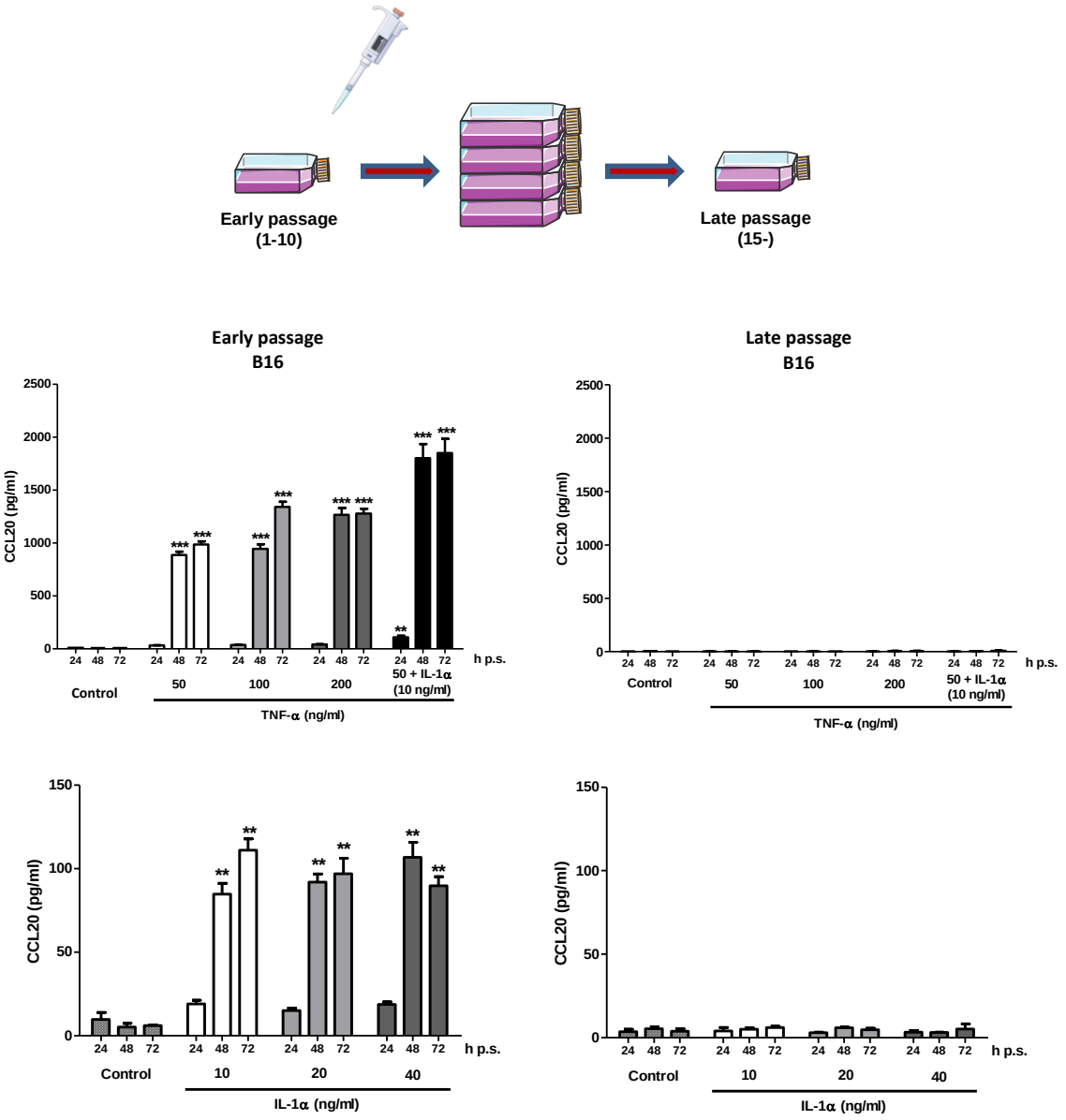


Figure 3. mCCL20 expression capability in melanoma cells is lost after several passages in vitro . B16-PMSCV cells were kept in culture for several passages. In the figure, mCCL20 expression capability was tested over several passages. Figure shows representative data from early (Left, passage 8) and late (right, passage 25) *in vitro* passages. $5x10^4$ cells were seeded in 24 well plates and stimulated with increasing concentrations of mTNF-α or co-stimulated with mTNF-α and mIL-1α. Medium was removed after 24, 48 and 72 hours respectively and mCCL20 amounts were measured by means of ELISA. (*h.p.s.*, hours post stimulation) Bar graphs represent mean ±SD from three independent experiments. Unpaired T-test *(* P<0.05, ** P<0.005, *** P<0.0005*)

5.2. B16 melanoma growth in C57BL/6J (Wild-type, Wt) and congenic CCR6 knock-out (CCR6$^{-/-}$) mice

Two different mouse strains were used for the in vivo experiments: C57BL/6J "Wild-type" mice and congenic CCR6 knock-out mice (C57BL/6J Background), that we will refer to subsequently as *Wt* and *CCR6$^{-/-}$* mice.

5.2.1. CCL20 levels in serum of Wt and CCR6$^{-/-}$ mice

We took serum samples from Wt and CCR6$^{-/-}$ animals and measured CCL20 levels by means of ELISA. Results showed that presence of CCL20 protein in the serum was minimal under homeostatic conditions and did not significantly differ between Wt and CCR6$^{-/-}$ mice **(Fig. 4).**

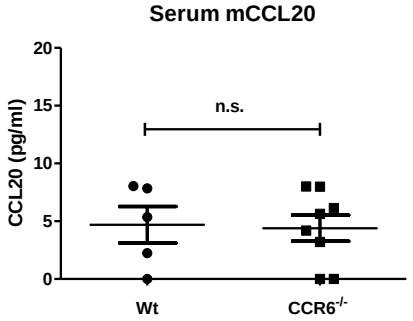


Figure 4. **Wt and CCR6$^{-/-}$ animals have low concentrations of mCCL20 in serum.** Blood was taken from healthy Wt and CCR6$^{-/-}$ animals and mCCL20 levels of the serum were measured by means of ELISA. Unpaired T-test.

5.2.2. Effect of CCL20 expression levels on B16 tumor growth in Wt and CCR6$^{-/-}$ mice

We inoculated 4x10^5 of early passaged (passage-8) B16 melanoma cells subcutaneously in the flank of Wt and CCR6$^{-/-}$ mice in order to monitor tumor growth over 15 days. mCCL20 expression capability of B16 melanoma cells was confirmed in vitro during the first days after inoculation **(Fig. 5A)**. In vivo, we observed significantly slower B16-tumor growth in CCR6$^{-/-}$ compared to Wt mice **(Fig. 5B).**

However no differences could be observed in tumor growth between the two experimental groups when using late passaged (passage-20) B16 melanoma cells. Additionally, tumors from both Wt and CCR6$^{-/-}$ grew significantly faster than in the previous experiments. In vitro analysis of the mCCL20 expression capacity of the late passage (>20 passages) B16 melanoma cells revealed, that mCCL20 expression capability was nearly lost in these cells **(Fig. 5C)**.

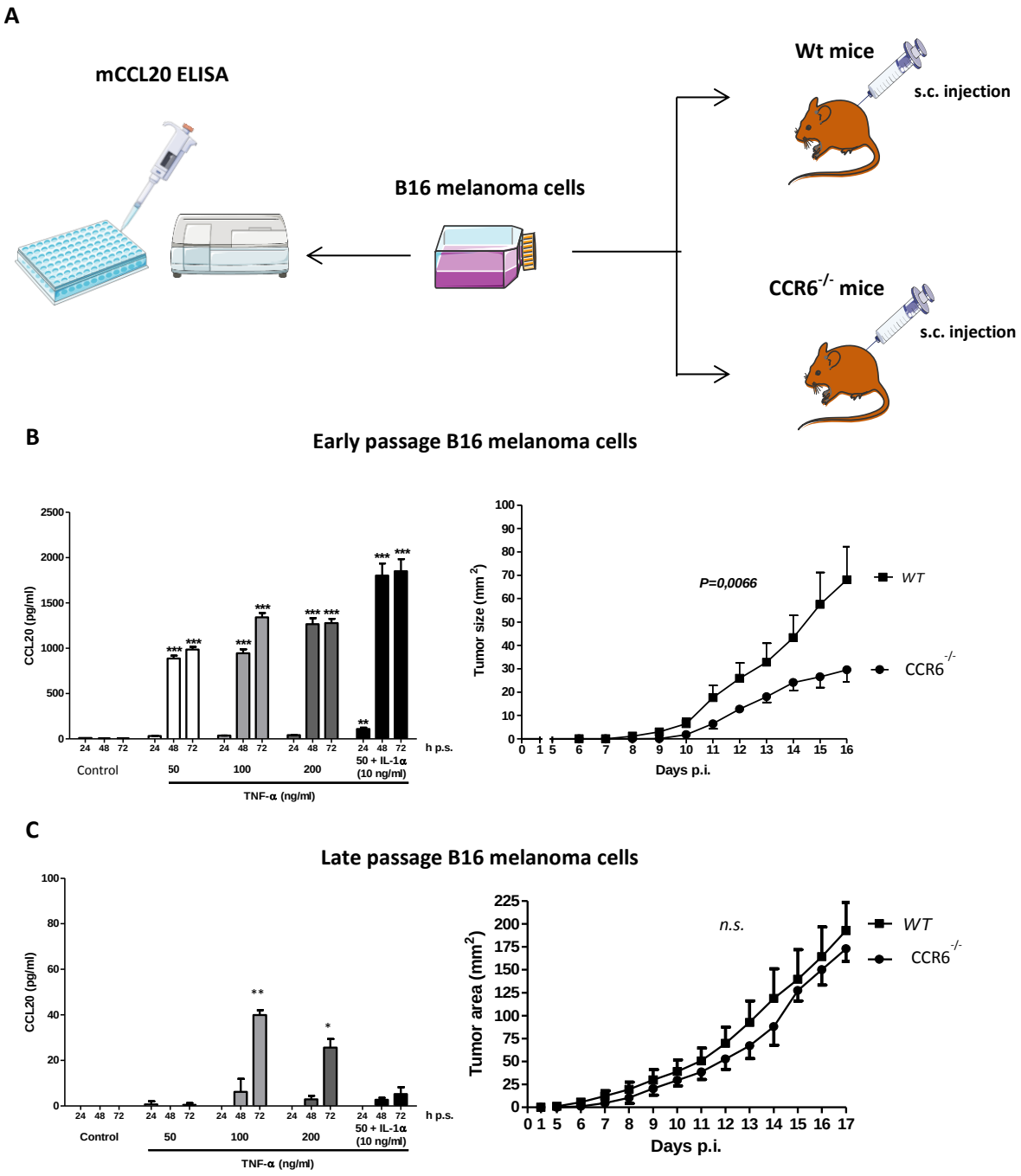


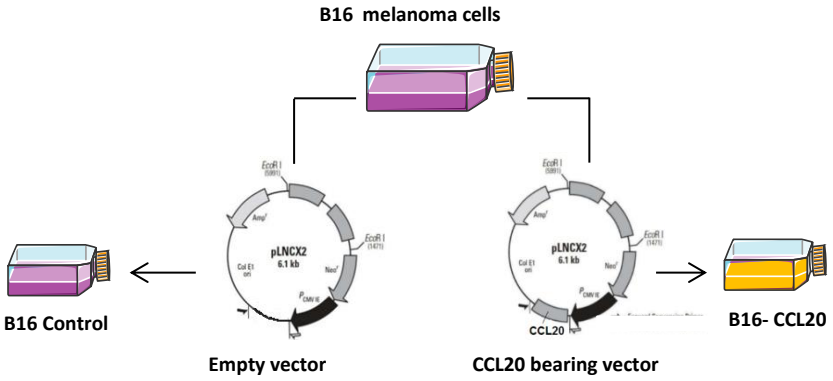
Figure 5. Tumor progression in Wt and CCR6$^{-/-}$ mice injected with early (passage-8) and late (passage-20) passages of B16 melanoma cells. 4x10^5 B16 melanoma cells were injected s.c. in the flank of the mice. Tumor growth was measured daily using a caliper. Tumor area = (major radius)*(minor radius)*π.. **A.** Illustrative scheme of the experimental setup **B.** mCCL20 production capability of inoculated B16-cells the day of the experiment after 8 passages in culture (Left) and progression of tumor growth over time (Right). **C.** mCCL20 production capability of inoculated B16-cells the day of the experiment after 20 passages in culture (Left) and progression of tumor growth over time (Right). Bar graphs represent mean ±SD from three independent experiments. Unpaired T-test *(* P<0.05, ** P<0.005, *** P<0.0005*). Dots represent means ±SEM of 5 mice in each group. Two-way ANOVA, Bonferroni post-test. (*h.p.s.*, hours post stimulation; *p.i.*, post inoculation)

5.3. Generation of retrovirally transduced mCCL20 expressing B16 melanoma cells

5.3.1. Aim and introduction

Due to the detected variations in mCCL20 expression during *in vitro* culture and the substantially effect *in vivo* tumor growth, accurate positive and negative controls were required for future animal experiments. On one hand a cell line capable of robust mCCL20 expression under homeostatic conditions and on the other hand one that had lost CCL20 expression capability completely.

With that aim, we cultured B16 cells in our lab for 15 passages until stimulation with mTNF-α could not trigger mCCL20 expression anymore. We obtained Retroviral transduction of B16 melanoma cells was carried out in the German Center for Cancer Research (DKFZ) in collaboration with Prof. Rainer Will, using pLNCX2 plasmid vectors from *Clontech*, one of which carried the murine gene for CCL20 (pLNCX2-CCL20). **(Scheme 1)**



Scheme 1. B16-Control and B16-CCL20 cell lines were generated from a parental B16 cell line**.**

5.3.2. mCCL20 expression in retrovirally transduced B16 clones

Once B16 cells were retrovirally transduced with control pLNCX2 or pLNCX2-CCL20 vectors, mCCL20 expression capability under homeostatic conditions was tested for six different clones from each new cell line by means of ELISA.

None of the pLNCX2-control B16 melanoma cells expressed CCL20 under homeostatic conditions **(Fig 6A, right)** while two out of six pLNCX2-CCL20 B16 clones expressed CCL20 under homeostatic conditions **(Fig 6A, left)**.

5.3.3. Selection of clones for *in vivo* experiments

Out of the positively transduced pLNXC2-CCL20 clones, the clone that showed higher mCCL20 expression levels under homeostatic conditions was selected for future in vivo experiments. In order to select an appropriate control counterpart for these cells, proliferation capability of all pLNCX2-control clones was tested by means of XTT assay and compared to the CCL20 expressing B16 cell line, choosing the clone that showed the closest similarity **(Fig. 6B).** These cell lines were renamed as *B16-CCL20* and *B16-Control* and used for the subsequent in vitro and in vivo experiments of this project. Stimulation with mTNF-α could not trigger significant mCCL20 expression in B16-Control cell lines and did not increase significantly mCCL20 production in B16-CCL20 cells **(Fig 7)** as measured by ELISA**.** Additionally, we tested whether, besides mCCL20 expression, the retroviral infection procedure had affected the expression of other pro-inflammatory cytokines by means of LEGENDPLEX assay. Both B16-Control and B16-CCL20 responded in a similar manner, as well as the parental B16 melanoma cell line **(Fig 8).**

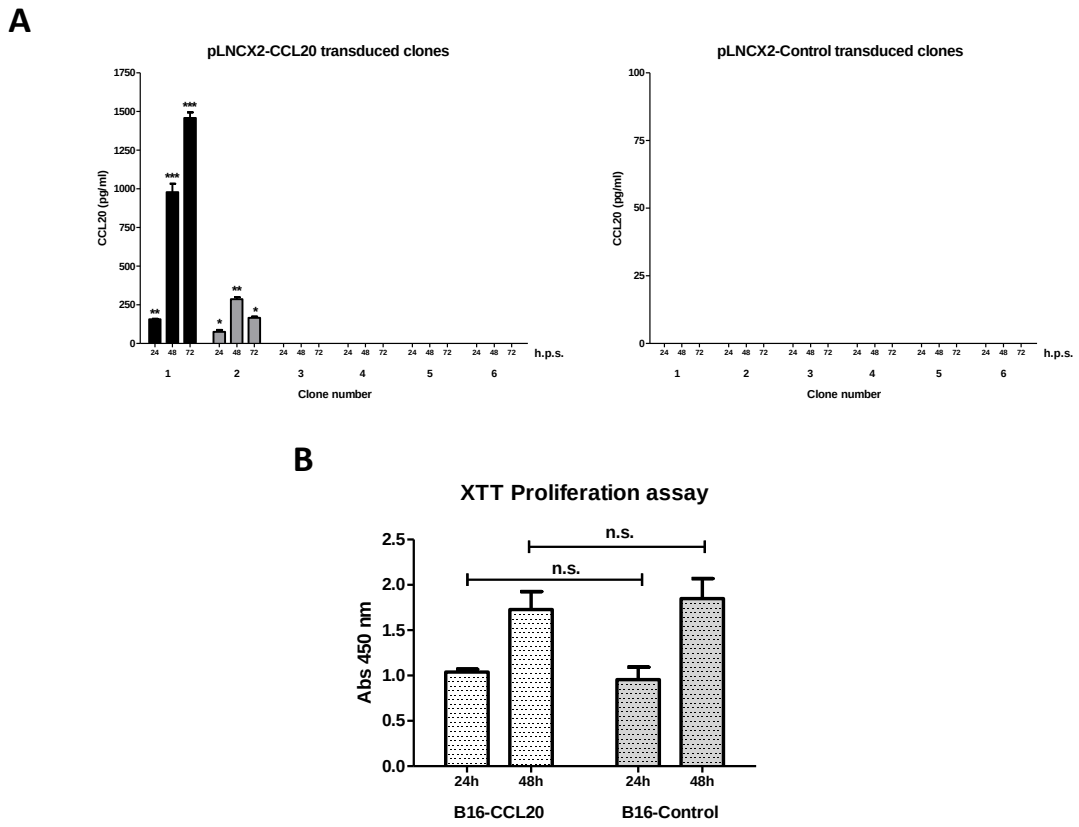


Figure 6. Selection of retrovirally transduced B16-CCL20 and B16-Control melanoma cells for future in vivo experiments. Retroviral transduction was carried out at the Genomics & Proteomics core Facility from the German Center for Cancer Research (DKFZ) under supervision from Prof. Rainer Will. pLNCX2-CCL20 clone 1 and pLNCX2-CCL20 clone 3 were picked up and renamed as B16-CCL20 and B16-Control **A.** All clones were cultured following the

same protocol in 3.1 and mCCL20 was detected by means of ELISA (*h.p.s.*, hours post seeding). Bar graphs show mean ±SD from three independent experiments. **B**. Results from XTT proliferation assay from B16-CCL20 and B16-Control cells. Bar graphs show mean ±SD from two independent experiments. Unpaired T-test *(* P<0.05,** P<0.005,*** P<0.0005).*

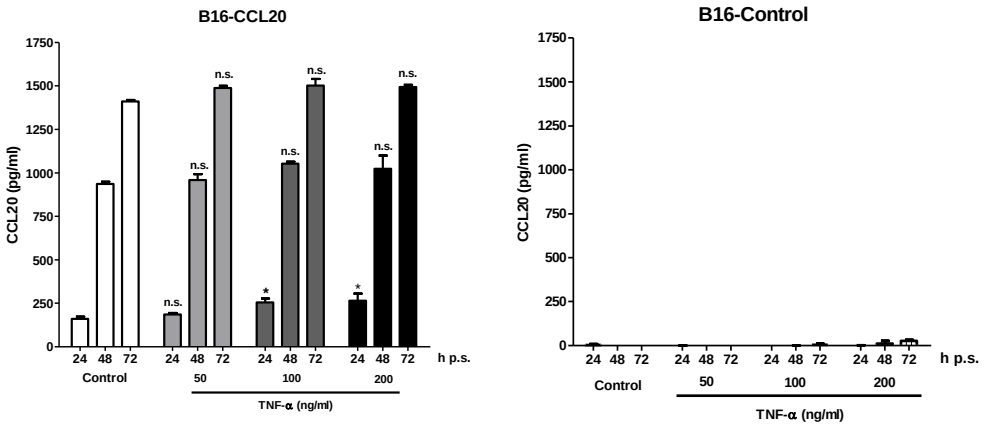


Figure 7. Stimulation with TNF- α cannot trigger significant mCCL20 expression in B16-Control cell lines. 5x10^4 B16-CCL20 and B16-Control melanoma cells were seeded in 24 well plates and stimulated with increasing concentrations of TNF-α. Medium was removed after 24, 48 and 72 hours respectively and CCL20 was measured by means of ELISA. Bar graphs represent mean ±SD from three independent experiments. Unpaired T-test *(* P<0.05)*

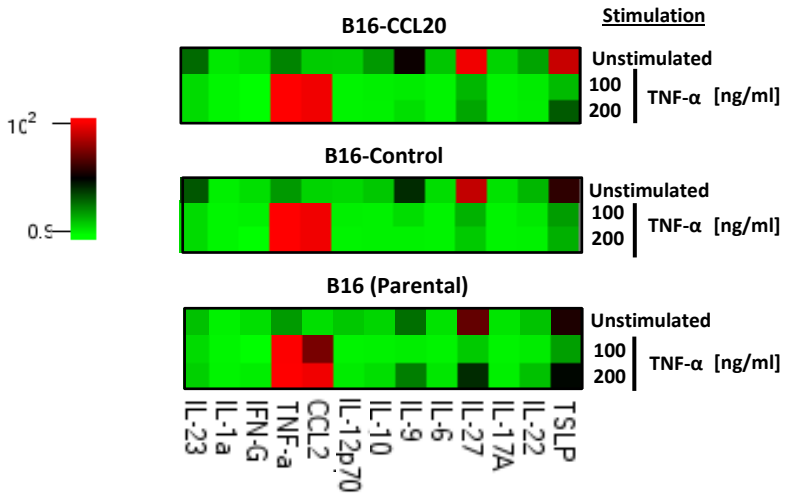


Figure 8. TNF-α triggers similar cytokine responses in retrovirally generated B16 melanoma cells. 5x10^4 B16-CCL20, B16-Control and B16 parental cells were seeded in 24 well plates and stimulated with increasing concentrations of TNF-α. Medium was removed after 48 hours respectively and concentrations of 13 different inflammatory cytokines were measured by means of LEGENDPLEX.

