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Functional Relevance of the Chemokines CCL17 and CCL22 for Dendritic Cell Biology

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TABLE OF CONTENTS

I. CONTENTS 2

II INDEX OF FIGURES

III INDEX OF TABLES

IV ABBREVIATIONS

APC	Antigen presenting cell
APHC	Allophycocyanin
BAC	Bacterial artificial chromosome
BAP	Bacterial alkaline phosphatase
bio	Biotin
bp(s)	Base pair(s)
BHI Medium	Brain heart infusion medium
BSA	Bovine serum albumine
CCR	CC chemokine receptor
cDNA	Complementary DNA
conc	Concentration
CR	C chemokine receptor
Cre	Causes recombination
CXCR	CXC chemokine receptor
CX_3CR	CX_3C chemokine receptor
DC	Dendritic cell
DMEM	Dublecco's Modified Eagle Medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Desoxy ribonucleic acid
dNTP	2-desoxyribonucleosidtriphosphate
eGFP	Enhanced green fluorescent protein
E.coli	Escherichia coli
EDTA	Ethylen diaminetetraacedic acid
EF cell	Embryonic fibroblast cell
ES cell	Embryonic stem cell
EMA	Ethidium monoazide bromide
EtBr	Ethidium bromide
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FRT	FLP recombination target
G-418	G-418 sulfate; GENETICIN®
GL	Genomic locus
HC	High concentration
HSV-TK	Herpes simplex virus thymidin kinase
i.p.	Intraperitoneal
Isoprop	Isopropanol
i.v.	intravenious
kb	kilo bases
KO	Knock out
LAH	Long arm of homology
LB Medium	Luria Bertani medium
LC	Langerhans cell
LD	Lethal dose
L-glu	L-glutamine

LIF	Leucemia inhibitory factor
L.m.	*Listeria monocytogenes*
L.m.ova	Ovalbumin expressing *L. monocytogenes*
loxP	Locus X-ing over P1 phage
M	Molar
mAb	Monoclonal antibodies
MDC	Macrophage derived chemokine
MCS	Multiple cloning site
MHC	Major histocompatibility complex
Na-Ac	Sodium acetate
neo	Neomycin phosphotransferase
o/n	Over night
ORF	Open reading frame
pBSIIKS$^-$	pBluescriptIIKS$^-$
PCR	Polymerase chain reaction
PE	R-phycoerythrin
PFA	Paraformaldehyde
pAb	Polyclonal antibody
RPMI	Roswell Park Memorial Institute (medium)
RT	Room temperature
SA	streptavidin
SB	Southern blot
SAH	Short arm of homology
SDS	Sodium dodecylsulfate
s/n	Supernatant
TAE	Tris acetate EDTA
TARC	Thymus and activation-regulated chemokine
Tris	Tris(hydroxymethyl)aminoethane
TV	Targeting vector
w/o	Without
wt	Wild type

1. INTRODUCTION

1.1 The Immune System – A Brief Overview

The immune system protects an organism against a remarkable spectrum of pathogenic microorganisms like bacteria, viruses, fungi, parasites and cancer cells. The cells and molecules of the immune system, which are generated in an enormous variety, form a highly dynamic and complex network which is able to recognize and eliminate pathogenic organisms as well as abnormal cells (e.g. cancer cells and virus-infected cells) and foreign cells (e.g. organ and tissue grafts). In the first step of immune reactions the immune system has to recognise an invading organism as foreign to mount an appropriate immune response which is called effector response. The recognition process is highly specific and enables the immune system to detect subtle chemical differences which distinguishes one foreign pathogen from another. Additionally, the immune system is able to discriminate between foreign and self-antigens. After a pathogen has been recognised by the immune system various immune cells and molecules are recruited to the site of infection to eliminate or neutralise the invading organisms. The immune system is able to mount an enormous variety of effector responses and each response has developed to eliminate a particular type of pathogen.

Vertebrates have developed two different types of immunity, innate and adaptive immunity, which are able to operate in a cooperative and interdependent way. The innate immune system builds a first line of defense against invading pathogens and is uniform in all members of a species as most components are preset before onset of infection. Cells of the innate immune system express so called pattern-recognition receptors (PRR's), e.g. Toll-like receptors (TLR's) or mannose receptors, which are able to recognise certain classes of molecules belonging to pathogenic organisms. These classes of molecules are called pathogen-associated molecular patterns (PAMP's). LPS, for example, is recognised by TLR-4 while bacterial CpG DNA is detected by TLR-9 (Akira and Takeda, 2004; Akira et al., 2001; Janeway and Medzhitov, 2002; Wong and Pamer, 2001).

Innate immunity shows a broad reactivity using many disease-resistance mechanisms that are not specific for a particular pathogen. These mechanisms consist of anatomic, physiologic, endocytic/phagocytic and inflammatory barriers including synthesis and release of antimicrobial components. The innate immune system is able to mount a fast response against invading pathogens and is very important during the critical period immediately after exposure of the host to the pathogen. In a healthy host, most invading microorganisms are cleared within days by innate responses before activation of the adaptive immune system occurs (Beutler, 2004; Carroll and Janeway, 1999; Medzhitov and Janeway, 2000).

Adaptive immunity is not uniform in all members of a species as most components are not preset but come into play after antigenic challenge. Adaptive immunity is characterised by antigenic specificity, diversity, self/non-self recognition and immunologic memory. Primary responses of adaptive immunity need several days to develop and have been shown to improve during the course of response. Secondary or memory responses are more rapid, amplified and more effective compared to primary responses. Innate and adaptive immunity do not operate independently. The activation of innate immune responses leads to activation, stimulation and direction of adaptive immune responses while adaptive immune responses are able to further enhance and influence innate immunity (Hoebe et al., 2004; Iwasaki and Medzhitov, 2004; Zinkernagel and Hengartner, 2004).

The immune system consists of primary and secondary lymphoid organs which are distributed all over the body. The lymphoid organs are connected by lymphatic vessels which transport lymph fluid that contains immune cells and antigens which have been collected throughout the body. The primary lymphoid organs, mainly the bone marrow and the thymus, are the site where immune cells develop, mature and become antigenically committed . Additionally, selection and elimination of self-reactive and useless immune cells takes place in the primary lymphoid organs. The secondary lymphoid organs trap antigen from defined tissues and vascular spaces and allow effective interaction of activated immune cells. There are several types of secondary lymphoid organs i.e. lymph nodes, spleen, mucosal-associated lymphoid tissue (MALT) and gut-associated lymphoid tissue (GALT) (Ohl et al., 2003).

All blood cells (leucocytes, red blood cells and platelets) are derived from pluripotent, self-renewing hematopoietic stem cells (HSC). Various hematopoietic growth factors (cytokines) induce proliferation and differentiation of the different types of blood cells. A graphical overview of this process, which is called hematopoiesis and takes place in the primary lymphoid organs is shown in Fig 1.1. Early in hematopoiesis HSC give rise to two different types of progenitor cells, the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP) which give rise to cells of either the lymphoid or the myeloid lineage (Appelbaum, 1996; Bellantuono, 2004; Passegue et al., 2003).