5.3.4. *In vivo* expression of mCCL20 by retrovirally transduced B16-PMSCV clones

Complementing our *in vitro* analysis, we tested the mCCL20 expression capability of B16-CCL20 and B16-Control *in vivo.* CCL20 could be detected in the supernatants from tumors of Wt and CCR6$^{-/-}$ mice subcutaneously injected with B16-CCL20 melanoma cells. By contrast, mCCL20 was barely detectable in supernatants from B16-Control tumors in Wt and CCR6$^{-/-}$ animals **(Fig. 9)**. mCCL20 could also be detected in the serum from Wt and CCR6$^{-/-}$ mice subcutaneously injected with B16-CCL20, but not in those injected with B16-Control cells. **(Fig 10)**

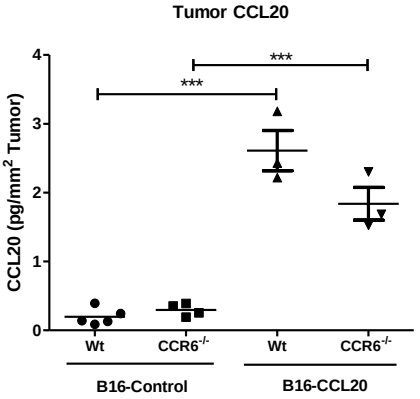


Figure 9. mCCL20 expression of retrovirally transduced melanoma cells in vivo. 4x10^5 cells from B16-Control or B16-CCL20 cells were subcutaneously injected at the flank of Wt and CCR6$^{-/-}$ mice. Tumors were removed and digested after 15 days. mCCL20 was measured from the supernatants by means of ELISA. Unpaired T-test *(*** P<0.0005)*

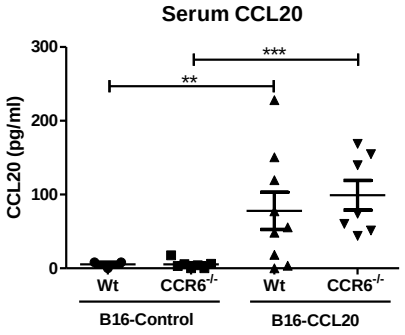
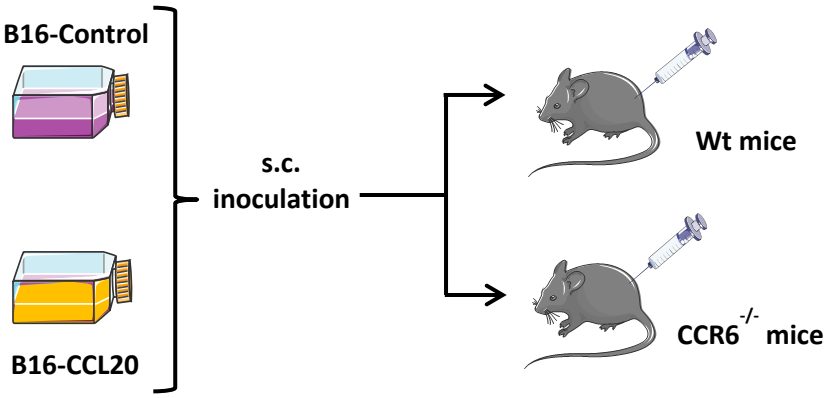


Figure 10. m**CCL20 can be detected in serum from B16-CCL20 injected mice.** Blood was taken from B16-Control and B16-CCL20 subcutaneously injected Wt and CCR6$^{-/-}$ animals 15 days after inoculation. mCCL20 in serum was measured by means of ELISA. Unpaired T-test *(** P<0.005, *** P<0.0005)*

5.3.5. Loss of mCCL20 expression in retrovirally transduced cell lines affects tumor growth in CCR6$^{-/-}$ mice

The experiment described in 3.2.3 was repeated by inoculating both cell lines in Wt and CCR6$^{-/-}$ animals in order to observe if overexpression of mCCL20 at the tumor site would create differences in tumor progression. Wt and CCR6$^{-/-}$ mice were divided into two groups each and subcutaneously injected either with B16-Control or B16-CCL20 melanoma cells **(Scheme 2)**



Scheme 2. Illustrative scheme showing established experimental groups. 10 Wt and 10 CCR6$^{-/-}$ mice were divided in two groups each and injected with B16-Control and B16-CCL20 melanoma cells respectively on each experiment.

In this first experiment no differences in tumor progression were observed between the B16-Control injected Wt and CCR6$^{-/-}$ groups. However, in the B16-CCL20 experimental groups, while Wt animals showed similar tumor growth rate than B16-Control injected groups, CCR6$^{-/-}$ mice developed tumors significantly slower **(Fig. 11A)**. Similar to our previous observations using B16 parental cells, Wt and CCR6$^{-/-}$ animals injected with B16-CCL20 cells showed no significant differences when using late passaged cells (>15 passages) **(Fig. 11B)** corresponding with a significant reduction of CCL20 expression capability as measured under homeostatic conditions in vitro **(Fig. 11C).**

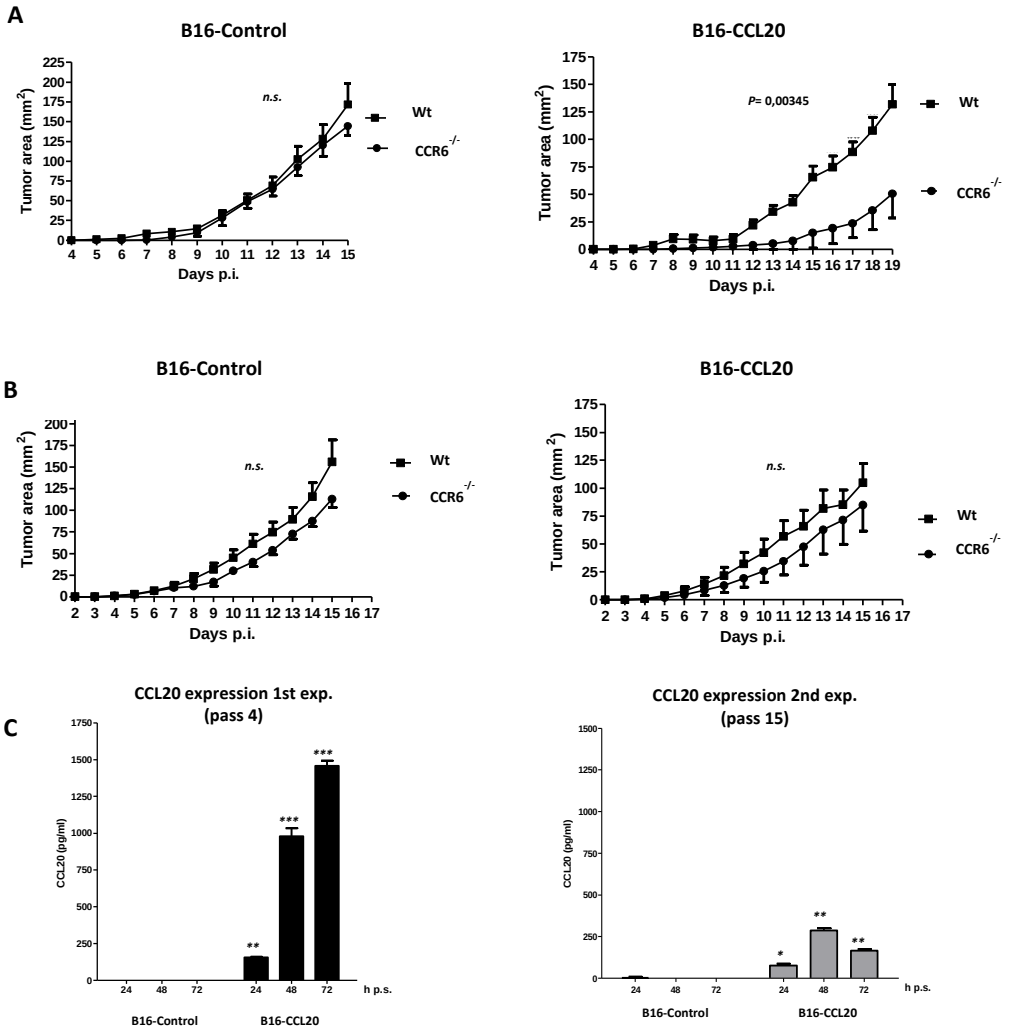


Figure 11. Reduction of mCCL20 homeostatic expression in B16-CCL20 cells affects tumor growth in CCR6$^{-/-}$ mice. 4x10^5 cells of B16-Control or B16-CCL20 were injected s.c. in the flank of Wt and CCR6$^{-/-}$ mice. Tumor growth was measured daily using a caliper. Tumor area = (major radius)*(minor radius)*π. Dots represent means ±SEM of 5 mice in each group. Two-way ANOVA, Bonferroni post-test. **A.** Tumor growth from early passaged B16-Control and B16-CCL20 injected mice **B.** Tumor growth from late passaged B16-Control and B16-CCL20 injected mice **C.** CCL20 expression under homeostatic conditions from early passaged (pass. 4) B16-Control and B16-CCL20 cells **D.** CCL20 expression under homeostatic conditions from late passage (pass. 15) B16-Control and B16-CCL20 cells. Bar graphs represent mean ±SD. Unpaired T-test *(* P<0.05, ** P<0.005, *** P<0.0005)*

5.4. CCR6$^{-/-}$ mice develop significantly slower melanoma tumors in presence of CCL20

5.4.1. Aim and introduction

In order to study the effect of mCCL20 overexpression during melanoma progression in Wt and CCR6$^{-/-}$ animals regarding tumor growth kinetics, survival rate and onset, experiments described in 3.3.6 were repeated using early passaged (pass 2-6) B16-Control and B16-CCL20 cells each time

5.4.2. Wt and CCR6$^{-/-}$ mice injected with B16-CCL20 melanoma cells show significantly smaller tumors compared to controls and Wt mice.

Again, four experimental groups as described in **Scheme 2** were established. Cells were inoculated subcutaneously in the flank of mice that had been shaved locally 24h before the experiment. Tumor size was monitored daily for a period of 15 days by using a caliper. Animals that did not develop tumors by this time point were kept under surveillance 3 extra days before being sacrificed in order to verify that no late-onset tumors would develop.

On day 15, animals injected with B16-CCL20 cells had smaller tumors than their B16-Control injected littermates. These differences were significantly higher in CCR6$^{-/-}$ mice.

In both experimental groups CCR6$^{-/-}$ animals displayed smaller tumors than Wt animals. In the case of B16-Control injected groups, CCR6$^{-/-}$ mice showed slightly significant differences compared to their Wt analogues. However B16-CCL20 injected CCR6$^{-/-}$ animals showed significantly smaller tumors than the B16-CCL20 injected Wt group (**Fig 12A and 12B**).

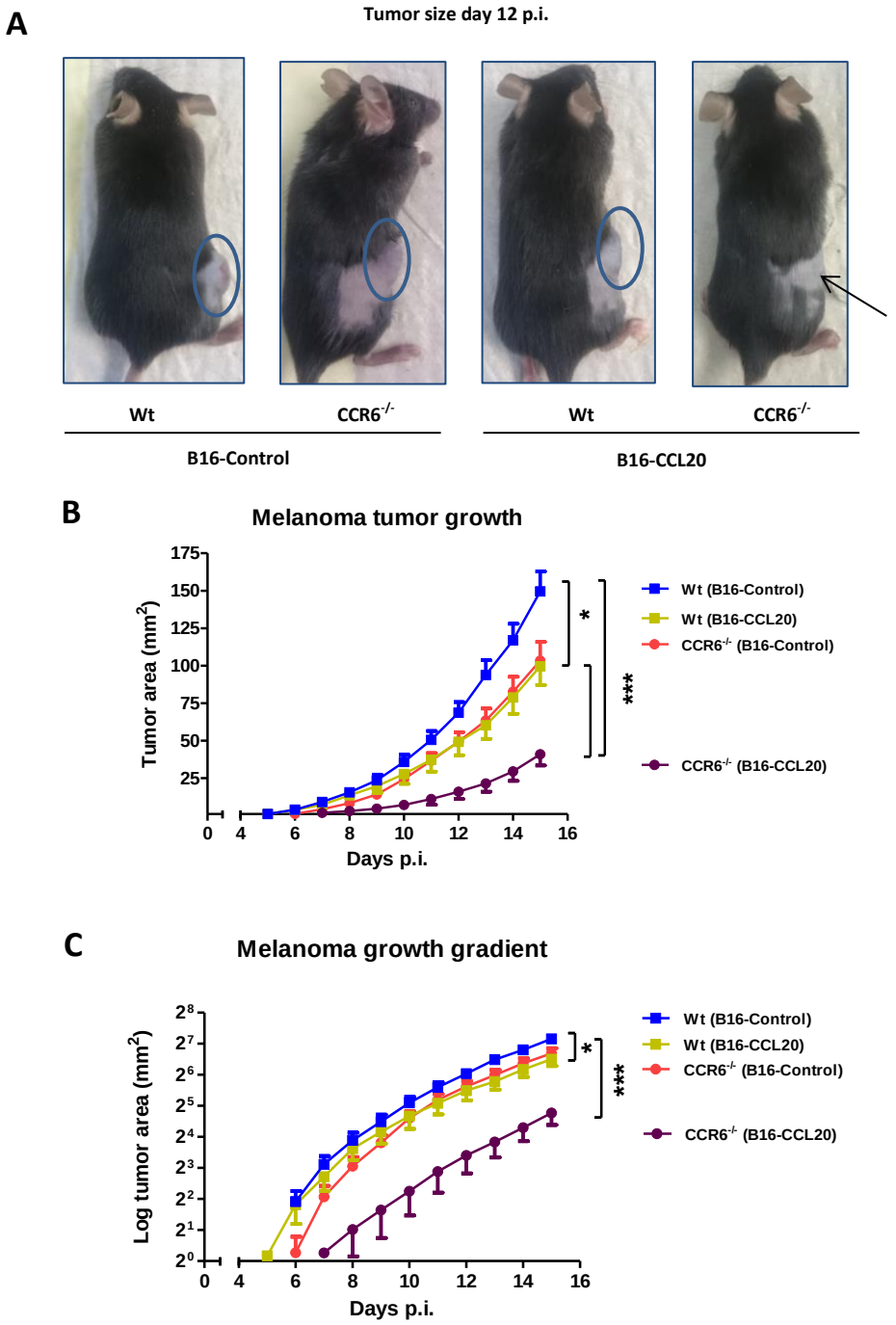


Figure 12. B16-CCL20 injected mice show slower melanoma tumor growth than B16-Control injected controls. 4x10^5 cells of B16-Control or B16-CCL20 were injected s.c. in the flank of Wt and CCR6$^{-/-}$ mice. Tumor growth was measured daily using a caliper. **A.** Illustrative pictures of melanoma tumors from Wt and CCR6$^{-/-}$ mice injected either with B16-CCL20 or B16-Control cells at day 12 after tumor inoculation. **B.** Tumor growth kinetics from all four experimental groups. Tumor area = (major radius)*(minor radius)*π. Dots represent means ±SEM of 25 mice in each experimental groups form 5 independent experiments. Two-way ANOVA, Bonferroni post-test *(** P<0.005,*** P<0.0005).* **C.** Tumor growth data from B transformed into log2 scale.

5.5. B16-CCL20 injected CCR6$^{-/-}$ mice showed differences in tumor growth rate, visual onset and incidence compared to controls and Wt mice

5.5.1. B16-CCL20 tumors in CCR6$^{-/-}$ mice maintain a constant tumor growth rate over time

In a recent publication Gegenbacher and collaborators highlighted the limitations of classical tumor data representation and suggested to display tumor size kinetics using logarithmic scales [307] Displaying tumor growth data by logarithmic scale we noted that tumors from B16-CCL20 injected CCR6$^{-/-}$ mice doubled their size every 48 hours once they became visible, kept this gradient over time and showed a delayed onset compared to the rest of the experimental groups. This phenomenon contrasts with the growth gradient of tumors from both Wt experimental groups and B16-Control inoculated CCR6$^{-/-}$ animals. In all these three experimental groups, tumors doubled their size every 24 hours during the first days after becoming visible, and every 48 hours from day 8 on. Based on this we divided tumor growth kinetics from Wt and B16-Control injected CCR6$^{-/-}$ animals into two different stages: One initial stage characterized by high growing gradient followed by a second stage of stable constant tumor growth **(Fig 12C).** Interestingly, while CCR6$^{-/-}$ experimental groups showed differences in tumor growth gradients and final tumor size, Wt experimental groups showed similar tumor growth gradients despite of the differences in final tumor size.

5.5.2. B16-CCL20 tumors from CCR6$^{-/-}$ mice showed later visual onset

Tumors arising in B16-Control and B16-CCL20 injected Wt animals were spotted visually (>2 mm Ø) at days 5-6, as well as tumors from B16-Control injected CCR6$^{-/-}$ mice. By day 9 after inoculation 80% of the animals on these three experimental groups had developed tumors. On the contrary, while first tumors from B16-CCL20 injected CCR6$^{-/-}$ animals could be detected between days 6-7, by day 9 the proportion of mice with visible tumors was only 20% (**Fig 12C and 13A**). Overall, visual onset of tumors arising in B16-CCL20 injected CCR6$^{-/-}$ mice was significantly delayed compared to both Wt experimental groups and B16-Control injected CCR6$^{-/-}$ group.

5.5.3. B16-CCL20 injected CCR6$^{-/-}$ mice have lower tumor incidence

In terms of tumor incidence almost 100% of B16-Control and B16-CCL20 injected Wt individuals had developed tumors by the end of the experiment (up to day 18). In the case of CCR6$^{-/-}$ mice, all of those injected with B16-Control cells developed tumors before day 18 after tumor inoculation. However, only 76% of the specimens from the B16-CCL20 injected CCR6$^{-/-}$ group had developed tumors by this time

point. Hence, almost 25% of the animals from this experimental group did not develop melanoma tumors at the inoculation site during the time of the experiment **(Fig. 13A and 13B)**

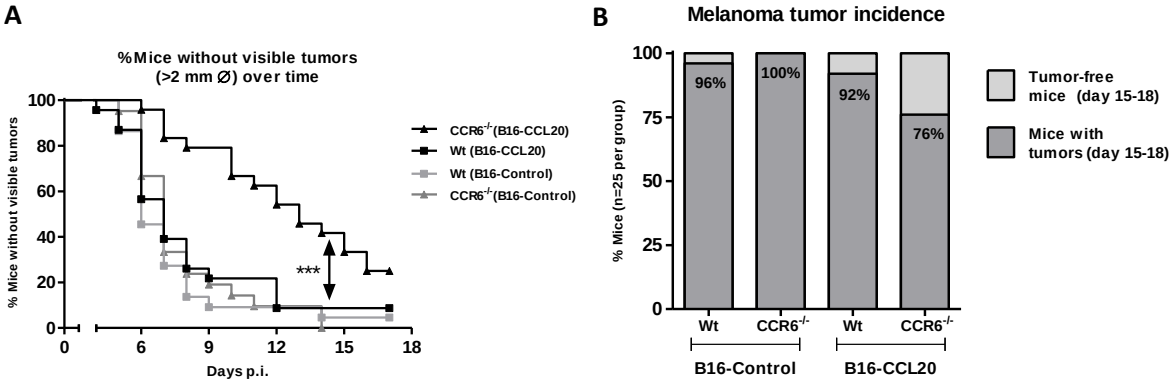


Figure 13. B16-CCL20 injected CCR6$^{-/-}$ mice show later melanoma onset and reduced tumor incidence. A. Percentage of individuals with visible tumors (>2 mm diameter) over time. Log-rank (Mantel-Cox) test (*** *P*<0.0005) **B.** % of animals that had developed tumors by day 18 after inoculation**.** In Both cases figures represent data from 25 mice on each experimental group taken from 5 independent experiments.

5.5.4. Local injections of rCCL20 decrease tumor growth in B16-Control inoculated CCR6$^{-/-}$ mice

Our previous experiments indicated that the constant presence of B16-derived CCL20 at the site of a growing tumor influenced tumor size and growing gradient. In order to test that these differences in growth were indeed caused by CCL20, we established a similar experimental model injecting B16-Control cells in Wt and CCR6$^{-/-}$ mice followed by local injections of recombinant murine CCL20 (rCCL20) every two days into the site of the tumor, increasing the injected amounts of rCCL20 proportionally as tumors were growing **(Fig 14A).**

In line with previous experiments no significant differences in tumor growth between CCL20 and PBS injected Wt mice were observed. However, rCCL20 injected CCR6$^{-/-}$ animals developed tumors significantly slower than their PBS injected littermates. The PBS injected CCR6$^{-/-}$ group showed no significant differences in tumor growth compared to both Wt groups **(Fig 14B).**

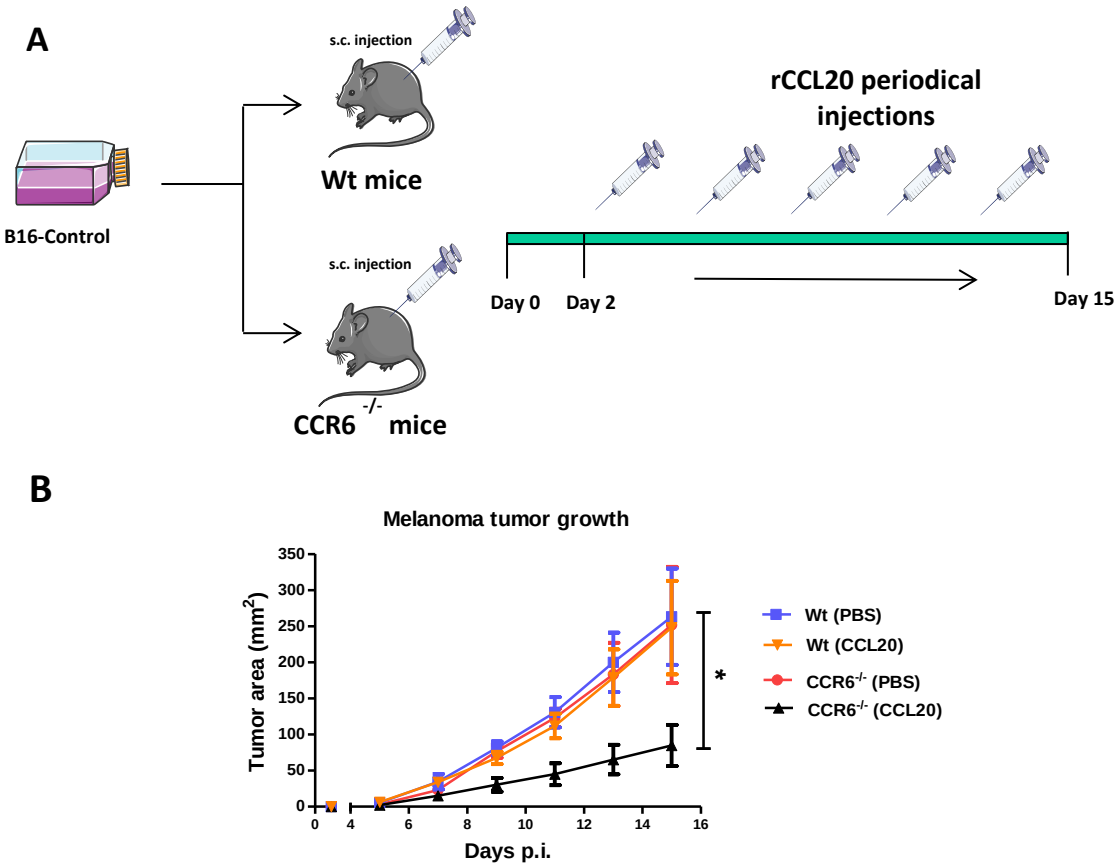


Figure 14. B16-Control tumors from inoculated CCR6$^{-/-}$ mice develop slower after local injections of rCCL20 when compared to controls. 4x10^5 cells of B16-Control cells were injected s.c. in the flank of Wt and CCR6$^{-/-}$ mice. Local injections of recombinant CCL20 or PBS were provided every two days after tumor inoculation. Tumor growth was measured daily using a caliper. **A.** Illustrative scheme of the experimental set up. Two additional groups of Wt and CCR6$^{-/-}$ mice were provided periodical injections of PBS after B16-Control melanoma cell inoculation as a control. **B.** Melanoma tumor growth from all four experimental groups. Tumor area = (major radius)*(minor radius)*π. Dots represent means ±SEM of 10 mice on each experimental groups of 2 independent experiments. Two-way ANOVA, Bonferroni post-test *(* P<0.05)*

5.5.5. Local injections of anti-CCL20 Ab slightly accelerate tumor growth B16-CCL20 injected CCR6$^{-/-}$ mice compared to controls.

We performed reciprocal experiments with local injections of an anti-mCCL20 antibody (anti-mCCL20 Ab) in order to determine the effect of local CCL20 neutralization on tumor growth and incidence in Wt and CCR6$^{-/-}$ mice. The capability of the anti-mCCL20 Ab to neutralize B16-CCL20 derived mCCL20 was tested in vitro before the in vivo experiment **(Fig 15B).**

We injected B16-CCL20 in Wt and CCR6$^{-/-}$ mice followed by local subcutaneous injections into the site of the tumor of anti-mCCL20 Ab every two days after tumor inoculation **(Fig 15A)**. While not reaching statistical significance, we observed a slight increase in tumor growth during the last days of the experiment in CCR6$^{-/-}$ mice that had been administrated periodically anti-mCCL20 Ab in comparison to PBS injected CCR6$^{-/-}$. Wt animals injected with PBS developed tumors slightly slower than their anti-mCCL20 injected littermates while no reaching statistical significance. **(Fig 15C)**.

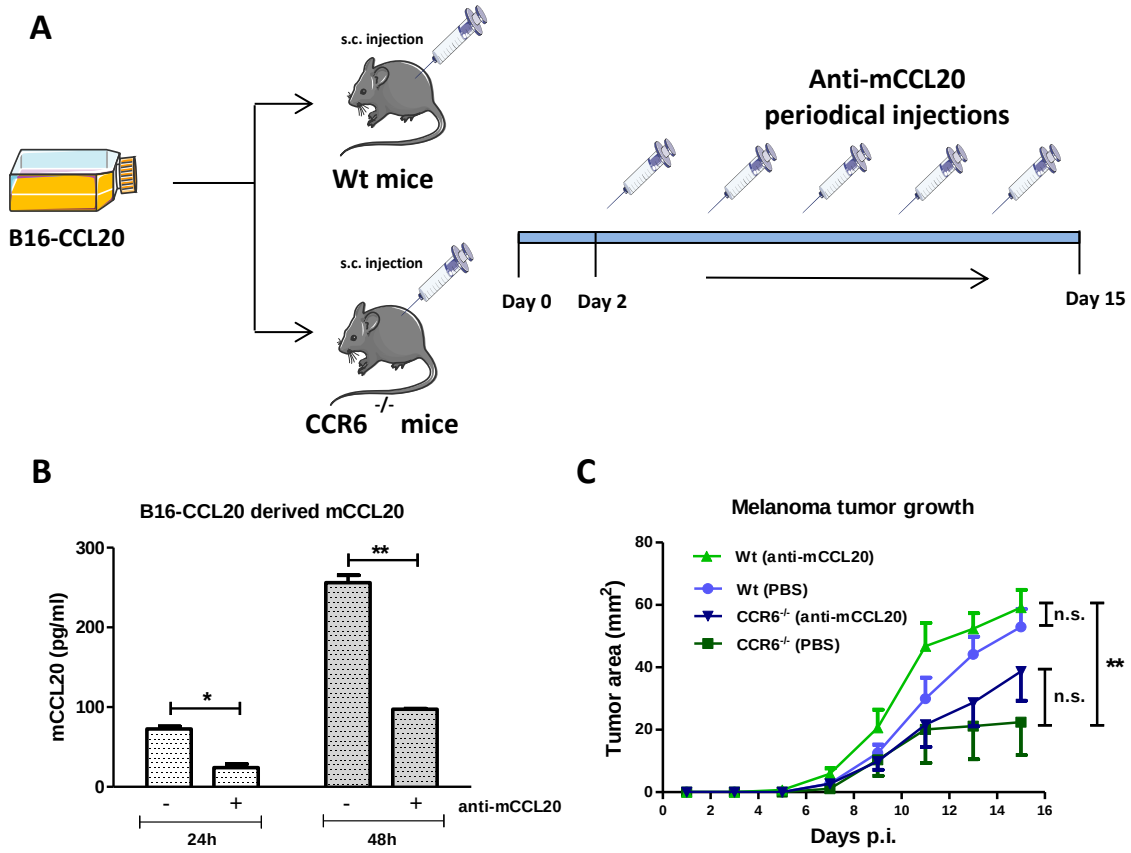


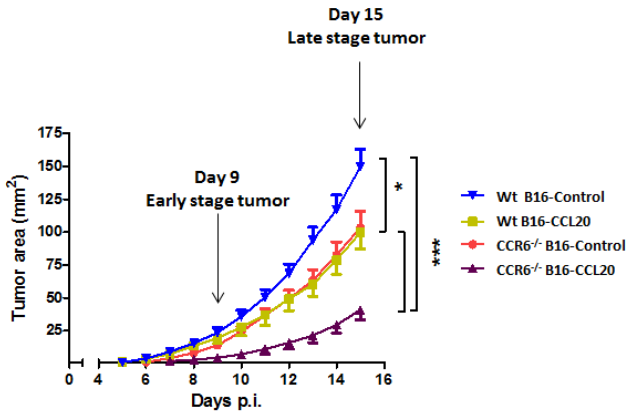
Figure 15. Neutralizing mCCL20 slightly accelerates tumor growth inB16-CCL20 injected CCR6$^{-/-}$ mice. 4x10^5 cells of B16-CCL20 cells were injected s.c. in the flank of Wt and CCR6$^{-/-}$ mice. Local intratumoral injections of anti-mCCL20 Ab or PBS were provided every two days after tumor inoculation. Tumor growth was measured daily using a caliper. **A.** Illustrative scheme of the experimental set up. Two additional groups of Wt and CCR6$^{-/-}$ mice were provided periodical injections of PBS after B16-CCL20 melanoma cell inoculation as a control **B.** anti-mCCL20 antibody is able to neutralize B16-CCL20 derived mCCL20. B16-CCL20 supernatants were incubated at 37ºC for 1 h with and without anti-mCCL20. mCCL20 was measured by means of ELISA. Unpaired T-test *(* P<0.05, ** P<0.005)* **C.** Tumor growth from B16-CCL20 injected Wt and CCR6$^{-/-}$ mice. Tumor area = (major radius)*(minor radius)*π. Two way ANOVA, Bonferroni post-test. Dots represent means ±SEM of 5 mice on each experimental group *(** P<0.005)*

5.6. Characterization of tumor infiltrating leucocytes (TILs) in B16-Control/B16-CCL20 melanoma tumors in Wt and CCR6$^{-/-}$ mice

5.6.1. Aim and introduction

As previous results indicated a role for the CCR6/CCL20 axis in melanoma, we set out to further characterize the immune cell infiltrate, in particular infiltrating CCR6+ leucocytes within tumors and DLN in our animal model **(Scheme 2)**.

Tumors were dissected from mice and immune cells were analyzed by FCM at two different time points: 9 days after tumor injection (once solid tumors were established at an early growing stage) and 15 days after tumor injection coinciding with the end of the experiment, when big tumors (> 1 cm Ø) were robustly established **(Scheme 3)**



Scheme 3. Illustrative figure showing experimental time points at which tumors got dissected and TILs were analyzed: Early (day 9 p.i.) and late (day 15 p.i.) stage tumors.

5.6.2. FCM gating strategy and identification of CCR6+ TIL subsets

We established two FCM gating panels that included different CCR6+ immune cell subsets alongside with other immune cell types known to have key roles in tumor progression and immunity. Due to experimental limitations it was not possible to functionally characterize all of them during each experiment and identification was phenotypically based on surface markers. Markers used to identify each subset are collected in **Table 1**.

Within the B-cell fraction, our FCM panel was designed to identify activated B-cells, B1 and Killer B-cells, Memory B-cells and B-regulatory cells. Among these CCR6+ subsets have been described for activated B-

cells [308], B-regulatory cells [309] and memory B-cells [308]**.** As for T-cells, our gating strategy included CD4+ and CD8+ fractions. Within CD4+ leucocyte subsets of T-regulatory and CD3+CD4+Foxp3- have been identified to express CCR6 [310]**.** On the other hand, no CD8+CCR6+ cells have been described in mice so far. However, given the key roles of CD8+ cells in tumor immunology [311] we decided to include this subset into our analysis. Regarding dendritic cells, we included plasmacytoid and myeloid types, of which CCR6+ subsets have been described [312]**.** Macrophages were also included in the final panel, as their role in melanoma is well described [259]. Our FCM gating strategy comprised two different panels based on the compatibility of the different detection antibodies used. Panel A included all B-cell subsets and CD8+ T-cells **(Fig 16)** while panel B included CD4+ T-cells, Dendritic cells and Macrophages. **(Fig 17)**

Immune Cell type	***Subset***	***Markers***				***Reference***
B-cells						
	B-regs	CD19+	CD45R+	CD5+		313
	Activated B-cells	CD19+	CD45R+	CD5-		314
	- CCR6+ Act. B-cells	CD19+	CD45R+	CD5-	CCR6+	
	Memory B-cells	CD19-	CD45R+	CD5-		314
	- CCR6+ Memory B-cells	CD19-	CD45R+	CD5-	CCR6+	
	B1/K cells	CD19-	CD45R-	CD5+		315
	- CCR6+ B1/k cells	CD19-	CD45R-	CD5+	CCR6+	
T-cells		CD3+				
	CD8+	CD3+	CD8+			316
	CD4+	CD3+	CD4+			317
	- CCR6+	CD3+	CD4+	Foxp3 -	CCR6+	318
	- T-regs	CD3+	CD4+	Foxp3+	CCR6-	319
	-CCR6+ T-regs	CD3+	CD4+	Foxp3+	CCR6+	320
DCs		CD11c+				321
	pDCs	CD11c+	CD45R+			322
	- CCR6+ pDCs	CD11c+	CD45R+	CCR6+		
	cDCs	CD11c+	CD45R-			323
	- CCR6+ cDCs	CD11c+	CD45R-	CCR6+		
Macrophages		$CD11c^{low}$	CD45R-			324

Table 1. Surface markers and phenotypical characterization of immune cell subsets.

Gating panel A (B-cells and CD8+ T-cells)

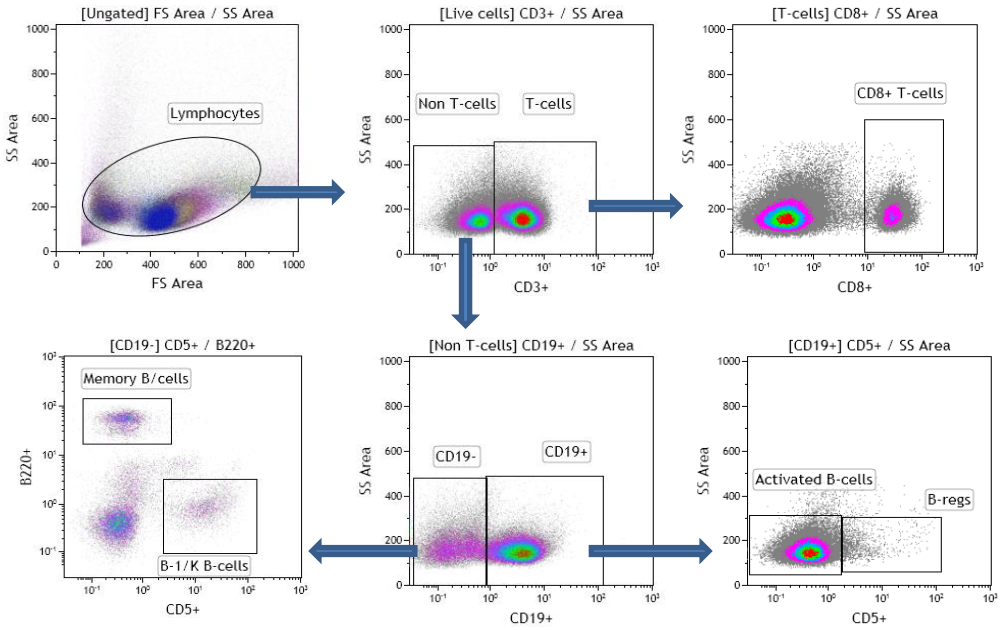


Figure 16. Overview of gating panel A. B-cell subsets (Memory B-cells, Activated B-cells, B1/K cells and B-regulatory cells) and CD8+ T-cells. Each subset was subsequently gated for CCR6+ signal.

Gating panel B (CD4+ T-cells, DCs and Macrophages)

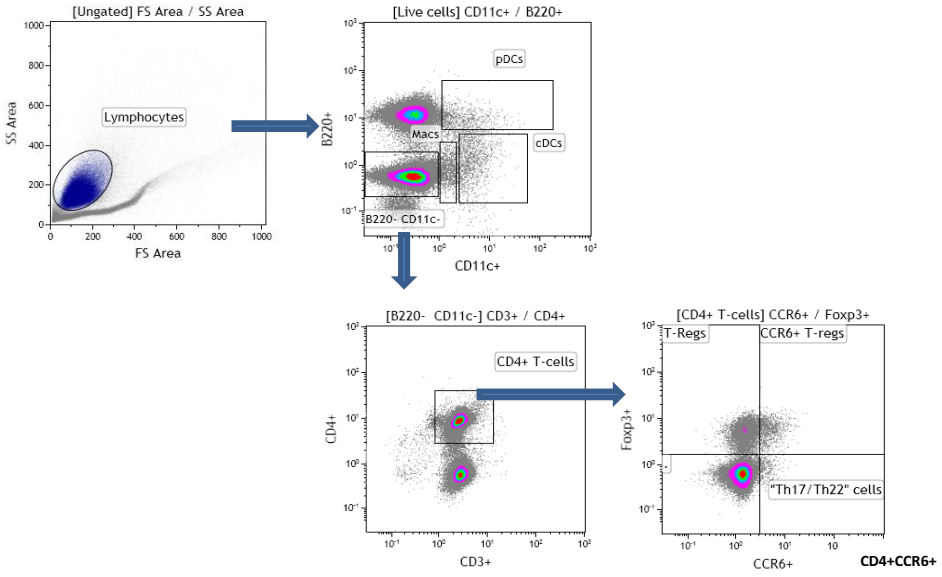


Figure 17. Overview of gating panel B. CD4+ T-cells (including T-regulatory cells), Dendritic cells (pDCs and cDCs) and Macrophages. Each subset was subsequently gated for CCR6+ signal.