CLP's can give rise to natural killer cells (NK cells), and T- and B-cell progenitors. T-cell progenitors further develop into T helper cells (T_H cells) or cytotoxic T cells (T_C cells) while B-cell progenitors differentiate into antibody producing B cells. T cells, which mature in the thymus and B cells which mature in the bone marrow are responsible for the adaptive immune responses. Interaction between free antigen and antigen-specific B cell receptors (BCR) as well as interaction with T_H cells and dendritic cells selectively induces activation and differentiation of B cell clones into antibody secreting plasma cells or memory cells. Therefore, B cells are responsible for the humoral branch of the adaptive immune system. Additionally, B cells can function as antigen-presenting cells which are able to activate memory T cells (Calame et al., 2003; Hardy and Hayakawa, 2001). T cells can be divided into two main subpopulations, the CD4-positive T_H cells and the CD8-positive T_C cells. T_H cells are activated upon recognition of antigen-derived, exogenous peptides which are bound to MHC II molecules on the surface of antigen presenting cells (APC's) via their T cell receptor (TCR). Activated T_H cells secrete a large variety of cytokines which regulate, direct and modulate immune responses. T_H cells can be further divided into T_H1 and T_H2 cells according to the cytokine profile they secrete. T_H1 cells characteristically secrete IL-12, IFNy, IL-2 and TNFß while T_H2 cells characteristically secrete IL-4, IL-5, IL-10 and IL-13. T_C cells are activated upon recognition of endogenous peptides which are bound to MHC I molecules on the surface of all nuclear cells via their specific TCR. Activated T_C cells display cytotoxic activity which leads to killing of e.g. virus infected cells or cancer cells (Bevan, 2004; Gemmell and Seymour, 1994; O'Garra, 1998; Sallusto et al., 2004).

NK cells, although of lymphoid origin, are not part of the adaptive immune system but of innate immune responses and display cytotoxic activity against a variety of tumor cells and virus infected cells (Moretta et al., 2001).

CMP's give rise to granulocyte-monocyte progenitor cells which differentiate into monocytes and neutrophils. Monocytes, which have phagocytic activity, circulate in the blood and can migrate into the bodies tissues. Upon migration, they differentiate into macrophages which also have phagocytic activity and are able to digest whole pathogenic microorganisms as well as injured or dead host cells, cellular debris and activated clotting factors. Macrophages also have the ability to function as antigen-presenting cells which are, like B cells, able to activate memory T cells. Phagocytosis of particulate antigen serves as initial activating stimulus which can be further enhanced by cytokines secreted by T_H cells or by mediators of the inflammatory response. Activated macrophages show greater phagocytic activity, increased ability to kill ingested pathogens, increased secretion of inflammatory mediators and increased ability to activate T cells. Neutrophils, which are also derived from granulocyte-monocyte progenitor cells, are the first immune cells to arrive at the site of infection. Like macrophages, they are active phagocytic cells but have a higher ability to kill ingested pathogenic organisms. Therefore, neutrophils are vital to control infections in the early phase. CMP's also give rise to eosinophil, basophil and erythroid progenitor cells and megakaryocytes which differentiate into eosinophils, basophils, erythrocytes and blood platelets, respectively. Eosinophils are motile phagocytic cells which play a role in defense against parasitic organisms. Basophils are non-phagocytic cells, which release pharmacologically active substances (Beutler, 2004; Carroll and Janeway, 1999; Janeway and Medzhitov, 2002).

Additionally, CLP's and CMP's can both give rise to dendritic cells. So far, there is limited knowledge about DC precursors and DC hematopoiesis *in vivo*. These topics are highly controversial and will not be discussed in my work. The subpopulations and functions of dendritic cells, which have been the main focus of this work, will be discussed in detail in chapter 1.2.

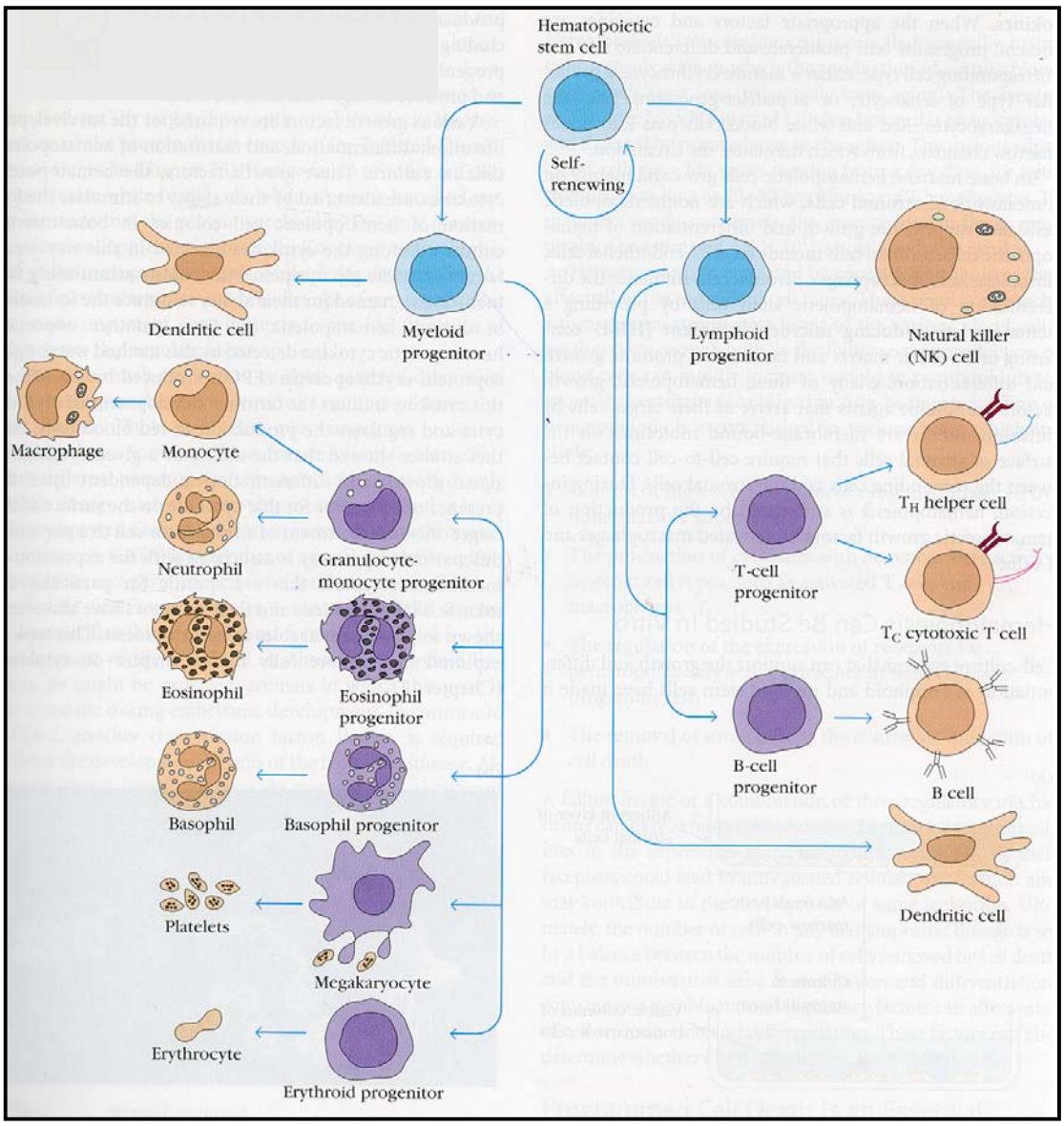

Fig. 1.1: Brief Overview of Hematopoiesis

Taken from Goldsby, Kindt, Osborne and Kuby, "Immunology", W.H. Freeman; 5th edition

1.2 Subpopulation and Functions of Dendritic Cells

Functions of Dendritic Cells:

DCs are unique antigen-presenting cells (APC's) as they are the only type of APC which is able to activate not only memory T cells but also naïve T cells. As a consequence, DCs are solely responsible for the initiation of primary immune responses which allow establishment of immunological memory (Banchereau and Steinman, 1998; Bell et al., 1999; Steinman, 1991). As previously described, secondary or memory immune responses can also be initiated by the other types of APC, the B cells and macrophages.