5.6.3. Characterization of TILS

5.6.3.1. B-cells

5.6.3.1.1. Activated B-cells (CD19+CD45R+CD5-)

Both Wt experimental groups showed low levels of activated B-cells at early and late time points regardless of the melanoma cell line injected. Interestingly, B16-Control injected CCR6$^{-/-}$ mice showed significantly higher levels of infiltrating active B-cells than B16-CCL20 injected CCR6$^{-/-}$ animals. Nevertheless, by day 15 activated B-cell infiltrate in this group had decreased to the same values of the B16-CCL20 group **(Fig 18B)**

5.6.3.1.2. Memory B-cells (CD19-CD45R+CD5-)

No significant differences were found in the amount of infiltrating Memory B-cells between the experimental groups from Wt and CCR6$^{-/-}$ animals. Interestingly, levels of this immune cell subset were slightly higher at day 15 in B16-CCL20 injected CCR6-/- **(Fig. 18C)**

5.6.3.1.3. Regulatory B-Cells (B-regs) (CD19+CD45R+CD5+)

Levels of B-regs were significantly higher at day 9 in B16-CCL20 injected CCR6ko$^{-/-}$ mice. This contrasts with low number of infiltrating B-regs in Wt experimental groups and B16-Control CCR6$^{-/-}$ group at both analyzed time points. **(Fig. 18D)**

5.6.3.1.4. B1/K cells (CD19-CD45R-CD5+)

With the exception of B16-Control injected Wt mice, B1/K cell levels decreased from day 9 to day 15 in the rest of the groups. No significant differences in B1/K cell infiltrate were detected between experimental groups **(Fig 18E).**

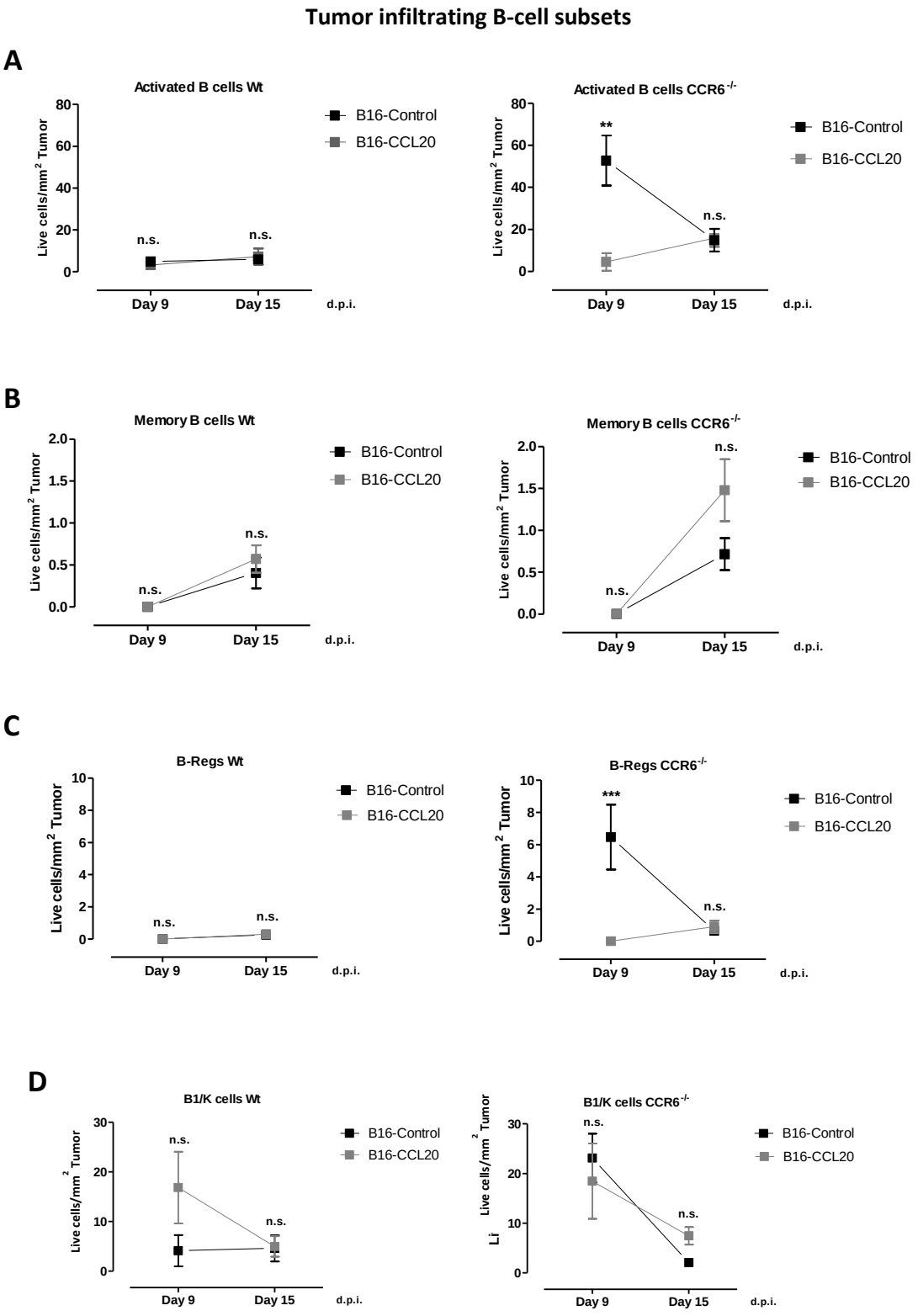


Figure 18. B-cell TILs subsets at early and late tumor growth stages. Tumors were dissected from Wt and CCR6$^{-/-}$ animals injected either with B16-Control or B16-CCL20 melanoma cells after 9 or 15 days after inoculation. After dissection and digestion the immune fraction was isolated by Percoll gradient and leucocytes were identified by

means of FCM analysis. **A.** Activated B-cells. **B**. Memory B-cells. **C.** B-regulatory cells (B-regs). **D.** B1 and Killer B-cells. Data was obtained from three independent experiments (n=15 mice). Values are expressed as mean ±SEM. Two-way ANOVA, Bonferroni post-test *(** P<0.005, *** P<0.0005*)

5.6.3.2. T-cells (CD3+)

We observed increased amounts of infiltrating T-cells within the tumors of $CCR6^{-/-}$ mice when compared to Wt mice at day 9 after tumor inoculation. While T-cell levels from B16-CCL20 injected $CCR6^{-/-}$ mice were significantly higher than B16-Control injected animals in this time point, by day 15 the amount of T-cells decreased drastically **(Fig 19A).**

5.6.3.2.1. CD8+ T-cells (CD3+CD8+)

No significant differences were found in the amount of infiltrating CD8+ T-cells between the experimental groups from Wt and $CCR6^{-/-}$ animals. Total amounts of this T-cell subset were considerably low over time **(Fig 19B).**

5.6.3.2.2. CD4+ T-cells (CD3+CD4+)

While levels of infiltrating CD4+ T-cells in Wt mice were as low as CD8+ cells at both time points analyzed, we observed highly significant amounts of CD4+ cells in $CCR6^{-/-}$ at day 9, decreasing dramatically by day 15. Interestingly, within both $CCR6^{-/-}$ experimental groups, B16-CCL20 mice showed significantly higher infiltrating CD4+ cells than their B16-Control counterparts **(Fig 19C).**

5.6.3.2.2.1. Regulatory T-cells (T-regs) (CD3+CD4+Foxp3+)

In line with our observations concerning total amount of infiltrating CD4+ T-cells, T-reg levels were significantly higher in the $CCR6^{-/-}$ experimental groups at day 9 time point compared with their Wt counterparts, decreasing afterwards. In this case, however, there were no significant differences between both experimental groups. Infiltrating T-reg levels in Wt were low at both time points **(Fig 19D).**

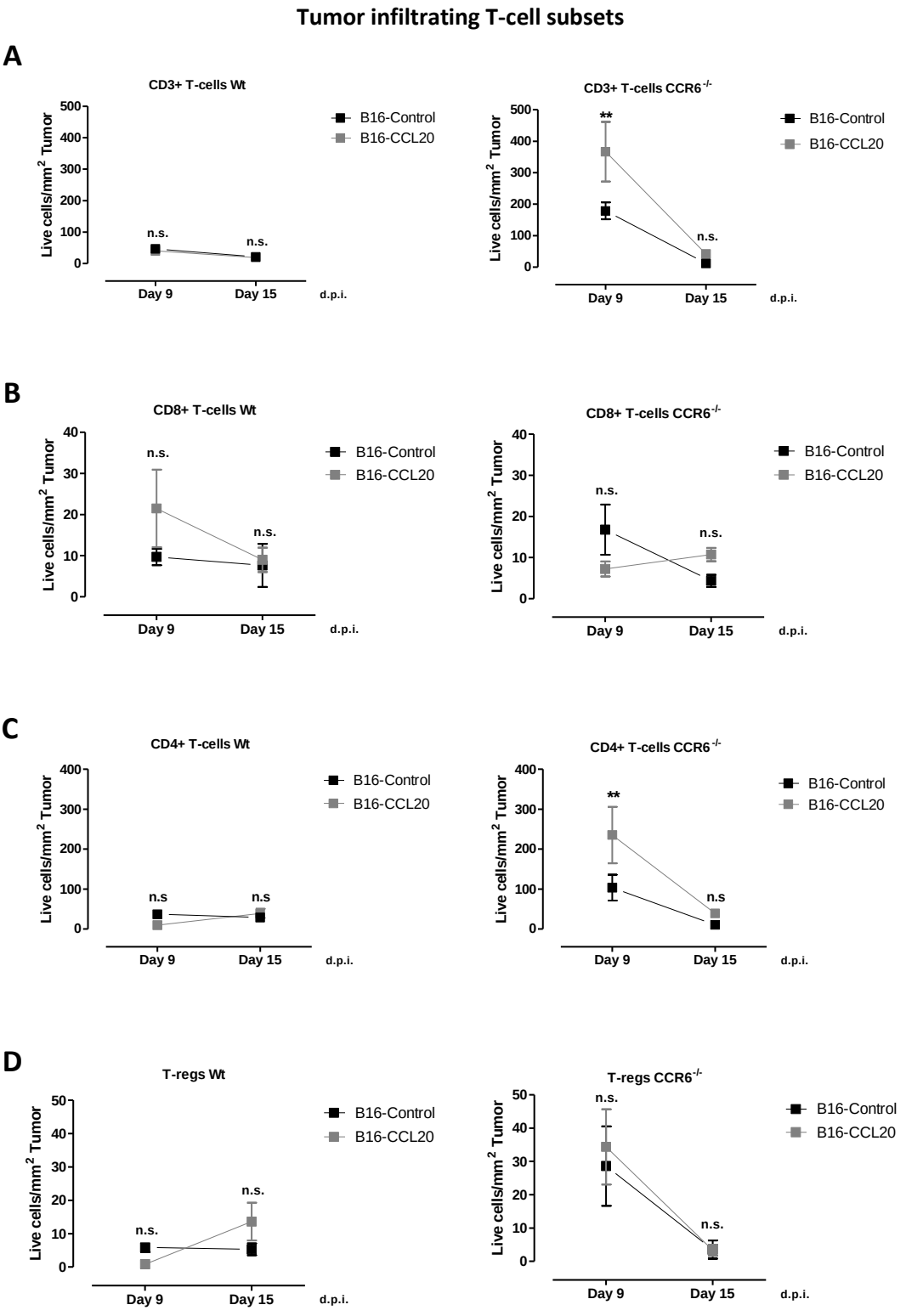


Figure 19. Levels of T-cell TIL subsets at early and late tumor growth stages. Tumors were dissected from Wt and CCR6$^{-/-}$ animals injected either with B16-Control or B16-CCL20 melanoma cells after 9 or 15 days after inoculation. After dissection and digestion the immune fraction was isolated by Percoll gradient and leucocytes were identified

by means of FCM analysis. **A.** Total T-cell number. **B.** CD8+ T-cells. **C.** CD4+ T-cells. **D.** T-regulatory cells (T-regs). Data was obtained from three independent experiments (n=15 mice). Values are expressed as mean ±SEM. Two-way ANOVA, Bonferroni post-test *(** P<0.005, *** P<0.0005*)

5.6.3.3. Dendritic cells

5.6.3.3.1. Conventional Dendritic cells (cDCs) (CD11c+CD45R-)

No significant differences were found between experimental groups within Wt or $CCR6^{-/-}$ animals. At day 9 however, levels of infiltrating cDCs were significantly higher in both $CCR6^{-/-}$ groups compared to their Wt counterparts **(Fig 20A).**

5.6.3.3.2. Plasmacytoid Dendritic cells (pDCs) (CD11C+CD45+)

Infiltrating rate of pDCs was weak or non-existent at analyzed time points in both $CCR6^{-/-}$ experimental groups and in B16-CCL20 injected Wt animals. Interestingly, levels of pDCs were significantly higher in B16-CCL20 inoculated Wt mice at day 9 **(Fig 20B).**

5.6.3.4. Macrophages ($CD11c^{low}$CD45R-)

Infiltrating macrophage rate was significantly higher in $CCR6^{-/-}$ experimental groups at day 9 time point compared to the Wt groups, whose macrophage amounts within the tumor kept more or less constant over time regardless of the melanoma cell line injected. At day 15 macrophage levels in the tumor of $CCR6^{-/-}$ mice decreased considerably **(Fig 20C).**

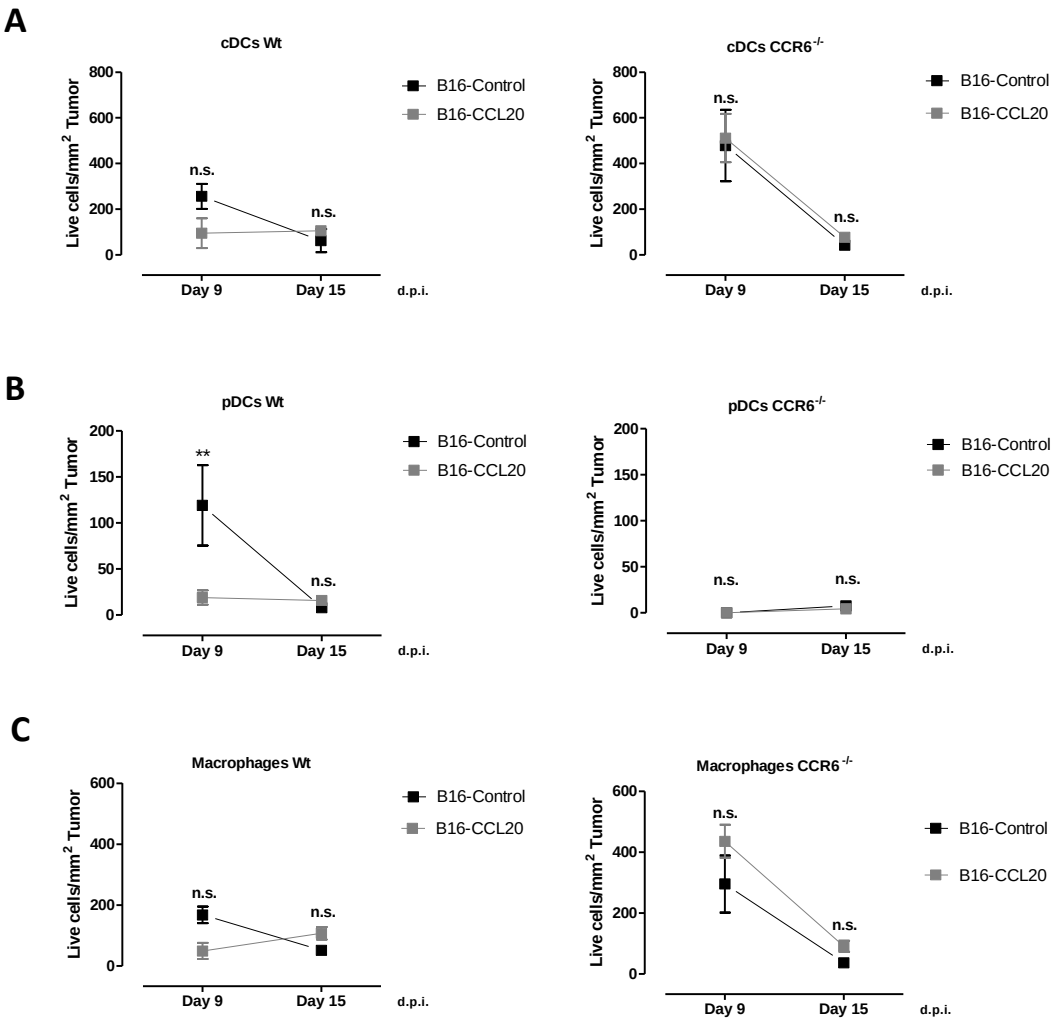


Figure 20. Tumor infiltrating Dendritic cells and Macrophages at early and late tumor growth stages. Tumors were dissected from Wt and CCR6$^{-/-}$ animals injected either with B16-Control or B16-CCL20 melanoma cells after 9 or 15 days after inoculation. After dissection and digestion the immune fraction was isolated by Percoll gradient and leucocytes were identified by means of FCM analysis. **A.** Conventional Dendritic cells (cDCs). **B.** Plasmacytoid Dendritic Cells (pDCs). **C.** Macrophages. Data was obtained from three independent experiments (n=15 mice). Values are expressed as mean ±SEM. Two-way ANOVA, Bonferroni post-test *(** P<0.005, *** P<0.0005*)

5.6.3.5. CCR6+ TILs in Wt mice

Throughout the analysis of TILs in B16-Control and B16-CCL20 injected Wt mice we detected CCR6+ subsets within CD4+ T-cells, T-regulatory, conventional DCs and plasmacytoid DCs. No CCR6+ infiltrate was detected in $CCR6^{-/-}$ mice (data not shown).

5.6.3.5.1. CD4+ CCR6+ T-cells

CD4+CCR6+ T-cell levels were significantly higher within tumors from B16-Control injected Wt mice at day 9, decreasing drastically at day 15. B16-CCL20 injected animals kept low levels of this CCR6+ immune cell subset **(Fig 21A).**

5.6.3.5.2. CCR6+ T-regs

Although infiltrating CCR6+ T-regulatory cell levels were similar at day 9 on both experimental groups, we observed a slightly significant increase at day 15 after tumor cell inoculation in B16-CCL20 injected mice **(Fig 21B).**

5.6.3.5.3. CCR6+ cDCs and pDCs

Regarding dendritic cells, we found significantly higher amounts of CCR6+ cDCs and pDCs in B16-Control injected groups at day 9 after tumor inoculation, followed by a considerable decrease by day 15. On the contrary, B16-CCL20 injected Wt animals showed relatively low levels of this infiltrating CCR6+ DC subsets **(Fig 21C).**

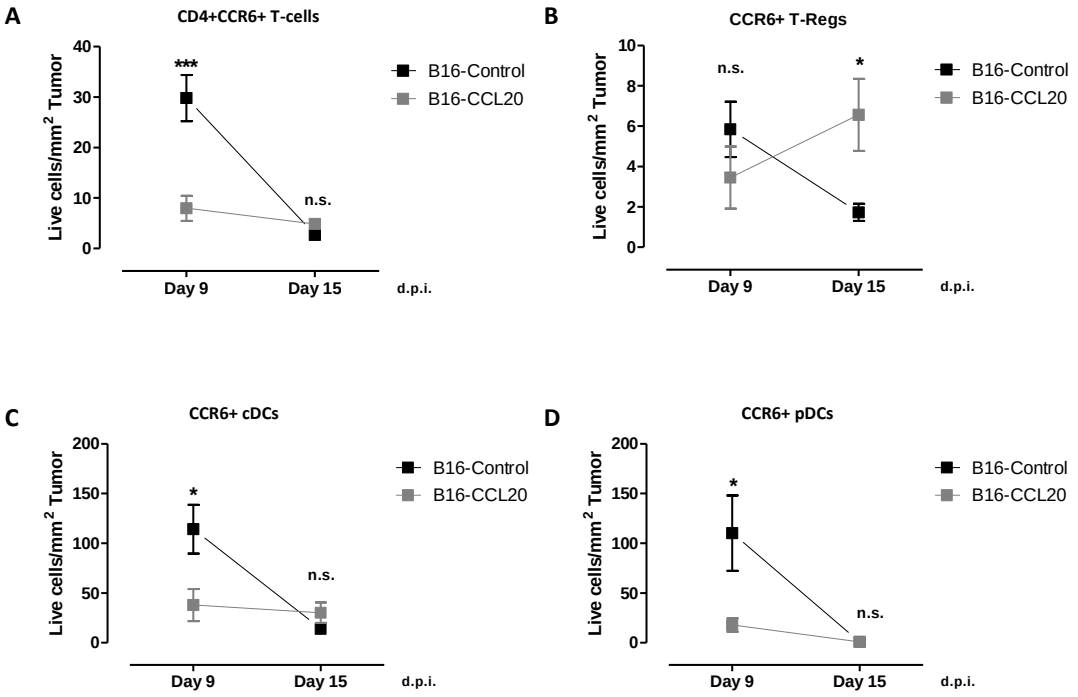


Figure 21. Tumor infiltrating CCR6+ cell subsets in B16-Control and B16-CCL20 injected Wt mice. Tumors were dissected from Wt animals injected either with B16-Control or B16-CCL20 melanoma cells after 9 or 15 days after inoculation. After dissection and digestion the immune fraction was isolated by Percoll gradient and leucocytes were identified by means of FCM analysis. **A.** CD4+CCR6+ T-cell fraction, including CD4+CCR6+ cells. **B.** CCR6+ T-regulatory cells. **C.** CCR6+ Conventional Dendritic cells. **D.** CCR6+ plasmacytoid Dendritic cells. Data was obtained from three independent experiments (n=15 mice). Values are expressed as mean ±SEM. Two-way ANOVA, Bonferroni post-test *(** P<0.005, *** P<0.0005*)

5.7. Ratios between TILs

5.7.1. Aim and introduction

In recent years, the importance that the balance between pro- and anti-tumor leukocytes has over tumor growth, promotion and regression has gained much attention**.** Several clinical studies have endorsed the hypothesis that ratios between different subsets can be good predictors of tumor prognosis**.** Most used ratios are CD8+/CD4+ (effector/helper), CD8+/Foxp3+ (effector/regulatory) and CD8+/pDCs (effector/suppressor) [325].

These three ratios were analyzed using data from the experiments described in section 5.6 in order to obtain a more comprehensive view of the events within the tumor.