DC progenitors which are located in the bone marrow give rise to DC precursor cells which leave the bone marrow to circulate the blood and lymphatics before they eventually home to different tissues of the body. There, they reside and function as "sentinels" which continuously sample the antigenic environment until they encounter microbial products or tissue damage (Shortman and Liu, 2002). It has been shown that DCs accumulate rapidly at the sites of antigen deposition where they can induce homing of effector cells to the site of antigen encounter (Lambrecht et al., 1998; McWilliam et al., 1994).

As immature DCs have a high phagocytic activity they can effectively capture antigens which have invaded the bodies tissues using different pathways for antigen uptake. One of the most important pathways is receptor mediated endocytosis using, for example, the mannose receptor Dec205 (Engering et al., 1997; Jiang et al., 1995; Sallusto et al., 1995) or the Fcy receptor types I and II (CD64, CD32) which allows the DCs to take up immune complexes or opsonized particles (Fanger et al., 1996). DCs can also phagocytose apoptotic and necrotic cell fragments (Albert et al., 1998; Rubartelli et al., 1997) as well as viruses, mycobacteria and intracellular parasites (Inaba et al., 1993; Moll, 1993; Rescigno et al., 1999).

After uptake, the antigens are degraded in the endosomes, the generated polypeptides are targeted to MHC class II rich compartments and are loaded onto MHC class II molecules while the DCs mature (Castellino et al., 1997; Inaba et al., 1998; Tan et al., 1997). The MHC II/peptide complexes are translocated to the surface where, in mature DCs, they are stable for days and are available for recognition of $CD4^+$ T cells. This process is called antigen processing (Inaba et al., 1997; Mellman and Steinman, 2001; Pierre et al., 1997).

After antigen uptake and during antigen processing, the immature DCs migrate to the draining lymphoid organs through the afferent lymph. This tightly regulated migration process requires the coordinated actions of several chemokines (see chapter 1.2.2) and is an important characteristic feature of DCs (Austyn et al., 1988; Banchereau et al., 2000; Kripke et al., 1990).

DCs mature during migration which leads to the transition from antigen-capturing cells to antigen-presenting cells. Various factors induce and/or regulate maturation and activation of DCs. Some of these factors are pathogen related molecules like LPS, bacterial DNA and CpG oligonucleotides as well as dsRNA which all act on distinct TLR's (Rescigno et al., 1999; Sparwasser et al., 1998; Verdijk et al., 1999). Other factors are the cytokines which are present in the local environment like TNF, IL-1, IL-6, IL-10, TGF-ß and prostaglandins or T cell derived signals (Banchereau et al., 2000; Cumberbatch and Kimber, 1995). It has been further demonstrated that innate cell types like NK cells, (δ T cells and NKT cells can mediate maturation of DC's by yet unknown pathways (Gerosa et al., 2002; Leslie et al., 2002; Vincent et al., 2002). It has also been shown that antigens from necrotic cells can activate DCs (Sauter et al., 2000). Pathogenic products like LPS and the local production of IL-1 and TNFα which mediate DC maturation also trigger the migration of peripheral DCs to the T cell areas of the lymphoid organs (Banchereau et al., 2000; Cumberbatch and Kimber, 1995). The maturation process leads to various changes of the DCs such as loss of the phagocytic activity, up-regulation of MHC class II molecules and co-stimulatory receptors like CD40, CD58, CD80 and CD86, changes in class II MHC and lysosomal compartments and a change in morphology (Banchereau et al., 2000; Steinman et al., 2003).

After arrival in the draining lymphoid organs, the mature DCs present the translocated MHCII/peptide complexes on the cell surface to antigen-specific $CD4^+$ T cells leading to T cell activation. Antigen-specific $CD4^+$ T cells in turn regulate immune effector cells like antigen-specific T_C cells and B cells, non-antigen-specific macrophages , eosinophils and NK cells (Banchereau et al., 2000).

DCs can also activate $CD8^+$ cytotoxic T cells by presenting antigenic peptide on MHC class I molecules. Antigenic peptides can be loaded on MHC I molecules via two different pathways - an endogenous pathway and an exogenous pathway (Pamer and Cresswell, 1998; Rock and Goldberg, 1999; Shen et al., 1997). In the endogenous pathway, cytosolic proteins from intracellular bacteria and parasites as well as viral proteins but also the cells own proteins are degraded to polypeptides. These polypeptides are loaded onto newly synthesized MHC class I molecules within the endoplasmatic reticulum (Banchereau et al., 2000). The presentation of endogenous peptides by DCs which have been infected with viruses or intracellular parasites and bacteria and the resulting activation of specific T_C cells enables the immune system to recognise and eliminate all infected cells.

The exogenous pathway is an alternative MHC class I pathways which only DCs and, to a lesser extend, B cells and macrophages possess. This pathway allows DCs to present polypeptides which are derived from exogenous antigens in context with MHC I. This pathway is called “cross-presentation” or “cross-priming”. (Bevan, 1976; Kovacsovics-Bankowski and Rock, 1995; Kurts et al., 1997); (Belz et al., 2002b)

Cross-presentation of exogenous antigens is an important function of DCs as it enables specific $CD8^+$ T_C cells to recognise and eliminate tumor cells and virus, bacteria and parasite infected cells in cases where the pathogen does not infect DCs. Additionally, cross-presenting DCs appear to be essential to maintain protective antiviral cytotoxic T cell memory (Ludewig et al., 1999).

Besides activating naïve T cells, DCs can directly activate naïve and memory B cells and contribute to their expansion. It has been shown, that DCs induce isotype switching towards IgA1 and IgA2 in CD40-activated naïve B cells (Fayette et al., 1997), help differentiation of activated B cells to plasma cells (Dubois et al., 1998) and enhance differentiation of CD40-activated memory B cells towards IgG secreting plasma cells (Dubois et al., 1997). Additionally it has been demonstrated that DCs induce CD40-independent immunoglobulin class switching through BAFF and APRIL (Litinskiy et al., 2002). It has been also been shown that DC provide B cells with CD40-independent proliferation signals and CD40-independent survival signals (Wykes and MacPherson, 2000).

In recent years there was growing evidence that DCs are not only important for the induction of immunity but that they are also play a role in the induction and maintenance of tolerance to self antigens (Shortman and Liu, 2002).

Central tolerance is a process which involves elimination of potentially self-reactive T cells in the thymus by induction of apoptosis. There is evidence, that this process is mediated by thymic DCs (Brocker et al., 1997). But as not all self-reactive T cells can be eliminated by central tolerance (Heath and Carbone, 2001), it is important that remaining self-reactive T cells are eliminated by mechanisms of peripheral tolerance. As DCs do not only transport foreign antigens but also self-antigens to the lymph nodes (Inaba et al., 1997), it is likely that DCs are responsible for tolerance as well as immunity.

Two mechanisms by which DCs might maintain peripheral tolerance have been proposed which are both supported by some experimental evidence and are therefore discussed controversely. The first mechanism proposes that a special regulatory subtype of DC is responsible for maintaining tolerance (Fazekas de St Groth, 1998; Suss and Shortman, 1996). The second mechanism proposes that all DCs can initiate tolerance or immunity depending on their maturation or activation state. The original concept proposed that mature DCs induce immunity while immature DCs induce tolerance by either destruction of T cells, induction of anergy or by inducing the generation of regulatory T cells (Dhodapkar and Steinman, 2002; Dhodapkar et al., 2001; Roncarolo et al., 2001; Steinman et al., 2000).

This concept has been recently challenged by the proposal that mature DCs which are in a quiescent state induce tolerance while only fully activated mature DCs induce immunity. As a consequence, immune responses can only occur if invading pathogens provide signals to trigger full activation of DCs (Albert et al., 2001; Shortman and Heath, 2001).

There is growing evidence that DC do not only induce proliferation and activation of T cells but that they also direct T cell responses. T cell activation and proliferation can result in tolerance or immunity by induction of either regulatory T cells or effector T cells. Effector T cells themselves can be polarized into T_H1 or T_H2 cells which secrete different classes of cytokines. The DC-derived signals, which regulate the T cell cytokine polarisation have not yet been completely determined. It has been shown so far, that the production of bioactive IL-12 by DC, which is tightly regulated, and DC-derived IFNy are important factors for the induction of TH1 cells (Hochrein et al., 2000; Langenkamp et al., 2000; Moser and Murphy, 2000). It has been suggested as well, that IL-23, which is produced by DCs, may also mediate T_H1 polarisation (Oppmann et al., 2000). DC also influence T_H2 polarisation as DC-derived IL-10 is required for optimal development of T_H2 responses (Maldonado-Lopez et al., 2001). Several pathogens like shistosomes, fungi and cholera toxin use DCs for the induction of T_H2 responses (Bozza et al., 2002; d'Ostiani et al., 2000; Gagliardi et al., 2000). It has been further demonstrated that histamine and lymphopoietin may act on DCs to induce T_H2 responses during allergic reactions (Mazzoni et al., 2001; Soumelis et al., 2002).

DCs also seem to be involved in establishment of immunologic memory. It has been shown that DC-derived IL-15 is involved in the maintenance of $CD8^+$ memory T cells and there is also evidence that DCs are able to select higher affinity memory cells (Hamilton and Harty, 2002; Ku et al., 2000; Livingstone and Kuhn, 2002).

Dendritic Cell Subpopulations:

DCs were discovered in 1973 by Steinman and Cohen and were first thought to be a homogenous cell type (Steinman and Cohn, 1973). Intensive research on DC biology revealed though that DCs are a highly heterogenous cell population which exists as a complex mixture of different subpopulations with different functions (Shortman and Liu, 2002; Wilson and O'Neill, 2003; Wilson and Villadangos, 2004). The functional diversity of the DC populations is related to their differentiation state, their location and the surrounding microenvironment (Ardavin, 2003; Banchereau et al., 2000). Characterisation of murine DCs using phenotypic and functional criteria led to the identification of six main subpopulations which are displayed in Table 1.1 (adapted from Heath et al., 2004). It can not be ruled out, though, that other organ or tissue specific DC subsets exist, which have not been identified yet (Heath et al., 2004; Henri et al., 2001; O'Keeffe et al., 2002). The organ and tissue distribution of the identified DC subpopulations is shown in Table 1.2 (adapted from Ardavin, 2003).

DC type	Surface phenotype						Derivation
	CD11c	CD8	CD4	CD205	CD11b	B220	
CD8α$^+$ DC	+	+	-	+	-	-	Blood
CD8α$^-$ CD4$^+$ DC	+	-	+	-	+	-	Blood
CD8α$^-$CD4$^-$ DC	+	-	-	-	+	-	Blood
Langerhans cell	+	-/low	-	Very high	+	-	Skin epithelia
Dermal/interstitial DC	+	-	-	+	±	-	Tissue
B220$^+$ DC (plasmacytoid DC)	Low	±	±	-	-	+	Blood/tissues

Table 1.1: DC Subsets, Surface Phenotype and Derivation (adapted from Heath et al, 2004)

Subpopulation	Thymus	Spleen	Lymph Node	Peyers Patch	Skin	Liver
CD8+ DCs	+	+	+	+	-	+
CD8- DCs$^{\#}$	*	+	+	+	-	+
Langerhans cells	-	-	-	-	+	-
Dermal/interstitial DCs	-	-	-	-	+	-
B220+ DCs	+	+	+	+	-	N.D

Table 1.2: Organ and Tissue Distribution of Murine DC Subpopulations (adapted from Ardavin, 2003); #includes CD4$^+$ and CD4$^-$ subpopulation; * CD8$^-$ DC can be detected in the thymus but constitue a minute proportion of thymic DCs; N.D. not determined

Although DC biology is a major topic of research, there is yet only limited knowledge of the function of the individual DC subsets. It has been shown, that $CD8\alpha^+$ DC are the predominant producers of IL-12 and can therefore induce strong T_H1 responses. Additionally, IL-12 is proposed to be important in T cell priming versus tolerance (Curtsinger et al., 2003; Maldonado-Lopez et al., 1999; Maldonado-Lopez et al., 2001). $CD8\alpha^+$ DC have also been demonstrated to play a central role in cross-priming and cross-tolerance (Belz et al., 2002a; Bennett et al., 1997; den Haan et al., 2000; Schulz and Reis e Sousa, 2002). The function of the $CD8\alpha^-$ $CD4^+$ and $CD8\alpha^-$ $CD4^-$ DC subset is poorly understood. $CD8\alpha^-$ DC have been reported to have the ability to induce B cell activation and plasmablast differentiation (Balazs et al., 2002; Garcia De Vinuesa et al., 1999). Additionally, there is some evidence, that $CD8\alpha^-$ $CD4^-$ DCs produce IFNy (Hochrein et al., 2001).

Dermal DCs were implicated to be the critical cell type for induction of $CD4^+$ T cell immunity to intradermally injected protein antigens (Itano et al., 2003). It was further indicated that $CD11b^+$ DCs, probably of dermal origin, play an important role in immunity against dermally injected *Leishmania major* (Filippi et al., 2003; Von Stebut et al., 2003).

$B220^+$ DC, which are often referred to as plasmacytoid DC, produce large amounts of IFNα and IFNβ upon encounter with various stimuli particularly TLR's or through detection of virions which probably contribute to the control of viral replication. The produced IFNα and IFNβ as well activate other types of DCs like those responsible for cross-priming (Asselin-Paturel et al., 2001; Cella et al., 1999; Nakano et al., 2001). Other types of DCs can also produce IFNα and IFNβ but only upon their own viral infection (Diebold et al., 2003). The Langerhans cells (LC), which are probably one of the best studied DC subsets will be discussed in more detail in section 1.5.

1.3 Structure and Function of Chemokines and Chemokine Receptors

Chemokines are a group of small proteins with a molecular weight of 8-14 kDa. Until today, more than 40 different chemokines have been identified in mouse and man, which are mainly secreted proteins that can act on their target cells in an autocrine, paracrine and exocrine fashion. The main biological function of chemokines is the induction and regulation of the chemotactic activity of the different leukocyte populations (Baggiolini, 1998; Horuk, 2001; Luster, 2002). However, some members of the chemokine family might also play a role in the regulation of hematopoiesis, angiogenesis, tissue architecture and organogenesis (Broxmeyer, 2001; Gale and McColl, 1999; Szekanecz and Koch, 2001).

Chemokines are divided into two major groups, which include the vast majority of the family members and two minor groups with one member each. The general structure of the four chemokine families is shown in Figure 1.2. Although the members of the chemokine family show a low level of sequence identity, their three dimensional structure is remarkably homologue (Proudfoot, 2002). All identified chemokines, with one exception, share four highly conserved cystein residues in their primary amino acid sequence which form two disulfide bonds (Cys1-Cys3; Cys2-Cys4) which are crucial for the structure and stability of the protein. Chemokines can be classified according to the organisation of the characteristic cysteine motif, which consists of the two N-terminal cysteine residues (McColl, 2002; Zlotnik and Yoshie, 2000).

The CC family, which contains the largest number of chemokines, is characterised by the fact that the two N-terminal residues of the conserved cyteine motif are adjacent. Most CC chemokine genes are located on the human chromosome 17 or murine chromosome 11, where they form a gene cluster. The second major group is CXC chemokine family whose members are characterised by the presence of one not conserved amino acid between the N-terminal cystein residues. Most CXC chemokine genes form a cluster on murine chromosome 5 or human chromosome 4.