5.7.2. CD8+/CD4+ Ratio

All four experimental groups showed low ratios of CD8+/CD4+ at day 9. By day 15 however, Wt mice had significantly higher CD8+/CD4+ ratios than $CCR6^{-/-}$ animals. These differences were a bit higher in B16-CCL20 injected Wt mice, although not statistically different from B16-Control injected Wt animals **(Fig 22A).**

5.7.3. CD8+/Foxp3+ Ratio

The ratio of CD8+ cells to Foxp3+ cells was low in $CCR6^{-/-}$ mice at both analyzed time points. In case of the Wt experimental groups, the ratios increased significantly by day 15. While no differences were observed between Wt groups at this time point in CD8+/Foxp3+ratios, they were significantly higher than in $CCR6^{-/-}$ animals **(Fig 22B)**

5.7.4. CD8+/pDCs Ratio

The CD8+/pDCs ratio increased dramatically in B16-CCL20 injected Wt mice by day 15. B16-Control injected Wt mice and both $CCR6^{-/-}$ experimental groups showed reduced values for this ratio at both analyzed time points during the experiment **(Fig 22C)**

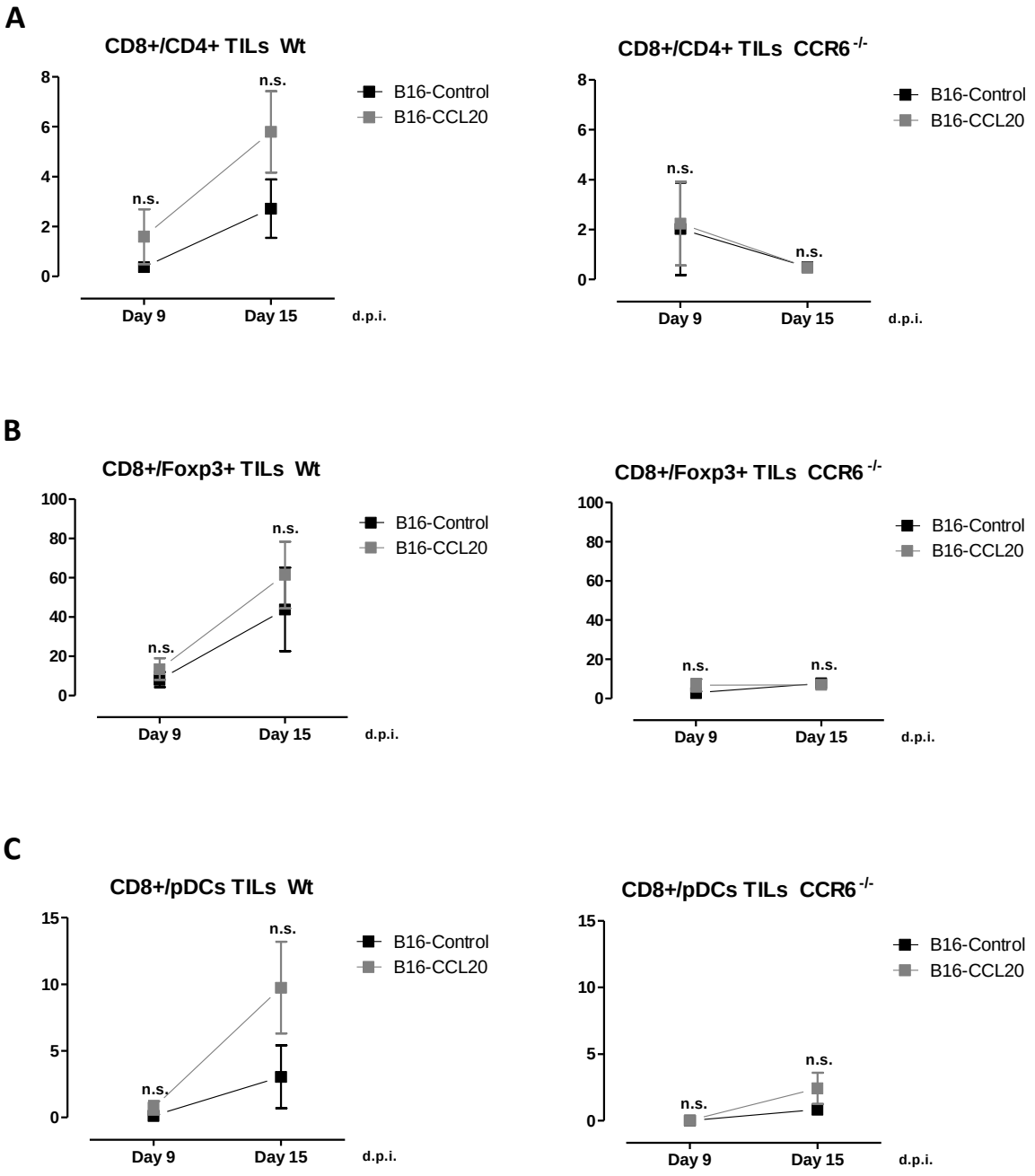


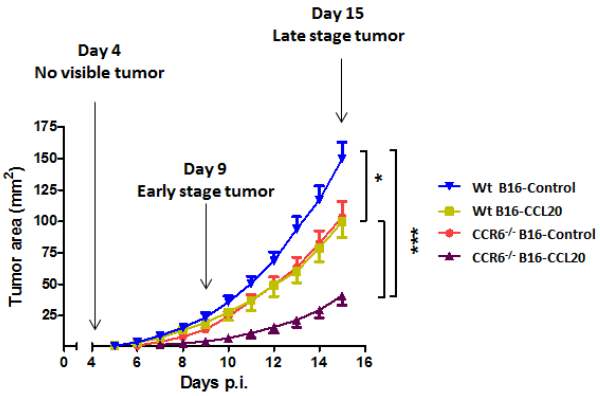
Figure 22. Leucocyte subset ratios in B16-Control and B16-CCL20 injected Wt and CCR6$^{-/-}$ mice. A. CD8+/CD4+ ratio. B. CD8+/Foxp3+ ratio. C. CD8+/pDCs ratio. Data taken from experiments described in 3.5. Values are expressed as mean ±SEM. Two-way ANOVA, Bonferroni post-test.

5.8. Characterization of the immune cell composition of tumor draining Lymph nodes (TDLN) from B16-Control/B16-CCL20 injected Wt and CCR6$^{-/-}$ mice

5.8.1. Aim and introduction

In addition to the characterization of the TILs, we were also interested in possible variations in the composition of these immune cell subgroups within the TDLN that could help us to understand the dynamics of the response from the immune system throughout the different stages of tumor progression. It has been described in previous studies that tumor-derived chemokines can be detected in the DLN [326], thus modulating the immune cell composition within [327].

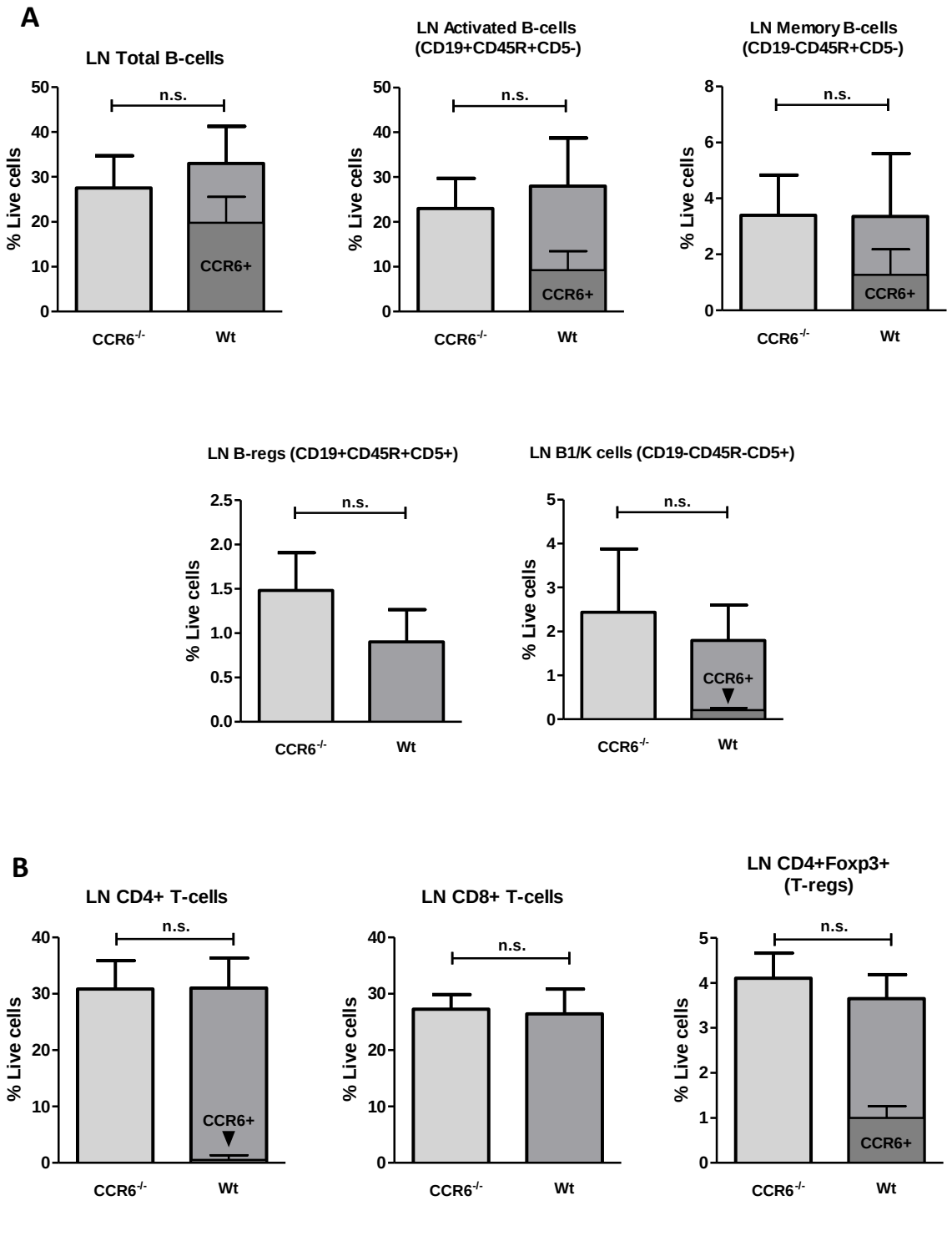
Following this idea we established three different time points to analyze the composition of the DLNs in B16 injected mice: An early time point (4 days after tumor inoculation), a second time point (9 days after tumor inoculation) when solid tumors have been established, and a late time point at day (15 days after tumor inoculation) at the end of the experiment **(Scheme 4).**



Scheme 4. Illustrative scheme showing experimental time points at which tumors got dissected and TILs were analyzed: Early (day 9 p.i.) and late (day 15 p.i.) stage tumors.

FCM gating strategy and identification of the different immune cell subsets described in this section was performed following the same procedure as in section 5.6.

Prior to this experiment, we characterized the inguinal LN immune cell composition from Wt and CCR6$^{-/-}$ individuals under homeostatic conditions (i.e. in the absence of a tumor) to ensure that there were no significant differences in the proportion of any of the selected leucocyte subsets **(Fig. 23)**


A
LN Total B-cells
LN Activated B-cells (CD19+CD45R+CD5-)
LN Memory B-cells (CD19-CD45R+CD5-)
LN B-regs (CD19+CD45R+CD5+)
LN B1/K cells (CD19-CD45R-CD5+)
% Live cells
n.s.
CCR6+
CCR6-/-
Wt
B
LN CD4+ T-cells
LN CD8+ T-cells
LN CD4+Foxp3+ (T-regs)

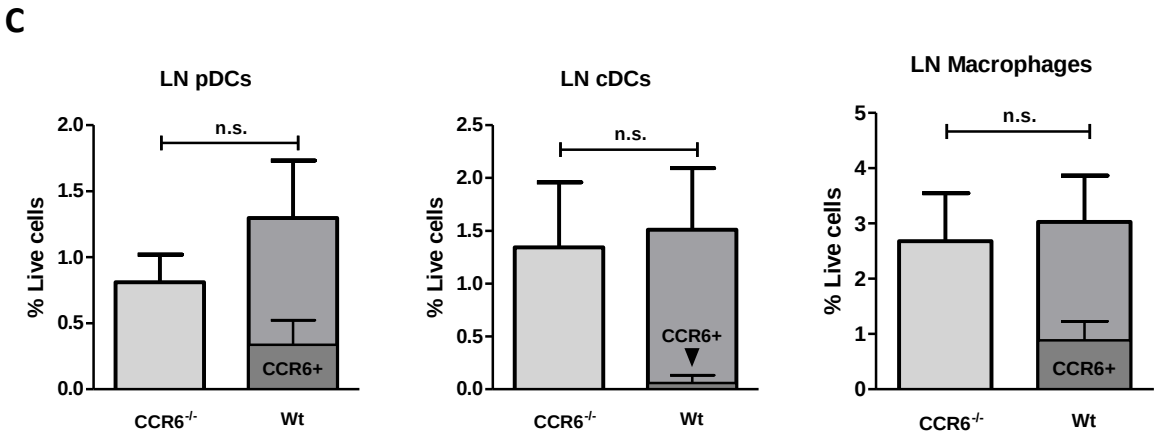


Figure 23. CCR6$^{-/-}$ immune cell proportions in the LNs do not differ from Wt mice under homeostatic conditions. LNs were dissected from Wt and CCR6$^{-/-}$ mice under homeostatic conditions. After mechanical disruption immune cell fraction was isolated and leucocytes were identified by means of FCM analysis. CCR6+ fraction of Wt mice derived cells is indicated as a superimposed bar graph. Each bar represents data taken from independent analysis of 10 mice in each group. Values are expressed as mean ±SEM. Two-way ANOVA, Bonferroni post-test. **A.** B-cell subsets **B.** T-cell subsets. **C.** Dendritic cells and Macrophages.

5.8.2. B-cells.

5.8.2.1. Activated B-cells (CD19+CD45R+CD5-)

With the exception of B16-Control injected CCR6ko mice, all experimental groups showed a slight increase on the proportion of activated B-cells that dropped significantly by day 15. However, the relative amounts of this immune cell subset were significantly similar in all experimental groups. In the case of $CCR6^{-/-}$ mice this contrasts with our previous findings, where we detected significantly increased amounts of this cells infiltrating the tumor at day 9 **(Fig. 24A).**

5.8.2.2. Memory B-cells (CD19-CD45R+CD5-)

The proportion of this B-cell subset in the draining LN decreased slightly compared to healthy individuals and remained constant as long as the experiment lasted. As we commented previously, we observed small amounts of Memory B-cells within the tumor by day 15 **(Fig. 24B).**

5.8.2.3. Regulatory B-Cells (B-regs) (CD19+CD45R+CD5+)

As in the case of Memory B-cells, the proportion of B-regs within the LN remained more or less constant over time and no significant differences were observed between the experimental groups. Interestingly, while no tumor infiltrating B-regs were detected on B16-CCL20 injected $CCR6^{-/-}$ animals by day 9, we observed a discrete increase of these cells within tumors from their B16-Control injected counterparts (Section 5.6.3.1.3) **(Fig. 24C).**

5.8.2.4. B1/K cells (CD19-CD45R-CD5+)

No differences in the proportion of B1/cells were observed in the lymph nodes of $CCR6^{-/-}$ experimental groups. In the case of Wt mice, both experimental groups showed similar amounts of this B-cell subset at early (day 4) and late (day 15) time points **(Fig. 24D).** However, B16-CCL20 injected Wt mice showed a significant increase on the proportion of B1/K cells by day 9. Interestingly, our analysis of TILs showed slightly higher amounts of these leucocytes in this experimental group as well (Section 5.6.3.1.4), although these differences were not statistically significant

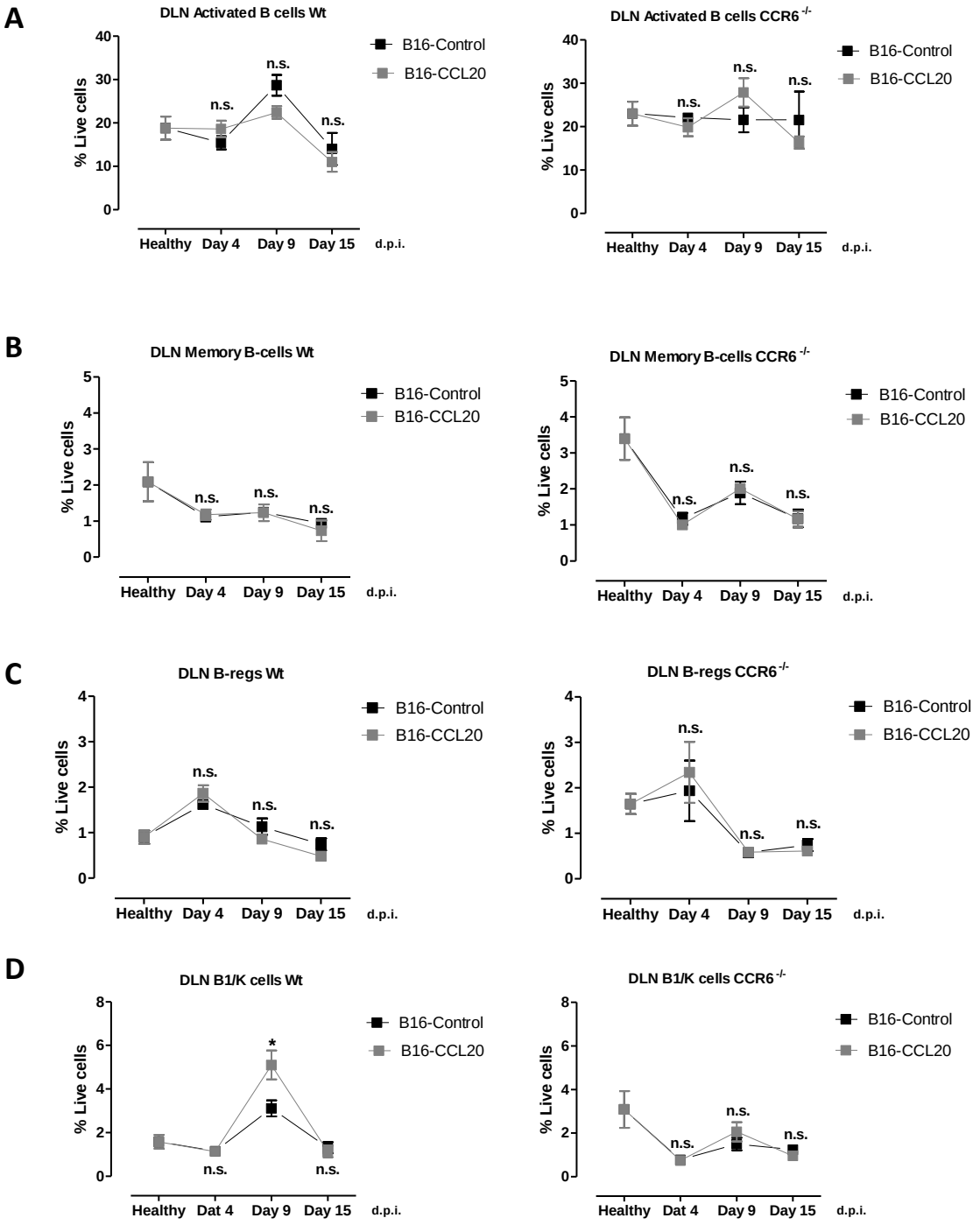


Figure 24. Percentage of B-cell subsets over time in TDLN from Wt and CCR6$^{-/-}$ mice. LNs were dissected from Wt and CCR6$^{-/-}$ animals injected either with B16-Control or B16-CCL20 melanoma cells after 4, 9 or 15 days after inoculation. After mechanical disruption immune cell fraction was isolated and leucocytes were identified by means of FCM analysis.. **A.** Activated B-cells. **B.** Memory B-cells**. C.** B-regulatory cells (B-regs). **D.** B1 and Killer B-cells. Data was obtained from three independent experiments (n=15 mice). Values are expressed as mean ±SEM. Two-way ANOVA, Bonferroni post-test (** P<0.05*)

5.8.3. T-cells (CD3+)

Although no significant differences were detected within $CCR6^{-/-}$ and Wt experimental groups, we observed that amount of total T-cells in B16-CCL20 injected animals behaved similarly over time, in contrast with B16-Control groups which relative amounts of this immune cell type fluctuated more.

It is to be mentioned that while B16-CCL20 injected $CCR6^{-/-}$ mice showed significantly higher amounts of T-cells infiltrating their tumors, this was not reflected in changes in relative amounts of this immune cell type in the lymph nodes in comparison with their B16-Control injected counterparts **(Fig. 25A).**

5.8.3.1. CD8+ T-cells (CD3+CD8+)

All four experimental groups had same relative amounts of CD8+ T-cells at all analyzed time points. As we commented previously, no significant differences in the amount of this T-cell subset were detected among TILs as well. In all cases the proportion of CD8+ T-cells increased significantly at day 9 after tumor inoculation, remaining at normal levels the rest of the time **(Fig. 25B).**

5.8.3.2. CD4+ T-cells (CD3+CD4+)

With the exception of a slight increase on the amount of CD4+ T-cells at the lymph nodes from B16-CCL20 injected $CCR6^{-/-}$ mice at day 4, proportions of these immune cells remained at similar amounts in all four experimental groups at all analyzed time points **(Fig. 25C).** Interestingly, although we detected higher amounts of tumor infiltrating CD4+ T-cells ad day 9 in $CCR6^{-/-}$ mice, the relative amounts of this T-cell subset were similar between Wt and $CCR6^{-/-}$ experimental groups (Section 5.6.3.2.2).

5.8.3.2.1. T-regulatory cells (T-regs) (CD3+CD4+Foxp3+)

Relative amounts of T-regs increased in all experimental groups after tumor inoculation. However, while this proportion decreased progressively over time in all groups, B16-CCL20 injected Wt animals kept constant levels of this T-cell subset.