The CXC Chemokine family can be further divided into ELR-containing and non-ELR containing chemokines dependent on the presence or absence of an glutamic acid/leucine/arginine motif. If present, the ELR motif is located on the N-terminal side of and in close proximity to the CXC motif. Two more chemokine classes have been identified, the C chemokine family and the CX_3C chemokine family which, until now, consist of one member each. The only known member of the C Chemokine family is XCL1/lymphotactin which contains only the second and the fourth cysteine of the characteristic motif. The gene that codes for XCL1 is located on murine and human chromosome 1. The only identified member of the CX_3C Chemokine family is CX3C1/fractalkine which is characterised by the presence of three unconserved amino acids between the two N-terminal conserved cysteine residues. The gene encoding CX_3CL1 is located on murine chromosome 8 and human chromosome 16 (Gale and McColl, 1999; McColl, 2002; Zlotnik and Yoshie, 2000).

CX_3CL1 was the first chemokine which was described to exist not only as secreted protein but also in a membrane bound form. The membrane bound form has a length of 373 amino acids and consists of an N-terminal chemokine module, a mucin-like stalk, a transmembrane domain and a short cytoplasmic tail. The secreted form only consists of the chemokine module which is separated from the membrane bound protein on the surface by peptidase mediated cleavage (Haskell et al., 2000; Imai et al., 1997b; Umehara et al., 2001). Only one further chemokine which exists in both forms has been identified which belongs to the CXC family and has been named CXCL16. It has been shown that the membrane bound form consists of 249 amino acids and has the same structure as CX_3CL1. The secreted form, the chemokine module, is separated from the membrane bound protein by peptidase mediated cleavage (Matloubian et al., 2000).

The rapid discovery of new chemokines led to the problem that several groups reported the same molecule under different names which caused significant confusion among scientists. For that reason, the nomenclature system which is described above was introduced in 1998. However, the original names are still commonly used. The systematic names of all chemokines which have been identified so far are listed in Table 1.3. together with the common alternative names in man and mouse.

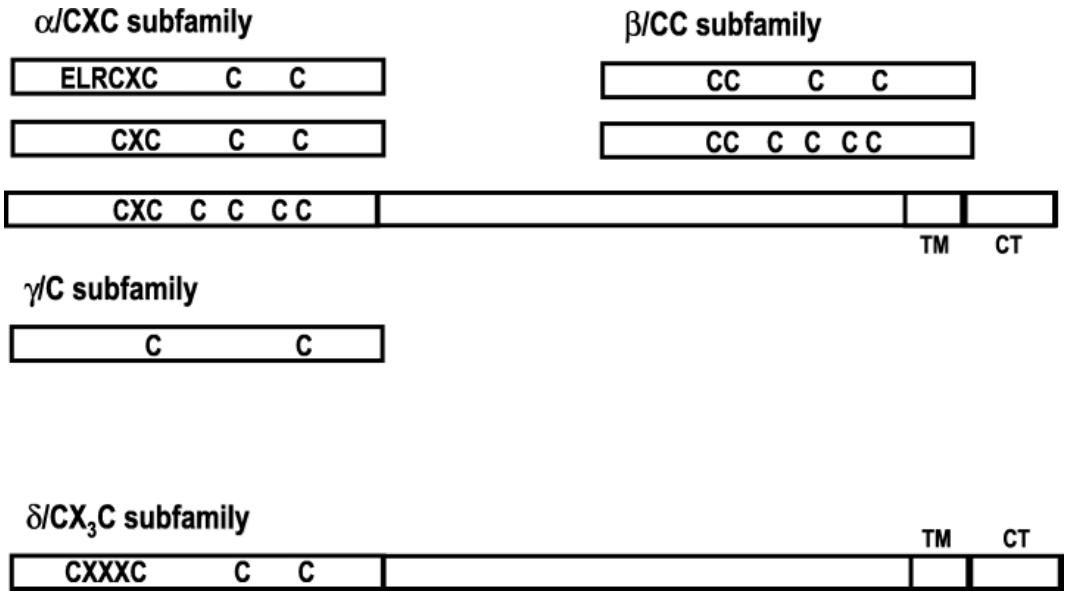

Figure 1.2: The General Structure of the Four Chemokine Subfamilies

Schematic representation of the conserved cysteine motif which allows the classification of all members of the chemokine family into four basic subfamilies. Schematic representation of the ELR motif which allows the subdivision of the CXC family. TM: transmembrane domain; CT: cytoplasmic tail (taken from McColl et al., 2002)

The members of the CC chemokine family can attract a wide range of cells including monocytes, eosinophils, basophils (Baggiolini and Dahinden, 1994; Baggiolini et al., 1997) and various subpopulations of lymphocytes including thymocytes, NK cells and DCs (Baggiolini et al., 1994; Bonecchi et al., 1998; Sozzani et al., 1997). It has been further shown that the members of the CXC chemokine family, which contain the characteristic ERL motif attract neutrophils while the non-ELR-containing CXC chemokines attract lymphocytes (Baggiolini et al., 1997; Gale and McColl, 1999).

Systematic name	Original name/s; Human	Original name/s; Mouse
CXCL1[1]	MGSA, Gro-α	KC
CXCL2[1]	MIP-2α, Gro-β	MIP-2*
CXCL3[1]	MIP-2β, Gro-(	*
CXCL4[2]	PF4	PF4
CXCL5[1]	ENA-78	LIX
CXCL6[1]	GCP-2	CKα-2
CXCL7[1]	NAP-2	?
CXCL8[1]	IL-8, NAF, NAP-1	?
CXCL9[2]	Mig	Mig
CXCL10[2]	IP-10	Crg2
CXCL11[2]	I-TAC	I-TAC
CXCL12[2]	SDF-1α, SDF-1β	SDF-1
CXCL13[2]	BLC, BCA-1	BLC, BCA-1
CXCL14	BRAK, bolekine	BRAK
CXCL15	?	Lungkine
CXCL16	CXCL16	CXCL16
CCL1	I-309	TCA-3
CCL2	MCP-1, MCAF	JE
CCL3	MIP-1α, LD78α	MIP-1α
CCL4	MIP-1β, LD78β	MIP-1β
CCL5	RANTES	RANTES
CCL6	?	C10, MRP-1
CCL7	MCP-3	MARC
CCL8	MCP-2	MCP-2
CCL9/10	?	MRP-2, CCF18, MIP-1 (
CCL11	Eotaxin	Eotaxin
CCL12	?	MCP-5
CCL13	MCP-4	?
CCL14	HCC-1	?
CCL15	HCC-2, Lkn1, MIP-1δ	?
CCL16	HCC-4, LEC	LCC-1
CCL17	TARC	TARC
CCL18	DC-CK1, MIP-4, PARC, AMAC-1	?
CCL19	MIP-3β, ELC, exodus-3	MIP-3β, ELC, exodus-3
CCL20	MIP-3α, LARC, exodus-1	MIP-3α, LARC, exodus-1
CCL21	6Ckine, SLC, TCA-4, exodus-2	6Ckine, SLC, TCA-4, exodus-2
CCL22	MDC, STCP-1	ABCD-1
CCL23	MPIF-1	?
CCL24	MPIF-2, eotaxin-2	?
CCL25	TECK	TECK
CCL26	Eotaxin-3	?
CCL27	CTACK, ILC	ALP, CTACK, ILC, ESkine
CCL28	CCL28, MEC	CCL28
XCL1	Lymphoctactin, SCM-1, ATAC	Lymphotactin
CX3CL1	Fractalkine	Neurotactin

Table 1.3: Systematic Names of Known Chemokines Including Most Common Alternative Names in Human and Mouse. (Taken from McColl, 2002 and Gale and McColl, 1999) *Human MIP-2α and MIP-2β are products of two different genes; only one murine MIP-2 gene has been identified; ?: not yet identified; [1]ELR-containing; [2]non-ELR-containing

The membrane bound CX_3CL1 protein mediates adhesion between endothelial cells and $CD8^+$ T cells, monocytes and NK cells while the soluble form induces chemotaxis of the same cell types (Imai et al., 1997b). XCL1 has been shown to selectively attract $CD8^+$ T lymphocytes (Kelner et al., 1994).

The system which is used to classify chemokines is exclusively based on structural characteristics and does not consider the functional role of the chemokines so that an alternative system has been developed which is based on the functional expression. This system divides all chemokines into two functional classes: the homeostatic/constitutive chemokines and the inflammatory/inducible chemokines (Baggiolini, 1998; McColl, 2002). The functional classification of chemokines is shown in Table 1.4. Homeostatic chemokines are constitutively expresses in lymphoid tissues and are responsible for the movement of thymocytes through the thymus during selection (Annunziato et al., 2001; Ansel and Cyster, 2001; Zlotnik and Yoshie, 2000). They are also responsible for the physiological trafficking of immune cells including lymphocytes and DCs to the secondary lymphoid organs in steady-state condition or during immune response (Baggiolini, 1998; McColl, 2002; Yoshie et al., 1997). Inflammatory chemokines are up-regulated at sites of inflammation where they are critically involved in the recruitment of effector cells which can mount an appropriate immune response (Gale and McColl, 1999; Godessart and Kunkel, 2001).

Chemokines mediate their biological effects on their target cells by binding to their specific chemokine receptor(s) (Horuk, 2001; McColl, 2002; Neote et al., 1993). Virtually every cell type expresses a unique combination of chemokine receptors which can also be classified as either homeostatic or inflammatory. Classification is based on the class of their binding partner (constitutive or inflammatory) and on the influence that ligation has on the target cell (D'Ambrosio et al., 2003; McColl, 2002) (see Table 1.4). All known chemokine receptors are G-protein coupled seven transmembrane domain receptors (GPCR) which use a flexible and complex network of intracellular signalling machinery (Baggiolini, 1998; Gale and McColl, 1999).

Engagement of the receptor leads to the induction of a coordinated signalling cascade which results in the generation of second messengers and activation of effector enzymes which is mediated by the interaction between the receptors and heterotrimeric G proteins. The signal transduction cascade leads to the activation of chemotaxis and different functions which are required for host defence including adhesion, respiratory burst, degranulation and lipid mediator synthesis (D'Ambrosio et al., 2003; Thelen, 2001).

Many chemokine receptors can bind to more than one ligand although the promiscuity is restricted to chemokines of one subfamily. CXC chemokines can only bind to CXC receptors while CC chemokines bind only to CC receptors. Several chemokines can also bind to more than one receptor which, together with the fact that many chemokine receptors have more than one ligand, leads to a high level of redundancy which grants a certain level of flexibility and security (D'Ambrosio et al., 2003; Proudfoot, 2002; Rossi and Zlotnik, 2000). The multiple receptor-ligand pairs which have been identified so far are shown in Table 1.4 (taken from McColl, 2003).

As most chemokine receptors are constitutively expressed on the cell surface, their activity needs to be regulated. Two major mechanisms of chemokine receptor regulation have been described so far. The first mechanism leads to the alteration of the surface expression profile of the cell by changing gene expression. It has been shown that naïve or immature cells, e.g. T lymphocytes and DCs, express a different set of chemokine receptors than mature or effector cells (Sallusto et al., 1998; Sozzani et al., 1999; Stumbles et al., 2001). The second mechanism of regulation leads to the desensitisation of the chemokine receptors. Homologous desensitisation is caused by internalisation of the receptors after ligand binding which leads to temporary unresponsiveness of the cell to the receptors ligands. Heterologous desensitisation occurs via transactivation which is caused by ligands which do not directly bind to the chemokine receptor which also leads to temporary unresponsiveness (Kraft et al., 2001; Le et al., 2001; Mashikian et al., 1999; Stanton et al., 1999).

The receptor-ligand pair CCR4-CCL17/CCL22 which has been a main focus of my work will be more intensively discussed in chapter 1.7.

Receptor	Ligand	Class
CXCR1	CXCL8, CXCL6	I
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8	I
CXCR3	CXCL9, CXCL10, CXCL11	I
CXCR4	CXCL12	C
CXCR5	CXCL13	C
CXCR6	CXCL16	?
CCR1	CCL3, CCL5, CCL7	I
CCR2	CCL2, CCL8, CCL13	I
CCR3	CCL11, CCL13, CCL5, CCL28	I
CCR4	**CCL17, CCL22**	C
CCR5	CCL3, CCL4, CCL5	I
CCR6	CCL20	C/I
CCR7	CCL19, CCL21	C
CCR8	CCL1	I
CCR9	CCL25	C
CCR10	CCL27, CCL28	I
CCR11	CCL25	C
XCR1	XCL1	I
CX3CR1	CX3CL1	?

Table 1.4: Receptor Ligand Pairs and their Classification (Taken from McColl, 2003)
C=Constitutive; I =Inducible; ?=not yet classified

1.4 Dendritic Cells and Chemokines

Dendritic cells are very important for the induction of immunity and tolerance. In order to carry out their functions it is vital that DCs of every maturation and activation state are at the right place at the right time. As a consequence, the migratory activity of DCs is highly important for their function as APC. DC migration is mainly coordinated and regulated by chemokines but is also influenced by cysteinyl leukotriens (McColl, 2002; Robbiani et al., 2000; Sallusto et al., 1999). The DC system is critically dependent on the ability of DCs to respond to different signalling patterns during each stage of their maturation. DC precursors are constantly recruited to the peripheral tissues which helps to maintain a sufficient DC density for effective immune surveillance in these tissues (Lanzavecchia and Sallusto, 2001; Mellman and Steinman, 2001; Steinman and Inaba, 1999).

At present, there is little information available about chemokine receptor expression and chemokine responsiveness of DC precursors in the peripheral blood. However, it has been implicated that CCR2 might be expressed by some precursor cell types and its ligands, CCL2, CCL8 and CCL13, could therefore be involved in the initial migration of these precursors into the peripheral tissue (Vanbervliet et al., 2002). It has been further implicated, that CXCR4 is expressed by some DC precursor cell types which would make them responsive for the chemokine CXCL12. As this chemokine is constitutively expressed in lymphoid tissue, it might be important for attraction of DC precursors and maintaining sufficient DC levels in these tissues (Zaitseva et al., 2002; Zou et al., 2001). Different tissues express different patterns of chemokines and it is likely that different DC precursors express different chemokine receptor patterns. As a consequence, the rate and type of DC precursors which enter a given tissue site can be orchestrated and controlled. At present, there is no evidence that chemokines play a direct role in DC differentiation. However, it is possible, that chemokines indirectly influence DC differentiation by directing DC precursors to specific microenvironments which are required for the various differentiation processes (McColl, 2002).

Immature DCs express receptors for a number of inducible chemokines including CCR1, CCR5, CXCR2 (Dieu et al., 1998; Stumbles et al., 2001; Vecchi et al., 1999). The ligands of these receptors (CCL3, CCL5 and CXCL8) are produced by tissue macrophages upon infection via ligation of TLR's with LPS, LTA or CpG DNA (Akira et al., 2001; Kaisho and Akira, 2002; Krieg, 2002). Additionally, macrophage-derived IL-1ß and TNFα induce expression of these chemokines which in turn induce migration of tissue DCs to the source of the chemokines which results in the accumulation of DCs at the site of infection. Enhanced expression of these chemokines probably also leads to enhanced recruitment of non-specific effector cells as neutrophils and monocytes (Sallusto et al., 1999). It is further speculated that up-regulation of these inflammatory cytokines leads to increased migration of DC precursors into these tissues to replenish the DC population (McColl, 2002). Immature DCs themselves produce different chemokines upon stimulation in the tissues including the inflammatory chemokines CCL3, CCL4, CCL5, CXCL8 and CXCL10 which enhance recruitment of neutrophils and monocytes. They also express the constitutive chemokines CCL17 and CCL22 on a basal level (Foti et al., 1999; Padovan et al., 2002; Yoneyama et al., 2002).