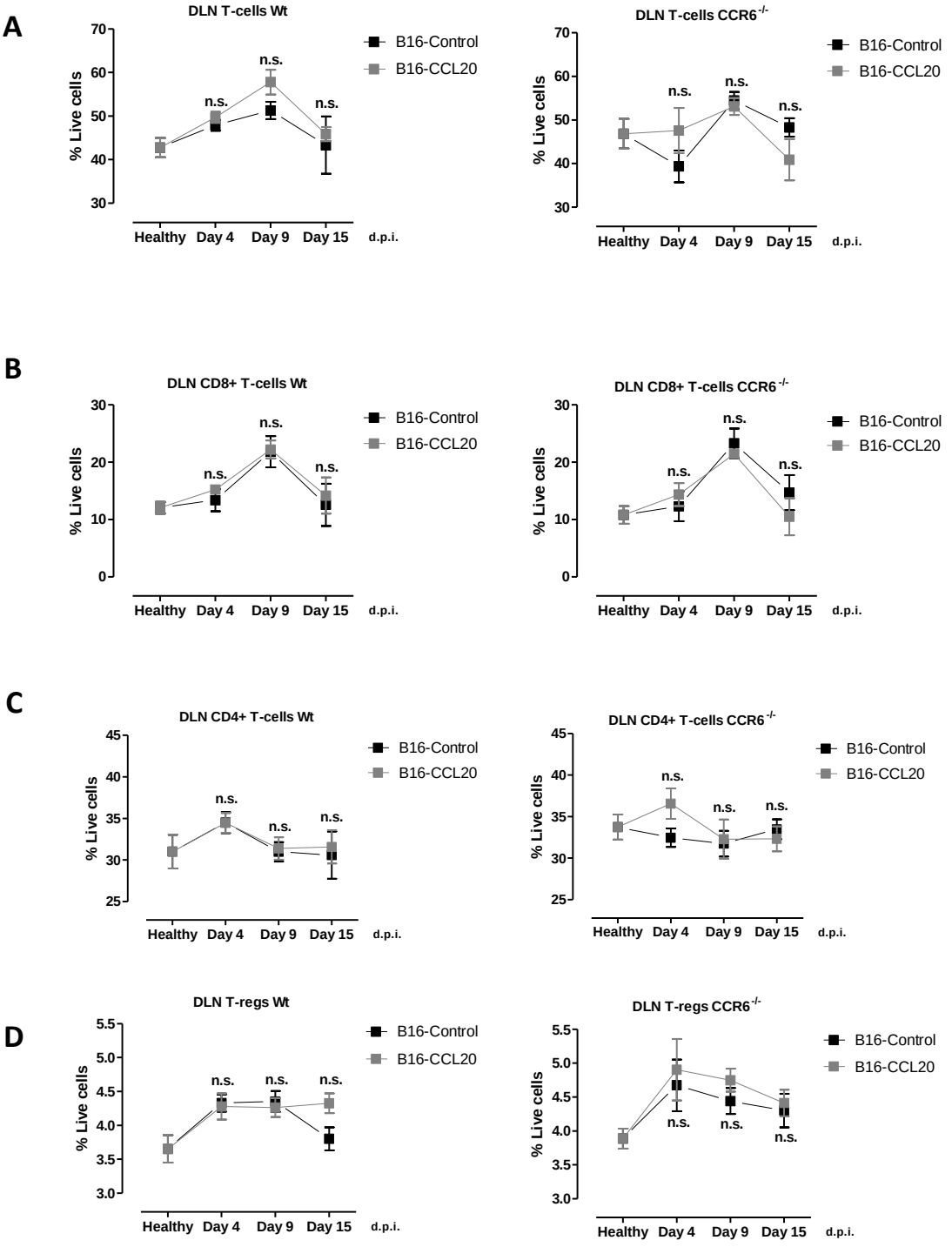


Figure 25. Percentage of T-cell subsets over time in TDLN from Wt and CCR6$^{-/-}$ mice. LNs were dissected from Wt and CCR6$^{-/-}$ animals injected either with B16-Control or B16-CCL20 melanoma cells after 4, 9 or 15 days after inoculation. After mechanical disruption immune cell fraction was isolated and leucocytes were identified by means of FCM analysis. **A.** Total T-cell number. **B.** CD8+ T-cells. **C.** CD4+ T-cells. **D.** T-regulatory cells (T-regs). Data was obtained from three independent experiments (n=15 mice). Values are expressed as mean ±SEM. Two-way ANOVA, Bonferroni post-test.

5.8.4. Dendritic cells

5.8.4.1. Conventional Dendritic cells (cDCs) (CD11c+CD45R-)

No significant differences were observed in the proportion of cDCs between the four experimental groups, although fluctuations over time were slightly different depending on the mice strain and the cell line injected. Our observations of infiltrating cDCs showed no significant differences between groups as well, although CCR6$^{-/-}$ animals presented higher levels of this immune cell subset at day 9 **(Fig. 26A).**

5.8.4.2. Plasmacytoid Dendritic cells (pDCs) (CD11C+CD45+)

The proportion of pDCs in B16-Control experimental groups increased significantly compared to their B16-CCL20 counterparts at day 4. Despite these early differences, the proportion of pDCs at days 9 and 15 was similar in all four experimental groups. Interestingly, although amounts of this immune cell subset fluctuated in the same way within the Lymph nodes of B16-Control injected Wt and CCR6$^{-/-}$, we observed an increase of tumor infiltrating pDCs only in the Wt group **(Fig. 26B).**

5.8.4.3. Macrophages (CD11c^{low}CD45R-)

No significant differences were detected between all four experimental groups in the proportion of macrophages within the DLN. In all cases the relative amount of macrophages decreased compared to healthy conditions **(Fig. 26C).**

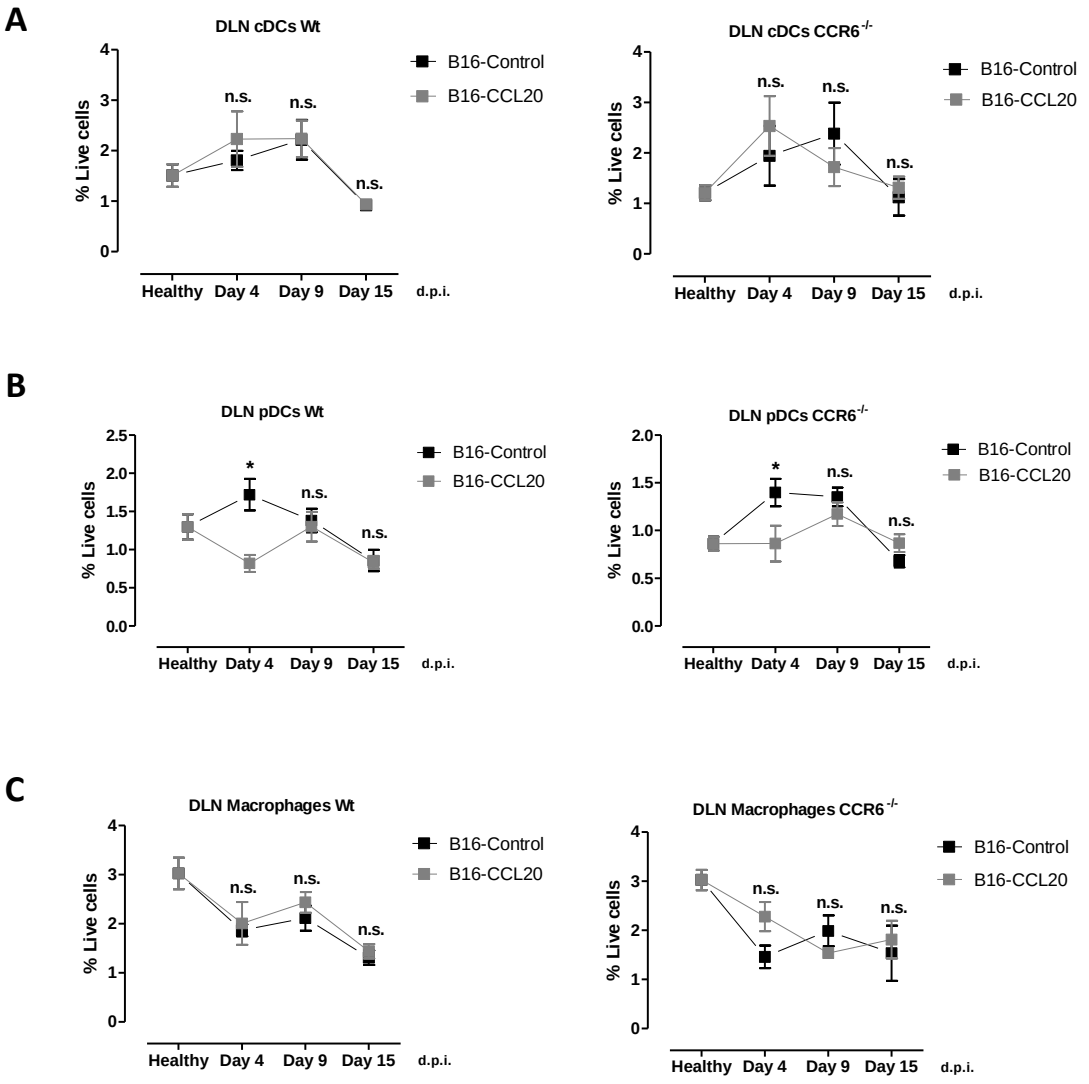


Figure 26. Proportion of Dendritic-cells/Macrophages in TDLN from Wt and CCR6$^{-/-}$ mice over time. LNs were dissected from Wt and CCR6$^{-/-}$ animals injected either with B16-Control or B16-CCL20 melanoma cells after 4, 9 or 15 days after inoculation. After mechanical disruption immune cell fraction was isolated and leucocytes were identified by means of FCM analysis. **A.** conventional Dendritic cells (cDCs). **B.** plasmacytoid Dendritic cells (pDCs). Data was obtained from three independent experiments (n=15 mice). Values are expressed as mean ±SEM. Two-way ANOVA, Bonferroni post-test. *(* P<0.05*).

5.8.5. CCR6+ immune cell subset composition of TDLN from B16-Control/B16-CCL20 injected Wt mice

While the infiltrating CCR6+ immune cell subsets were limited to CD4+ T-cells and dendritic cells, within the lymph node we detected a bigger array of leucocyte types expressing this receptor. In addition to the previously commented, we detected different types of B-cells (Activated, B1/K and memory B-cells) and Macrophages. Interestingly, no CCR6+ cDCs were detected on the lymph nodes.

5.8.5.1. CCR6+ Activated B-cells

Levels of CCR6+ Activated B-cells decreased drastically at day 9 and increased significantly by day 15 in both Wt experimental groups. However, no significant differences were detected between B16-Control and B16-CCL20 injected mice **(Fig. 27A).**

5.8.5.2. CCR6+ B1/K cells

CCR6+ B1/K cell proportion increased progressively till day 9 and decreased slightly by day 15, coming back to the values of healthy individuals. As in the case of Memory B cells, no significant differences were found between both Wt experimental groups **(Fig. 27B).**

5.8.5.3. CCR6+ Memory B-cells

The proportion of CCR6+ Memory B-cells within the Lymph node decreased drastically at day 4 after tumor inoculation compared to healthy conditions. The relative amounts of these immune cell subsets increased slightly by day 9. This phenomenon was observed in both B16-Control and B16-CCL20 injected Wt mice **(Fig. 27C).**

5.8.5.4. CD4+ CCR6+ T-cells

The amount of CD4+CCR6+ T-cells increased drastically in the lymph node at day 4 compared to healthy conditions. However, while in B16-Control injected mice the proportion of this T-cell subset kept decreasing progressively, the relative numbers of CD4+CCR6+ T-cells found in B16-CCL20 injected animals were significantly higher by day 15 **(Fig. 27D).**

5.8.5.5. CCR6+ T-regs

The proportion of CCR6+ T-regs was constant over time after tumor inoculation. However, we observed significant increase of this cell subset in B16-CCL20 injected Wt mice by day 15 **(Fig. 27E).**

5.8.5.6. CCR6+ pDCs

Levels of CCR6+ pDCs decreased drastically after tumor inoculation in both Wt experimental groups. Despite a slight increase on the amount of this cell subset in B16-Control injected animals around day 9, no significant differences were detected between the groups while the experiment lasted **(Fig. 27F).**

5.8.5.7. CCR6+ Macrophages

Similar to CCR6+ pDCs, the relative amounts of macrophages within the DLN of Wt animals decreased drastically compared to healthy conditions and kept low levels until the end of the experiments. Injection of B16-Control or B16-CCL20 cells created no observable differences in the proportion of this immune cell subset **(Fig. 27G).**

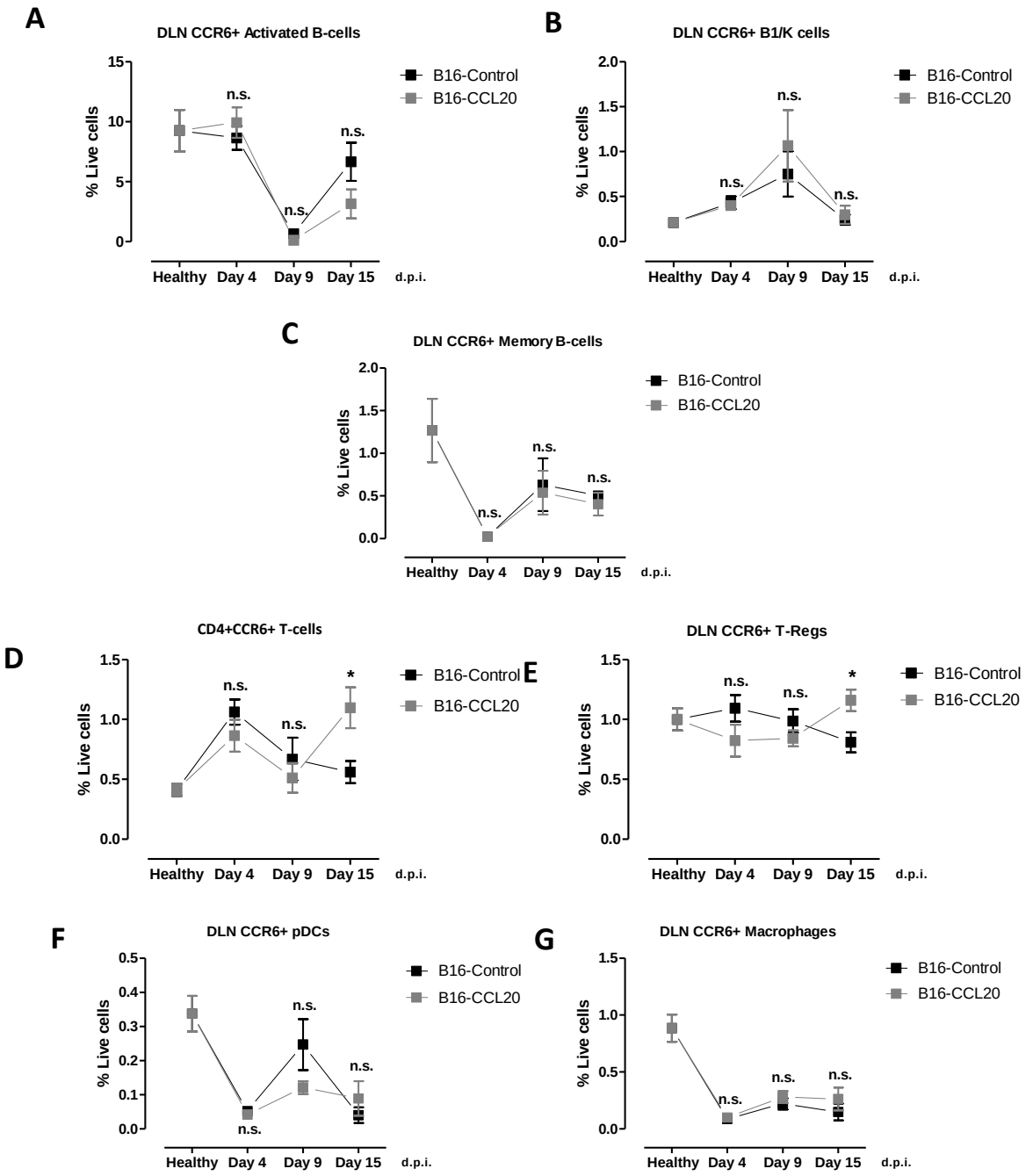


Figure 27. Proportion of CCR6+ immune cell subsets at TDLNs from Wt and CCR6$^{-/-}$ mice over time. LNs were dissected from Wt and animals injected either with B16-Control or B16-CCL20 melanoma cells after 4, 9 or 15 days after inoculation. After mechanical disruption immune cell fraction was isolated and leucocytes were identified by means of FCM analysis. **A.** CCR6+ Activated B-cells. **B.** CCR6+ B1/K B-cells. **C.** CCR6+ Memory B-cells. **D.** CCR6+ CD4+ T-cells. **E.** CCR6+ T-regulatory cells. **F.** CCR6+ pDCS. **G.** CCR6+ Macrophages Data was obtained from three independent experiments (n=15 mice). Values are expressed as mean ±SEM. Two-way ANOVA, Bonferroni post-test *(*P<0.05)*

5.9. Characterization of the inflammatory cytokine composition of B16-Control/B16-CCL20 tumors arising in Wt and CCR6$^{-/-}$ mice

5.9.1. Aim and introduction

The presence or absence of certain cytokines in the microenvironment can affect the immune system acting directly over the leucocytes, or indirectly by modifying the conditions of the tumor microenvironment [328]. The absence of proper chemoattractants or immune cell activators within the tumor may hamper the migration and/or activation of certain immune cells. It has been observed that the immune effector function can be inhibited by suppressor factors secreted by tumor cells [329]**.** In this context, variations in the concentration of CCL20 at the site of a growing tumor may affect the cytokine composition of the tumor microenvironment.

This part of the project sought to analyze the composition of soluble components of the tumor microenvironment in B16 injected experimental animals in order to detect possible imbalances in the levels of 13 different murine cytokines: IL-23, IL-1α, IFN-ɣ, TNF-α, CCL2, IL12p70, IL-10, IL-9, IL-6, IL-27, IL-17α, IL-22 and TSLP

5.9.2. Proportion of IFN-ɣ within the tumor microenvironment is significantly lower in B16-CCL20 injected CCR6$^{-/-}$ mice

Supernatants from dissected and digested tumors of B16-Control and B16-CCL20 injected Wt and CCR6$^{-/-}$ were extracted and analyzed the following day by means of LEGENDPLEX assay **(Fig 28A)**

Data was normalized to tumor size. We observed no significant differences in the levels of the majority of cytokines between the four experimental groups. However, B16-CCL20 CCR6$^{-/-}$ mice showed significantly lower levels of IFN-ɣ when compared to Wt groups and B16-Control injected CCCR6$^{-/-}$ animals **(Fig 28B)**

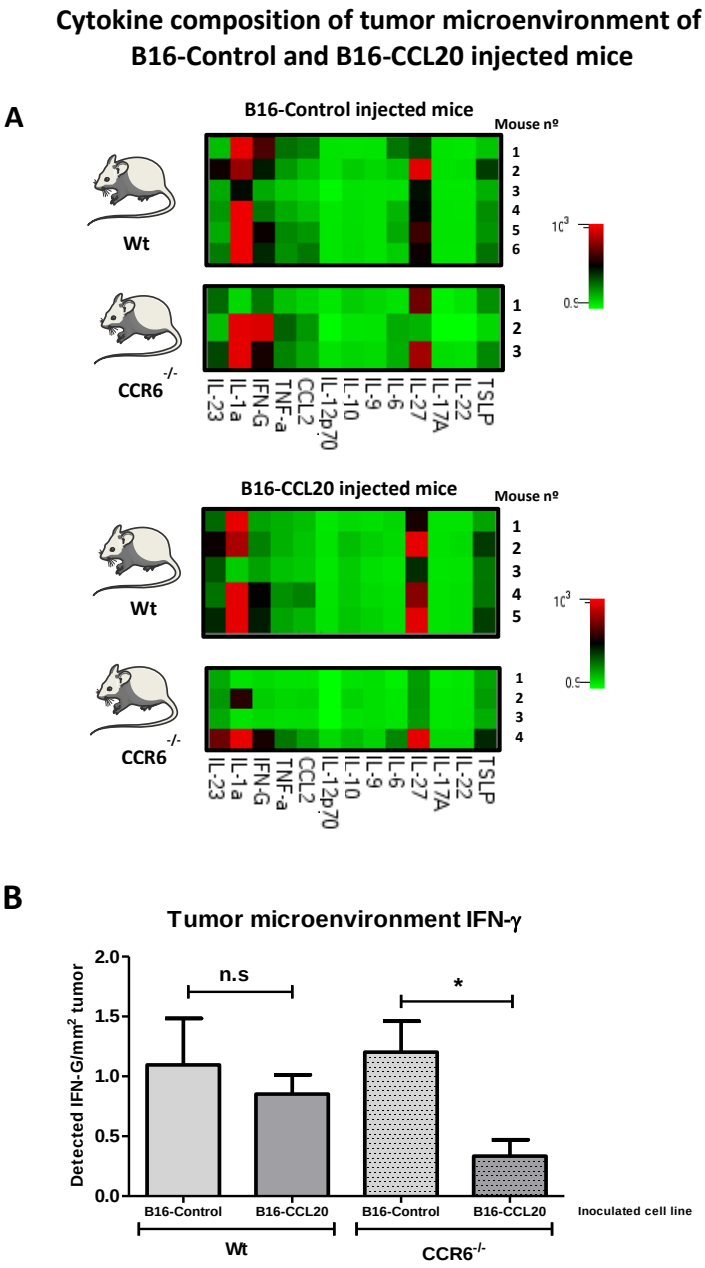
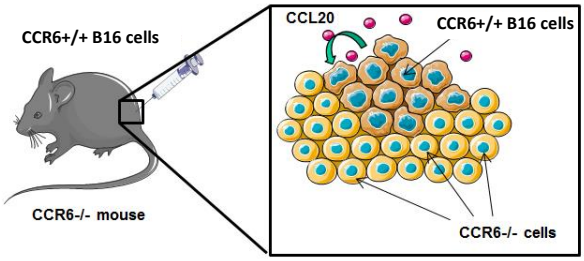


Figure 28. Pro-inflammatory composition of the tumor microenvironment from B16-Control and B16-CCL20 injected Wt and CCR6$^{-/-}$ mice. Solid tumors were dissected at day 15 after B16 cell injections. Tumors were digested and cytokine composition from the supernatants was analyzed by means of LEGENDPLEX. **A.** General overview in form of heatmap from the 13 different cytokines identified (bottom). Rows represent different individuals from each experimental group. Data was normalized to individual tumor size. **B.** Concentration of IFN-γ per tumor mm^2. No significant differences were observed for the remaining 12 cytokines described. Unpaired T-test (** P<0.05*).