During maturation, DCs loose surface expression of CCR1, CCR5 and CXCR2 and as a consequence the ability to respond to CCL3, CCL5 and CXCL8 (Sallusto et al., 1998; Sozzani et al., 1999; Vecchi et al., 1999). At the same time, maturing DCs up-regulate surface expression of CCR7 which leads to responsiveness to the chemokines CCL19 and CCL21. These steps are important for the directed migration of maturing DCs to the draining LN (Sallusto et al., 1999; Sallusto et al., 1998). It has been shown that CCL19 and CCL21 are constitutively expressed in the T cell zones of the draining lymphatics and by high endothelial venules (HEV) which line the draining lymphatics (Baekkevold et al., 2001; Luther et al., 2000; Saeki et al., 1999). Lack of either CCL19 and CCL21 or CCR7 leads to lack of DC migration from peripheral tissues to the draining lymph nodes (Nakano and Gunn, 2001; Saeki et al., 1999). CCR7 is also vital for the homing of naïve and activated T cells to the secondary lymphoid organs (Forster et al., 1999). As CCL19 and CCL21 are constitutively expressed in the T cell areas of the draining LN and CCR7 is expressed on T cells and mature antigen-loaded DC it is possible for these cell types to meet and activate each other which allows initiation of immune responses.

Mature DCs in the lymphoid organs loose the capacity to produce the inflammatory chemokines CCL3, CCL4, CCL5, CXCL8 and CXCL10. They up-regulate CCL17 and CCL22 and start to produce CCL18 and CCL19 which attract B and T lymphocytes to the lymphoid organs and allow activation of these immune cells (Imai et al., 1999; Sallusto et al., 1999; Vulcano et al., 2001).

Taken together, normal immune function is critically dependent on the intact interaction of the DC system and the chemokine system.

1.5 Langerhans Cells and Langerhans Cell Migration

Langerhans cells (LC), which are one of the best described subpopulation of DCs, are located in the epidermis where they form an organised network and serve as sentinels of the skin by surveillance of the local microenvironment. LC transport foreign antigen to the draining LNs and present these antigens to responsive T lymphocytes which leads to T cell activation. Therefore, the LCs are vital for the initiation of cutaneous immune responses.

The LCs were first discovered by Paul Langerhans in 1868. They were described as dendritically shaped cells in the epidermis and were first thought to be cells of the nervous system (Langerhans, 1868; Wolff, 1991). In the 1950s experimental evidence led to the conclusion that LCs were worn-out melanocytes which had lost the capacitiy to produce melanin pigments (Wolff, 1991). In contrast to these findings, Birbeck et al. described in 1961 that LCs were found in areas of the epidermis which lack melanocytes. Birbeck also described rod- and tennis racket-shaped organelles inside LCs which were named after him (Birbeck MS, 1961). Birbeck granules were also detected in histiocytosis X, which is today known as LC histiocytoses (Basset et al., 1966). These observations led to the conclusion that LCs might be mesenchymal cells or histiocytes or even macrophages.

Shortly afterwards, it was shown that LC expressed ATPase which was known to be expressed by leukocytes which provided further evidence of a relationship of LCs with immune cells (Bradshaw M, 1963; Mustakallio, 1962; Wolff, 1963). In 1976 it was reported that contact sensitizers which were applied to the skin led to LC accumulation and close contacts to lymphocytes which strongly suggested a role of LCs in antigen transport and induction of contact allergy (Silberberg et al., 1976). In the late 1970s it was reported that LCs express MHC class II molecules, Fc-receptors and complement receptors on their cell surface which proved that LCs indeed were cells of the immune system (Klareskog et al., 1977; Rowden et al., 1977; Stingl et al., 1977).

In the early 1980s it was demonstrated that LCs have an outstanding capacity for antigen presentation and were therefore thought to be a kind of specialised macrophage (Bjercke et al., 1984; Braathen and Thorsby, 1980; Green et al., 1980).

In 1985, it was shown by Steinman and Schuler that LCs are in fact a member of the dendritic cell family (Schuler and Steinman, 1985). From that moment on, LCs were used as the preferred model to study dendritic cell biology which made the Langerhans cell a well described dendritic cell type. The fact that GMCSF is a crucial survival and growth factor for all dendritic cell types was established by using LCs as model cells (Heufler et al., 1988; Romani et al., 1989). The process of DC maturation was studied and described using LCs which showed the reorganisation of cell surface phenotype, increase in T-cell stimulatory capacity, the downregulation of phagocytosis and antigen-processing properties and the translocation of MHC class II molecules to the cell surface upon maturation (Lee et al., 1993; Reis e Sousa et al., 1993; Schuler and Steinman, 1985). As not all properties of LCs are yet understood, they are still intensively studied and remain an important topic of research.

LCs can be well distinguished from other DC subtypes. First of all, the Birbeck granules, which are unique cytoplasmic organelles and are visible under the electron microscope, can only be found in LCs (Birbeck MS, 1961; Romani et al., 2003). Second, LCs show a characteristic surface phenotype. LCs express CD11b, CD33 and the adhesion molecule E-cadherin (Iwama et al., 2002; Lenz et al., 1993; Schuler and Steinman, 1985).

Upon maturation and migration, LCs can up-regulate CD8α (Anjuere et al., 2000; Merad et al., 2000). LCs also express the C-type lectins CD206 (MMR), CD205 (Dec-205), Dectin-1 and Dectin-2, which are also expressed by other DC subpopulations. CD206, CD205 and Dectin-2 are involved in antigen uptake while Dectin-1 participates in T cell interactions (Ariizumi et al., 2000a; Ariizumi et al., 2000b; Jiang et al., 1995). The only identified C-type lectin which is exclusively expressed on LCs is CD207 (Langerin) which has been shown to be involved in the formation of Birbeck granules (Figdor et al., 2002; Valladeau et al., 2000). Antibodies against CD207 will be a good tool for identification of LCs as soon as they are available.

Langerhans cells, like all immune cells, are derived from stem cells in the bone marrow (Katz et al., 1979; Perreault et al., 1984). During ontogeny, LC precursors populate the epidermis and acquire imunologically important molecules like MHC class II, CD45 and CD207. The characteristic Birbeck granules also develop during this process (Foster and Holbrook, 1989; Tripp et al., 2004). The mechanism by which bone marrow derived LCs migrate into the epidermis is yet unknown but is has been shown that TGF-ß and CCL20, which binds to CCR6, are absolutely vital for this migratory step (Borkowski et al., 1997; Dieu-Nosjean et al., 2000; Manome et al., 1999). It has been further shown that CCL13, which binds to CCR2, is involved in the re-population of inflamed epidermis by LCs (Merad et al., 2002). It has been demonstrated that the turn-over rate in the epidermis is not high as the resident LCs are long lived (Czernielewski et al., 1985; Kamath et al., 2002; Merad et al., 2002). In the steady-state there is hardly any immigration of LC precursors into the skin and the few dividing LCs are derived from unknown skin precursors and not from precursor cells which circulate the blood. Only in the inflamed condition there is recruitment from blood-born precursors into the epidermis (Merad et al., 2002).