5.10. Autocrine signaling pathway in melanoma cells.

5.10.1. Aim and introduction

As shown in previously, we found that overexpression of CCL20 by B16 cells resulted in a significant reduction in tumor progression in CCR6$^{-/-}$ mice. Besides possible effects in the tumor microenvironment, we wanted to study if CCL20 could affect B16-cells in an autocrine manner. Since the different B16 murine melanoma cell lines used in our experiments were CCR6$^{+/+}$, the aim of this part of the project was to assess whether they could be expressing CCR6, since they could be the only CCR6+ bearing cells in these animals **(Scheme 5).**



Scheme 5. Illustrative scheme of CCR6$^{+/+}$ B16 melanoma cells of possible autocrine signaling of CCL20 on CCR6 expressing melanoma cells in CCR6$^{-/-}$ mouse skin.

5.10.2. B16-CCL20 cells express CCR6 in vitro

We analyzed CCR6 expression by means of FCM under homeostatic conditions in our different melanoma cell lines B16-Control, B16-CCL20 and their parental B16 cell line. Our results showed that while B16-parental and B16-Control cells had weak/non-existent expression of CCR6, B16-CCL20 cells showed significant expression of the chemokine receptor **(Fig 29)**

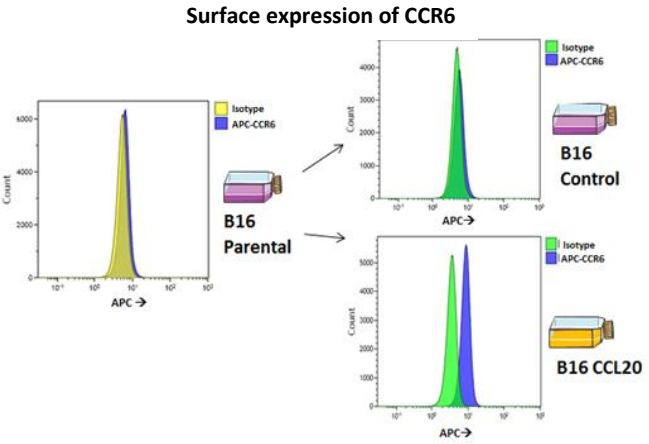


Fig. 29. B16-CCL20 cells express CCR6 in vitro . B16-Control, B16-CCL20 and B16-parental Cells were cultured in 24-well plates for 48h under homeostatic conditions and CCR6 expression was measured by means of FCM. **Yellow.** APC-isotype control signal. **Blue.** APC-CCR6 antibody signal.

5.10.3. rCCL20 does not affect the proliferation capacity of B16-CCL20 and B16-Control cells in vitro

In order to test if recombinant murine CCL20 (rCCL20) may affect the proliferation of our retrovirally transduced cell lines we cultured B16-Control and B16-CCL20 in presence of rCCL20 and measured proliferation after 24 and 48h by means of XTT. Results showed that presence of rCCL20 in the medium had no effect in proliferation when compared to control conditions in both cell lines **(Fig. 30).**

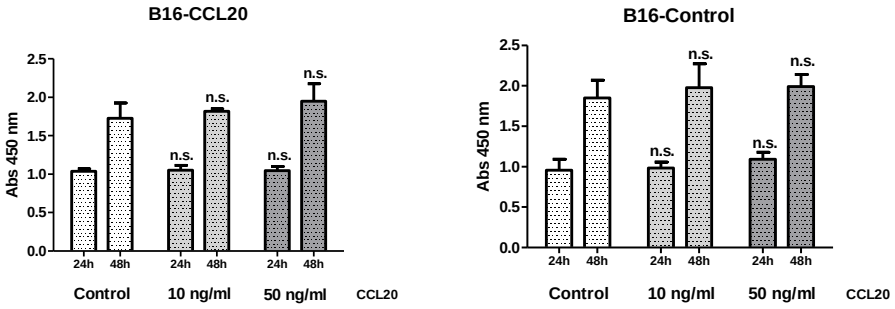


Fig 30. CCL20 stimulation does not modify proliferation capability of B16-Control and B16-CCL20 cell lines. Results of XTT proliferation assay from B16-CCL20 and B16-Control cells under control and CCL20 stimulation conditions for 24 and 48 hours. Bar graphs show mean ±SD from two independent experiments.

5.10.4. rCCL20 reduces expression of certain cytokines of by B16-CCL20 and B16-Control cells in vitro

We analyzed the cytokine composition of supernatants from B16-Control and B16-CCL20 cells cultured in presence or absence of rCCL20 and compared them with the original B16 parental cell line by means of Legendplex. High concentrations of rCCL20 were able to downregulate the expression of some cytokines (such as TNF-α, Il-10, IL-9 and IL-27) in the three cell lines **(Fig 31)**.

5.10.5. Stimulation with rCCL20 increases wound healing ratio of B16-CCL20 but not B16-Control melanoma cells in vitro

The effect of rCCL20 on the migratory capability of B16-CCL20 and B16-Control was tested by means of wound healing assay. Under homeostatic conditions, unstimulated B16-CCL20 showed higher wound healing ratios than B16-Control cells. Further, an in support of an effect of autocrine CCR6/CCL20 interaction the wound healing ratio of CCR6+ B16-CCL20 increased significantly 24h after wound scratch in the presence of high concentrations of rCCL20. However, the presence of rCCL20 did not alter the migration capability of CCR6- B16-Control cells **(Fig 32)**.

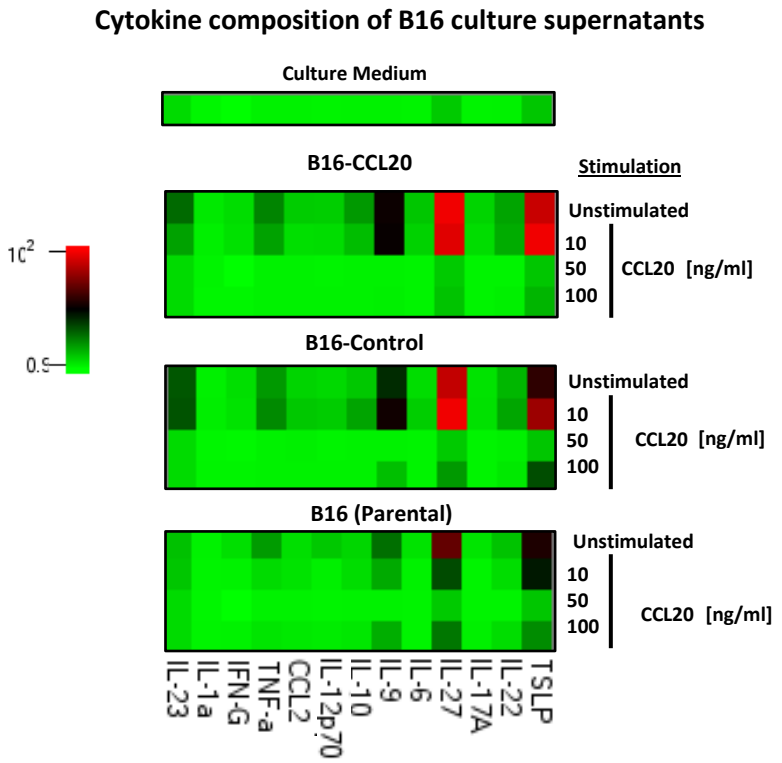


Figure 31. B16-Control and B16-CCL20 respond in the same way to CCL20 and TNF-α stimulation in terms of cytokine expression. B16, B16-Control and B16-CCL20 cells were cultured with and without CCL20 and TNF-α stimulation for 48h. Supernatants were collected and concentrations of 13 different murine pro-inflammatory cytokines were measured by means of LEGENDPLEX. **A.** Expression with and without different concentrations of rCCL20. **B.** Expression with and without different concentrations of TNF-α

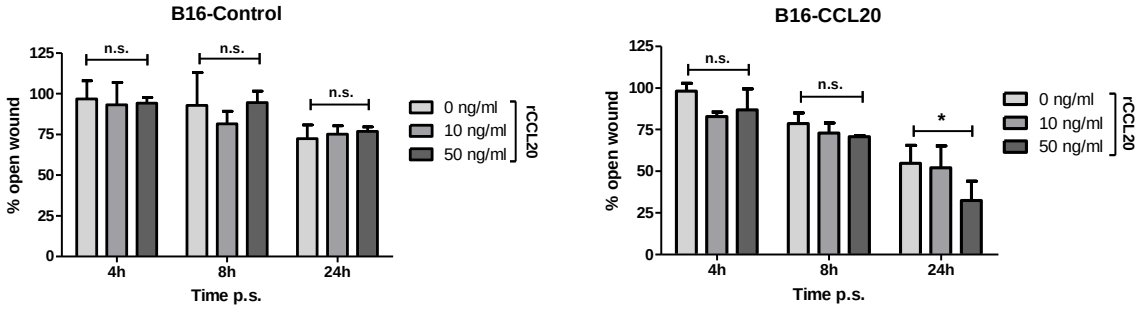


Figure 32. Wound healing rates of B16-Control and B16-CCL20 cells. Bar graphs show mean ±SD from three independent experiments. Student T-test *(*P<0,05)*.

Discussion

6. DISCUSSION

Melanoma tumor initiation, growth and metastasis are regulated by a series of chemokine-controlled mechanisms and various chemokine axes have been studied as potential therapeutic targets for the control of melanoma [330,331,332]. In this context, the CCR6/CCL20 axis is an attractive target due to the mutual exclusivity of CCL20 and CCR6. However, and despite promising results obtained during the study of this chemokine axis in other types of cancer, the precise role of CCR6 and CCL20 in melanoma pathogenesis is still a matter of controversy.

To quote an example of this dissonancy, in 2016 Matsuo and collaborators focused on the impact of CCR6-deficiency on antitumor immunity using a subcutaneous B16 injection model finding no differences between Wt and $CCR6^{-/-}$ mice in terms of tumor progression, relative amounts of TILs and TDLN leucocyte composition [300]. Two years later Samaniego and his group described a relation between CCL20 expressing tumor infiltrating macrophages and poor tumor prognosis [141]**.** The fact that CCR6 is expressed in a huge array of immune cells with very diverse functions, together with the fact that several kinds of cells are able to secrete CCL20 originates most of these controversies.

Groups like the mentioned above have emulated CCL20-deficiency conditions by using anti-CCL20 antibodies to regionally hamper the interactions of this chemokine with its environment. However, while examples like this make a good approach, still leave some loopholes open. First, CCL20 is expressed under homeostatic conditions by several tissues [97]**.** Blocking this chemokine in certain anatomical locations might modify the chemotactic gradient, thus influencing on the migration of certain immune cells towards surrounding tissues. Second, since CCL20 can come from different sources within the tumor (such as tumor cells, stromal components, TILs), CCL20 decrease may trigger its expression in any of these components by autocrine or paracrine feedback loops. Further, CCR6+ subsets have been described among both, immune cell types that drive the activation and those that mediate down-regulation of immune responses.

To illustrate the dilemma derived from this experimental models for chemokine-axes we can take a look at CCR7 in cancer. When expressed by B16 cells, CCR7 increases tumorigenecity and metastatic rate of melanoma tumors in mice. However, when expressed by tumor infiltrating dendritic cells, these immune cells migrate towards regional lymph nodes and activate cytotoxic CD8+ T-cells, resulting in a strong anti-

tumor response [270]. Hence, treating melanoma tumors with CCR7 blockers/inhibitors will not result in a clear predictable outcome.

Complementing previous studies, in this project we made a combined approach of both CKR-knock out animals and local over- and under-expression of the chemokine at the tumor site to clarify the role of the CCR6/CCL20 axis in melanoma tumor progression. In addition to the study of CCR6 deficiency using $CCR6^{-/-}$ animals we have focused on the characterization of the effects of tumor derived CCL20 in melanoma pathogenesis and progression.

6.1. In vitro characterization of CCL20 expression in human and murine melanoma cell lines under homeostatic and inflammatory conditions

Unlike the majority of chemokines, CCL20 can be expressed under both homeostatic and inflammatory conditions. In the skin, its antimicrobial properties support the skin's barrier function against occasional bacterial infections [333]. Under homeostatic conditions CCL20 is expressed in the skin mainly by keratinocytes [334]. On the other hand, upon inflammatory conditions CCL20 is overexpressed in the epidermis functioning as a chemo-attractant for CCR6+ immune cells. This mechanism has been described as a major pathogenic factor in inflammatory skin diseases such as psoriasis [162].

CCL20 expression by human melanocytes upon inflammatory stimuli has been described as well [335]. In melanoma, various groups have reported inducible expression of CCL20 by tumor cells [297].

In line with these observations, we have observed that under homeostatic conditions *in vitro* CCL20 expression is weak or non-existent, but could be triggered under stimulation with pro-inflammatory cytokines such as TNF-α or IL1-α in both the murine and human melanoma cell lines we tested.

TNF-α and IL1-α are found at high concentrations within the tumor microenvironment, thus potentially facilitating migration of CCR6+ immune cells towards CCL20 expressing tumors. Nevertheless, in vitro conditions may not properly reflect how these cells would behave *in vivo*. The cytokine milieu within the tumor microenvironment comprises many different factors, and CCL20 expression by tumor cells could vary depending on the combination of these. For instance, it has been reported that TGF-β, a cytokine reported to be present in melanoma tumors [336] downregulates CCL20 expression [337].

6.1.1. Loss of chemokine expression in vitro or the "passage effect"

In vitro CCL20 production capability of human and murine melanoma cells decreased progressively over passages up to a point where stimulation with pro-inflammatory cytokines like mTNF-α could not trigger its expression anymore.

It has been reported that in vitro culture conditions may modify the expression of several genes over time [338] and cells that have been overly cultured show malfunctions, reduced proliferation or get senescent [305,339]. In our case, loss of CCL20 expression capability started to take place after a few passages.

Collaborator Professor Fran Balkwill from the Barts Cancer institute in London had been experiencing similar phenomena in different cell lines regarding the expression of several cytokines/chemokines, and suggested the use of early passaged cells for experimental use. Reasons of chemokine expression loss over time are not yet well understood. Some groups like Pivarcsi and collaborators have suggested that as the tumor is growing cells downregulate the expression of chemokines through MAPK-signaling pathways in order to evade host antitumor immune responses [265]. Other groups have observed similar phenomena in CKR expression. After isolating bone marrow stromal cells and culture them for several passages, Honzarenco and his team reported loss of expression from some CKRs, although in this case this reaction was followed by a decrease in the proliferation capabilities and higher apoptotic rates [340].

Aside from the molecular mechanisms that reduce chemokine expression capability in tumor cells, we observed that these fluctuations of CCL20 expression could influence our in vivo experiments. We saw that there was a correlation between the decrease in the CCL20 production capability of B16 cells and tumor growth and progression in Wt and $CCR6^{-/-}$ mice. These differences were significantly higher between $CCR6^{-/-}$ individuals.

Although our first experiments gave us some hints on how CCL20 and CCR6 could influence melanoma progression, the variations in CCL20 expression capability of B16 melanoma cells affected the accuracy of our experimental injection model.

Generation of B16-Control and B16-CCL20 cells responded to the need of accurate positive and negative controls to study the effect of CCL20 expression by B16 melanoma cells in tumor progression and

immunosurveillance. Once generated and tested, inoculation in Wt and CCR6$^{-/-}$ mice gathered all the conditions to study the role of CCR6 and CCL20 in vivo, alone and combined. Hence, on one hand we could study the effect of CCL20 in melanoma progression in presence/absence of CCR6 and on the other hand we used the low/none CCL20 expressing B16-Control cell line to short out the influence of CCR6 under CCL20 deficiency conditions. In any case, although B16-CCL20 cells could express high amounts of CCL20 under homeostatic conditions, its expression was lost after several passages as well. Therefore in pursuit of accuracy, early passage cells had to be used for experimentation.

6.2. Characterization of CCR6/CCL20-dependent immune responses in a transplantable B16-melanoma model using WT and CCR6$^{-/-}$ mice.

6.2.1. Impact of CCL20-overexpression by B16 cells on tumor growth kinetics.

Our mouse model for the study of murine melanoma using B16-CCL20 and B16-Control injected mice corroborated our conjectures that CCL20 could influence tumor growth. The outcome of the effect was different between Wt and CCR6$^{-/-}$ mice.

On one hand, Wt mice showed slightly smaller tumors at the final stages of the experiment upon CCL20 overexpression of melanoma cells. The presence or absence of this chemokine did affect neither the onset nor the early tumor growth. It was in the last stages of the experiment that tumor growth slowed down in B16-CCL20 injected Wt animals.

On the other hand, B16-CCL20 injected CCR6$^{-/-}$ mice showed significantly reduced tumor growth compared to their control littermates as well. However, the observed differences in final tumor were initiated at early tumor progression stages (days 4-8). Tumor onset in B16-Control injected CCR6$^{-/-}$ mice did not differ significantly from Wt experimental groups. In fact, tumor sizes from B16-CCL20 injected Wt mice at day 15 were almost similar to the ones from B16-Control CCR6$^{-/-}$ animals at day 15. In these three experimental groups tumor growth gradient was significantly higher during the first days after becoming visible and got stable over time.

Injecting B16-Control and emulating the conditions of B16-CCL20 tumors by administration of rCCL20 periodically could reproduce these observations. Moreover, and despite not reaching statistical significance, emulating the opposite situation using anti-mCCL20 antibodies in order to hamper CCL20 effects in B16-CCL20 tumors resulted in a slight increase of tumor growth in CCR6$^{-/-}$.

On the contrary tumors from B16-CCL20 injected CCR6$^{-/-}$ mice did not become visible till 4-5 days later than the rest of the experimental groups and showed a constant tumor growth gradient after that. Hence we can differentiate two effects caused by CCL20: An effect on tumor progression in Wt mice and on tumor initiation in CCR6$^{-/-}$ mice. Two questions arise from these observations. First, how is it possible that CCL20 has such a noticeable impact on animals that lack its unique and exclusive receptor? And second, why does CCL20 not cause a similarly significant effect in Wt mice?

6.3. The tumor microenvironment

6.3.1. Cellular components of the tumor microenvironment

6.3.1.1. TILs in WT mice

We observed that B16-CCL20 injected Wt mice developed smaller tumors than their control littermates. Interestingly the relative amounts of the overall analyzed TILs were similar in both experimental groups at the end of the experiment (day 15 after tumor inoculation) with the only exception of increased amounts of CCR6+ T-regulatory cells in B16-CCL20 injected mice. More differences were observed between groups at day 9 after inoculation where levels of overall pDCs and CCR6+ fraction of some immune cell subsets (pDCs, cDCs, CD4+ T-cells and T-regs) were significantly higher in B16-Control injected animals.

It could be possible that an accumulation of these immune cell subsets during early stages of tumor development could hamper the anti-tumor immune response or simply enhance tumor growth. In a recent human study, a correlation between lower amounts of circulating pDCs and negative tumor prognosis was observed [341]. It could be possible that pDCs accumulate at the site of the tumor, resulting in a pro-tumorigenic outcome. Accumulating amounts of T-regs would also contribute to this outcome due to their immunosuppressive function.

On the other hand, the fact that we found increased amounts of CCR6+ immune cells within early stage tumors of B16-Control injected mice caught our attention. Regardless of the differences in the outcome on melanoma progression, we anticipated higher migration of CCR6+ leucocytes in B16-CCL20 injected mice (as it is the case for CCR6+ T-regs). It has been reported however, that in some cases CCR6 expression by immune cells does not imply a migratory-response to the presence of CCL20 [113].

However, there are also other considerations that could help finding an explanation for our observations. The absence or low concentrations of CCL20 at the tumor site leave CCR6 free of any ligand, thus making it detectable by FCM antibodies, while CCR6 turnover after CCL20-binding in B16-CCL20 tumors would be enhanced reducing the time gap when the receptor is free to bind detection antibodies. In line with this idea, it is possible that in B16-CCL20 tumors, CCR6+ immune cells migrate to regional LNs downregulating CCR6 expression after ligand binding, as reported for some APCs in melanoma were downregulation of CCR6 is followed by CCR7 expression, which leads the cells towards

regional lymph nodes. A passive diffusion of tumor-produced chemokines towards the LN could be the cause of this migration, as has been described in some studies [326].

Another possible explanation to this phenomenon would be that the differences in CCL20 concentration within the tumor microenvironment could be modifying the conditions (inducing the secretion of other chemokines, for example) that trigger the migration or activation of different immune cell subsets. In this situation CCR6+ immune cells could migrate following CCR6-independent chemo-attractant gradients were CCR6 would play a secondary role.

6.3.1.2. TILs in CCR6$^{-/-}$ mice

As it was the case in both experimental groups of Wt mice, the relative amounts of the analyzed TILs were similar between the CCR6$^{-/-}$ experimental groups at day 15 after inoculation. This was also the case for most of the analyzed immune cell subsets at day 9 with the exception of activated B-cells, B-regs, overall T-cells and CD4+ T-cells, which were significantly higher in B16-control injected mice at this time point.

Considering that the only difference between B16-Control and B16-CCL20 tumors is the release of mCCL20 to the tumor microenvironment, differences in TILs could again be explained by changes in the cytokine composition of the former. Previous studies have reported similar situations. For instance, some years ago Liao and collaborators observed that B-Cells responded to CCL20 without expressing CCR6 at protein or mRNA level [114]**.** This type of observations can be due to indirect effects caused by CCL20, or direct effects triggered by different chemokines binding to CCR6, like CCL18 or CCL5 [110].

Regarding the differences we observed in for some TIL subsets, B-cells have been associated with good melanoma tumor prognosis due to their role in the activation of T-cells [250]. Surprisingly increased amounts of these cells, alongside with T-cells, resulted in increased tumor progression, unlike in B16-CCL20 injected CCR6$^{-/-}$ mice. In recent years, infiltrating B-cells are increasingly being reported in melanoma and other tumor types, and their contribution to the final outcome has led to antagonistic conclusions [342]**.** It is being debated that the possible origins of this controversy could originate from differences in the specific micro-anatomical locations infiltrated by B-cells, the different surface markers used and the assumption that B-cells migrate as a result of a tumor antigen-specific immune response or simply as a response to inflammatory signals [343].

In our study, we found increased levels of B-regs within the B-cell fraction. High amounts of this subset could be the cause of increased tumor growth due inhibition of the immune responses, in the same way that it has been reported in various cancers [344].

As described in section 5.7, no differences were found in the studied immune cell ratios within Wt or CCR6$^{-/-}$ experimental groups. CD8+/CD4+, CD8+/Foxp3+ and CD8+/pDCs ratios were higher in Wt than in CCR6$^{-/-}$ groups at day 15. However, despite of these differences B16-CCL20 injected Wt and B16-Control injected CCR6$^{-/-}$ groups displayed similar tumor sizes at the end of the experiment. On the other hand, despite of the similarities in the analyzed immune cell ratios, tumor onset, growth and progression were significantly different between the two CCR6$^{-/-}$ experimental groups.

While we cannot conclude that CD8+ ratios with respect to CD4+ T-cells, T-regs and pDCs are irrelevant based on these observations, additional research is required to clarify the impact of these immune cell ratios on melanoma progression. It should be mentioned as well that detecting cells within the tumor gives valuable information but state of the activation of those cells may need to be analyzed in order to more accurately elucidate the magnitude and type of the immune response in each case.

6.3.2. Soluble components of the tumor microenvironment

Besides changes in the composition of TILs, differences in tumor growth caused by CCL20 could also be explained by changes in the tumor microenvironment. As commented previously during the introduction, it is well known that CCL20 can interact with various elements of the tumor microenvironment. For instance, Hasan and collaborators showed in 2006 that cathepsins present within melanoma are able to bind, cleave and inactivate CCL20 [297]**.** In the same line, it is highly likely that CCL20 could bind one or more proteins or elements within the tumor microenvironment and activate/block certain signaling pathways.

In our experimental model, CCL20 concentration in the microenvironment of B16-CCL20 injected CCR6$^{-/-}$ mice would create an imbalance of certain components that would end up affecting tumor growth like, for example, the expression or downregulation of specific growth factors necessary for tumor progression, or by altering the adhesion to collagen [345]. On the contrary, in Wt mice surrounding CCR6 expressing cells/tissues would take CCL20, thus reducing its concentration at the tumor microenvironment. The only difference we could detect between the TMEs of our experimental groups

was a reduction on the concentration of INF-ɣ in B16-CCL20 injected CCR6$^{-/-}$ mice. Nevertheless further research is necessary in order to test this hypothesis.

Finally, changes in the composition of the tumor microenvironment could be the indirect cause of differences in TIL composition between B16-CCL20 and B16-Control injected mice. It is highly likely that in addition to the detected changes in INF-ɣ other cytokines could be affected, thus attracting or activating different subsets of immune cells.

6.4. TDLN Immune cell compositions

Besides analyzing TIL in our four experimental groups, we were also interested in the immune cell composition of the TDLN. It has been observed that tumor produced cytokines and chemokines can diffuse from the skin to the regional lymph nodes [346] and as already commented, TILs like some APCs migrate towards regional lymph nodes once they have reside within the tumor in order to enhance the antitumor immune response [326]. In a recent study, researchers were able to enhance the anti-tumor immune response in mice by injecting with a DNA vaccine consisting on the CCL20 sequence tagged with the melanoma antigen Gp100. In treated mice, enhanced APC activation of CD4+ and CD8+ resulted in a better prognostic outcome [299]. In sum, relation between CCL20/CCR6 axis and LN related immune responses had to be analyzed in our project as well.

6.4.1. TDLN immune composition in Wt mice

At day 4 after tumor inoculation, the TDLN immune cell composition of B16-Control and B16-CCL20 injected mice was vastly similar. The only observed differences were found in B16-Control Wt animals, which showed significantly higher numbers of pDCs. Interestingly amount of TIL pDCs in this experimental group was significantly higher at day 9 as well.

Similar findings were observed at day 9 after tumor inoculation, where the only observed differences were in terms of B1/K cells, which were significantly higher in the B16-CCL20 injected group. Later on at day 15, a higher accumulation of CCR6+ CD4+ T-cells and CCR6+ T-regs was observed in this group as well. The accumulation of CCR6+ immune cells in regional lymph nodes of B16-CCL20 injected mice could point out that B16 derived CCL20 could have been diffused from the skin towards the LN as it has been previously reported for skin-derived CCL27 [326].

6.4.2. TDLN immune composition in CCR6$^{-/-}$ mice

Interestingly, despite highly significant differences in final tumor size, the onset and progression, immune cell composition of TDLN of B16-Control and B16-CCL20 injected CCR6-/- mice was highly similar for all immune cell subsets selected at all time points analyzed. The only exception was observed at day 4 after tumor inoculation, where B16-CCL20 injected animals showed higher levels of pDCs compared to controls.

This phenomenon was also observed at day 4 in TDLN from B16-Control injected Wt mice. This could point out to a critical role of pDCs at early tumor stages. As we have observed before, B16-Control injected CCR6$^{-/-}$ mice had higher relative amounts of certain T-cell and B-cell subsets at day 9 after tumor inoculation. It can be hypothesized that early accumulation of pDCs in TDNL of B16-CCL20 injected CCR6$^{-/-}$ mice would hamper the migration of these subsets towards the tumor, leading to a slowdown of tumor growth.

Additional research is required in order to analyze the precise role of those T-cell and B-cell subsets in the overall tumor outcome at early stages. Nevertheless, the fact that increased CCL20 expression at the tumor site promotes early accumulation of pDCs in the TDLN of CCR6$^{-/-}$ opens up new questions that would require further research as well.

6.5. Study of potential autocrine effects of CCR6/CCL20 interactions in melanoma

Besides functioning as a chemoattractant of immune cells through CCR6, CCL20 can play different roles within tumors interacting/affecting other components or processes, or even the same tumor cells.

6.5.1. Can CCL20 stimulate B16 tumor cells in an autocrine manner?

Co-expression of CCR6 and CCL20 by malignant tumor cells has been reported in various epithelial cancers (nasopharyngeal tumors, colorectal cancer, lung and pancreatic adenocarcinomas), pointing to autocrine self-stimulation pathways [176]. Regarding melanoma, in a recent publication Samaniego and collaborators suggested that CCL20 interacts with cells in the tumor stroma through a paracrine signaling loop. While this may provide a plausible mechanism that could explain our observations in Wt mice, it cannot be applied to the $CCR6^{-/-}$ model [141].

As commented previously, the B16 melanoma cell lines used in this project were $CCR6^{+/+}$. Thus, this opens the possibility for a hypothetical autocrine signaling pathway in which B16-derived CCL20 would bind CCR6 receptors on the surface of the same tumor cells triggering a signal affecting the tumorigenic capability of melanoma cells. In line with this hypothesis, other chemokine axes have been found to affect melanoma progression in an autocrine manner. This is the case of CXCL1, CXCL2 and CXCL8, that regulate melanoma tumor progression influencing tumor growth, angiogenesis and metastasis through CXCR1 and CXCR2 [347]; or CXCR4 and CXCL12, which can regulate local invasion and metastasis [277]. In the case of CCL20, Several reports have pointed out autocrine interactions of this chemokine with cancer cells such in bone metastasis [348], lung adenocarcinoma [349], colorectal liver metastases [350] or human erythroid leukemia [351] for example. Additionally, CCL20 appears to have a direct role over the regulation of CSCs [352].

To test this hypothesis in our model, we first studied CCR6 expression in our melanoma cell lines. We were able to detect CCR6 expression by B16-CCL20 cells, but not by B16-parental or B16-Control cell lines. However CCL20 did not affect in vitro growth in neither of the cell lines and, although upon high concentrations of CCL20 in the medium the expression of certain pro-inflammatory cytokines was reduced, the effect was the same in both cell lines.

Pointing to a possible CCL20 autocrine-loop in B16-CCL20 tumors, B16-CCL20 showed higher migration rates than B16-Control cells in a wound healing assay in vitro , and stimulation with CCL20 increased the wound healing rate even more in B16-CCL20 cells but had no effect at all in B16-Control cells.

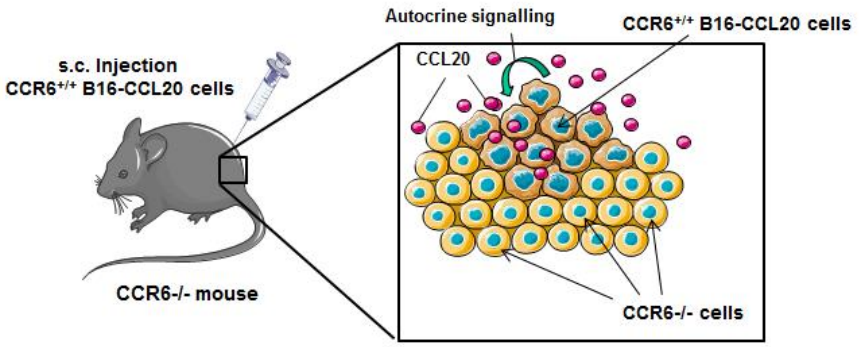
6.5.2. Does CCL20 upregulate CCR6 expression?

When B16-Control injected CCR6$^{-/-}$ mice are inoculated periodically with recombinant CCL20, we observe significant differences in tumor growth compared to controls. It seems possible that upon high concentrations of CCL20 in the tumor microenvironment, B16 cells would upregulate CCR6 expression. CCR6 upregulation upon CCL20 stimulation has been reported in hepatocellular carcinoma [353]. In our case, the only evidence we have so far of a direct effect of CCL20 on B16-Control cells is that in presence of the former, the expression of some cytokines are downregulated in the latter. This effect could also be observed in B16-CCL20 cells and the parental B16 cell line.

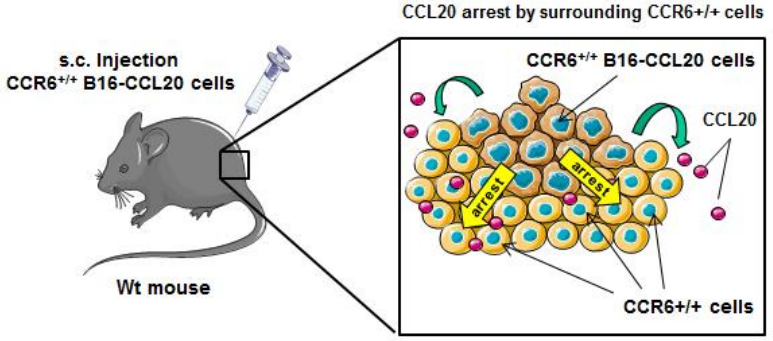
However, if CCL20 would affect tumorigenic capabilities of B16 cells through an autocrine loop, why do we not observe delayed onset and reduced tumor growth in Wt animals as well? Theoretically, in Wt mice the low amounts of CCL20 produced by early stage tumors could be taken up by CCR6-expressing cells from surrounding tissues, such as fibroblasts or keratinocytes in contrast to the CCR6-deficient microenvironment CCR6$^{-/-}$ mice. This hypothetical situation in which B16 produced CCL20 would be arrested and taken up by surrounding tissue would emulate conditions in B16-Control injected Wt and CCR6$^{-/-}$ animals where CCL20 presence is weak or non-existent **(Scheme 6)**

In order for this hypothesis to be tested, further research is needed, in particular on the precise effect of a hypothetical autocrine-signaling pathway. Generation of a CCR6$^{-/-}$ CCL20-expressing B16 melanoma cell line, alongside with tumor injection models using immune-deficient mice could contribute to elucidate this hypothesis.

CCL20 autocrine signaling in B16-CCL20 melanoma cells in CCR6$^{-/-}$ mice



CCL20 capture by CCR6-expressing tumor microenvironment in Wt mice



Scheme 6. Theoretical Model for B16 derived mCCL20 targets in B16-CCL20 injected Wt and CCR6$^{-/-}$ mice. (Top) In CCR6$^{-/-}$, B16-derived CCL20 accumulates at the tumor site, opening the possibility for a self-stimulatory, autocrine pathway. **(Bottom)** In Wt mice, B16-derived CCL20 is being captured by a CCR6-expressing microenvironment, thus reducing its concentration and self-stimulatory properties at tumor site.

6.6. Conclusions and future insights

We have demonstrated that B16-derivede CCL20 is able to reduce tumor progression in Wt mice and plays a key role on tumor onset, growth and progression in $CCR6^{-/-}$ mice, pointing to a decisive role of this chemokine for melanoma pathogenesis. Additionally, with this project we have contributed to more comprehensively depict the influence of CCR6 and CCL20, alone and combined, on the cellular and soluble immune milieu within melanoma tumors and the regional skin-draining lymph nodes. While our contribution will help to clarify some aspects of complexity that still surround the CCR6/CCL20 axis in malignant diseases the role of this chemokine axis in melanoma is still far from being completely understood and we have to acknowledge the limitations of our study. The absence of a commercially available antagonist for CCR6 together with the lack of a reliable $CCL20^{-/-}$ mouse model hampers the study of the CCR6/CCL20 axis. Further research is required, in particular in order to elucidate the precise role and function of described CCR6+ immune cell subsets, the identification of the effects and targets of CCL20 expressed in the melanoma microenvironment and a possible impact on CSCs, tumor angiogenesis and metastasis.

Acknowledgements

7. Acknowledgements

Supervisors and collaborators

This work could not have been possible without the funding and support of the Research Training Group (RTG) 2099 “Hallmarks of Skin Cancer” and the Deutsche Forschungsgemeinschaft (DFG).

I would like to thank my project supervisor Dr. med. Anke Lonsdorf for her continuous support during the development of this project and related research, for her motivation, patience and guidance. I would also like to thank my thesis supervisor Prof. Dr. med. Alexander H. Enk for giving me the opportunity of working in this laboratory and for his advice during lab meetings.

Besides my supervisors, I would like to thank the rest of my thesis committee members, Prof. Dr. Peter Angel, Prof. Dr. Frank Lyko and Dr. Guoliang Cui for their time and their support.

My sincere thanks also go to Dr. Rainer Will for his contribution to this project by generating the retrovirally transduced melanoma cells that had been a key part of the most successful experiments I have performed. Special thanks as well to Prof. Balkwill for her advice on chemokine expression in cell culture.

Lab People

I would like to thank to all my co-workers and friends that have accompanied me during this years. This project could not have ended successfully without your help. Your help in the lab was as important as your contribution for making me feel welcome and comfortable at all times. Thanks specially…

… to Steffi, for being my “*sister in arms*” all this time, since we started our PhDs more or less at the same time. Thanks for your help, time, advice, patience and all those German to English translations. But most of all, for supporting all my crazy ideas and joining all adventures without doubting. Without you I could have never reached this far.

... to Felix, my “*patrol companion*” and RTG colleague, for joining me in all the adventures (from the suburbs of London till the top of Germany) and help me in the most stressy/saturated moments as well as for being real friend.

…to Cinthia, my desk-neighbor, for helping me in my professional life, as well as in the personal one. For all the shared moments and for teaching me a new version of my own language.

… to Stefan, my "*German father-in-law*", for coming with me to the Friseur, taking me to watch soccer, and in general, for making me feel cozy in Germany

… to Lukas, for all those interesting discussions, for contributing to generate a good atmosphere in the lab and for keeping the safety in the streets of Heidelberg

… to Fareed, for your honest and hearty way of being and for all the cultural shocks

… to Carmen, for all your help with the paperwork and bureaucracy, always with a smile

… and of course to Oherly, Florina, Akira, Hao, Jing, Frauhammer, Adriana, Luana, Osman, Danny, Silvie, Galina, Britta, Steffi M, Karsten, Sabine, Florian, Priscila, Halina, Josefine, Silvio, Uschi, Randy, Sam Durrance, Christian B. Fod, and of course, Mr. Lee.

Special Thanks

Special thanks to Mireia for encouraging me to come to Heidelberg and try new adventures, for supporting me in the bad as well as in the good moments, for believing in me, for being always next to me. Mireia, Fa anys vam decidir arriscar-ho tot i aquí seguim malgrat totes les desventures. Eskerrik asko nire bizitzan agertzeagatik.

Special thanks to Jose, my trench partner and battle companion. This adventure could not have been possible without you. Thanks for joining me in the decadency as well as in the glory: From the suburbs of Mannheim to the skies of Hamburg, from soviet bunkers to luxurious restaurants. But most of all, thanks for being a true friend. Por los malos momentos, lo siento… España y yo somos así.

Special thanks for my parents, Claudio and Pilar as well as to my sister Andrea for their unconditional support, moral help and for taking care of me. For sending me the best Jamón as well as the best wishes. Thanks as well to my family, especially to Felisa, Sonia, Cristina, Angel and Ane. Esta tesis va dedicada a todos vosotros.

Special thanks to my friends in Bilbao, for their moral support and the good moments, and for remaining my friends after all. Thanks for making me feel like I never left every time I have been visiting. Thanks specially to Larri, Caba, Iker, Asi, Sofi, Sara, Sandra, Ivan, Cacha and Edu (you always considered yourself from Bilbao).

Acknowledgements

Special thanks to my friends in Barcelona, especially to Haritz, Marta and Blanca, for keeping contact and make distances appear shorter. Also to Cati, Marta and Alba, for abiding my presence into your house every two weekends during the most difficult moments, always with a smile. And of course, to Ismael, who took care of the yisus-calendar all this time and came to Heidelberg to visit me.

Special thanks to all my flat mates during this time. To Das and Moneo, for joining me out of the nest into the unknown. To Oscar and Jokin for coming after and expanding my horizons. To Johanna and Sergio, for representing the inflection point of this adventure and have made all this adventure worthwhile.

…And last but not least

As youngsters that had grown up watching American university movies and heavily influenced by Hollywood culture, we all expected something else. However, instead of a bright garden area there was a puddled parking lot; instead of a cafeteria full of cheerleaders and rugby players, there were fatty tortilla pintxos next to some people organizing an *asamblada*; instead of a professor imbuing wisdom in a lab full of state-of-the-art technology, there was Opilionman teaching us how to use MS-DOS in 2012.

Yes, life was not as movies had made us believe. And still, there we were.

- I still cannot believe all the things that had happened this semester…

Young Oscar was walking down the hallway next to me. I remember it as if it was today. He said that sentence one afternoon of January in 2009, when the first semester of university was over. Last months had been full of crazy and weird anecdotes and adventures. We didn't know that the best moments were yet to come. If someone would have come from the future to tell us all the stuff that was about to happen, we could not have believed it. And in the end, despite of all the decadency at the good old UPV, those became the best years of our lives.

Thanks to my Leioa family for all those years. The limit is the sky.

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