LCs internalise and process foreign antigen after encounter in the skin which leads to migration from the epidermis of the skin to the draining lymph nodes where they carry out their function as APC. Migration of Langerhans cells is mainly studied by skin sensitisation experiments using chemical allergens like FITC (see section 2.9.4). It has been shown that IL-1ß, IL-18 and TNFα are required to induce mobilisation and migration of LCs in skin sensitisation experiments (Cumberbatch et al., 2001; Cumberbatch et al., 1997). It is proposed, that IL-18 which acts upstream of IL-1ß and TNFα, is needed to sustain elevated expression of these cytokines during contact sensitisation. So far, there is no evidence that IL-18 itself can induce mobilisation and migration of LCs (Cumberbatch et al., 2001). IL-1ß can act on LCs in an autocrine fashion which is an important signal to induce migration. Additionally, IL-1ß induces increased expression of TNFα by keratinocytes which in turn gives an additional migratory signal to the LC via TNF-R2 (Ryffel et al., 1991; Wang et al., 1997; Wang et al., 1996).

To prevent excessive and unnecessary LC migration and maintain tissue homeostasis, LC migration has to be counter-regulated. It has been demonstrated that IL-10, TGF-ß1 and lactoferrin act either individually or in concert to inhibit LC migration (Cumberbatch et al., 2000; Riedl et al., 2000; Wang et al., 1999). During the migration process, the LCs mature which results, as previously described, in the loss of surface expression of CCR1, CCR5 and CXCR2 and the ability to respond to CCL3, CCL5 and CXCL8 (Sozzani et al., 1999; Vecchi et al., 1999). Like all maturing DCs, LCs up-regulate the surface expression of CCR7 which leads to responsiveness to CCL19 and CCL21 and directed migration from peripheral tissues to the draining lymph nodes (Sallusto et al., 1999; Sallusto et al., 1998).

1.6 CCL17 (TARC), CCL22 (MDC) and CCR4

CCL17, which is also known as TARC or ABCD-2, and CCL22, which is alternatively named MDC or ABCD-1, are members of the CC chemokine family. The genes that encode CCL17 and CCL22 are not localised in the chemokine cluster on chromosome 11, like most CC chemokines genes, but on chromosome 8 where they form a little chemokine cluster of their own together with CX_3CL1 (Zlotnik and Yoshie, 2000).

The CCL22 gene contains three exons which are separated by two introns of 1.2 kb and 2.7 kb length. The CCL22 gene gives rise to a 2.2 kb transcript which contains an open reading frame (ORF) of 276 nucleotides which encodes a 24 amino acid long leader peptide and a mature protein with a length of 68 amino acids with a molecular mass of 7,8 kDa (Schaniel et al., 1998). The CCL17 gene also contains three exons which are separated by two introns of 614 bp and 456 bp, respectively. The CCL17 gene gives rise to three different transcripts which is due to the fact that different transcriptional start sites are used. All three transcripts contain an ORF which encodes a 70 amino acid long processed secretory protein of approximately 8 kDa with either a 61 or 23 amino acid long leader peptide (Lieberam and Forster, 1999; Schaniel et al., 1999).

CCL17 and CCL22 bind with high affinity to the chemokine receptor CCR4 which is the only identified and confirmed receptor for both chemokines although some experimental data imply an additional receptor for CCL17, which is thought to be CCR8, and CCL22, which is unknown (Bernardini et al., 1998; Imai et al., 1997a; Imai et al., 1999; Struyf et al., 1998). Until today, no further chemokine ligands for CCR4 have been identified although there is experimental evidence that CCR4 might also bind to CCL3(Chvatchko et al., 2000).

CCL17 and CCL22 share a similar expression profile and overlapping biological functions which implies a high degree of redundancy. Both chemokines are expressed on a basal level by immature DCs and are strongly up-regulated upon maturation so that DCs are the main producer of both chemokines (Alferink et al., 2003; Lieberam and Forster, 1999; Ross et al., 1999; Schaniel et al., 1998). CCL17 and CCL22 have also been shown to be expressed by LCs (Alferink et al., 2003; Tang and Cyster, 1999). CCL17 and CCL22 are not expressed by naïve, activated and memory T cells, NK cells and granulocytes (Kanazawa et al., 1999; Ross et al., 1999; Schaniel et al., 1998; Schaniel et al., 1999). It has been shown that CCL17 is not expressed in monocytes and macrophages while CCL22 expression has been shown in human but not in murine monocytes and macrophages (Godiska et al., 1997; Ross et al., 1999; Schaniel et al., 1998). CCL17 and CCL22 are not produced by naïve B cells but there is experimental evidence that CCL22 but not CCL17 can be expressed by activated B cells (Schaniel et al., 1998). The expression profile of CCL17 and CCL22 is summarised in Table 1.5.

	CCL17	CCL22
Immature DC	**+/-**	**+/-**
Mature DC	**+ +**	**+ +**
T cells	**-**	**-**
Naive B cells	**-**	**-**
Activated B cells	**-**	**+**
Monocytes	**-**	**+ (human)** **- (mouse)**
Macrophages	**-**	**+ (human)** **- (mouse)**

Table 1.5: The Expression Profile of CCL17 and CCL22

+/-: basal expression; ++: high expression; -: no expression

Analysis of CCL17-eGFP reporter mice and additional *in vitro* studies have shown that CCL17 is expressed in various lymphoid and non-lymphoid tissues. CCL17-expressing cells were detected in the thymus, lymph nodes, lung and the intestine but, interestingly, not in the spleen. Even after systemic microbial challenge or after *in vitro* stimulation of splenocytes, CCL17 expression is not induced. Analysis of the CCL17 expressing cells showed, that they exclusively belong to the $CD11c^{+}$ $CD11b^{+}$ $CD8\alpha^{-}$ $CD205^{+}$ subsets which also include Langerhans cells. CCL17 is expressed on a basal level in immature $CD11c^{+}$ $CD11b^{+}$ $CD8\alpha^{-}$ $CD205^{+}$ DC. During maturation, CCL17 expression is constantly up-regulated so that mature DCs express high levels of CCL17. It has been shown that CCL17 expression can be further up-regulated by stimulation of CCL17 expressing cells with TLR-ligands like LPS and CpG DNA. Analysis of the CCL17-eGFP homozygous reporter mouse, which lack CCL17, demonstrated that these mice show diminished T-cell-dependent contact hypersensitivity responses and deficiency in rejection of allogenic organ transplants (Alferink et al., 2003; Lieberam and Forster, 1999).

CCR4, the corresponding receptor, is expressed by a lot of different cell types which allows CCL17 and CCL22 to act on a wide range of target cells. CCR4 has been shown to be expressed by activated T cells, preferentially cells of the T_H2 and the T_C2 subtype, and by the CLA+ subset of skin homing memory T cells (Biedermann et al., 2002; Bonecchi et al., 1998; D'Ambrosio et al., 1998). CCR4 is also expressed LC's which, as they also express the receptors ligands CCL17 and CCL22, can lead to autrocrine stimulation (Katou et al., 2001; Randolph, unpublished data). Additionally, CCR4 is found on the surface of monocytes, macrophages, NK cells and blood platelets in mice and man (Andrew et al., 1998; Maghazachi, 2003; Power et al., 1995).

Analysis of the CCR4 knock out mice, which develop normally, showed disrupted chemotactic response to the chemokines CCL17 and CCL22 as expected. Surprisingly, the mice also show no chemotactic response to the chemokine CCL3 which was shown to bind to CCR3 and not CCR4. It is therefore possible that CCL3 additionally binds to CCR4 and that CCR3 and CCR4 signalling somehow influence each other. It was further shown that CCR4 deletion had no effect on T_H2 differentiation *in vitro*. CCR4 knock out mice showed resistance to LPS-induced endotoxin shock in high and low dose models which might be due to a significant decrease in the $F4/80^+$ population in the knock out mice (Chvatchko et al., 2000). CCR4 knock out mice were also analysed in a murine model of chronic fungal asthma and it was shown that $CCR4^{-/-}$ mice exhibited an aggressive anti-fungal response which was characterised by enhanced neutrophil function and increased macrophage recruitment into the airways. The inability of the recruited neutrophils and macrophages to kill the fungus led to severe invasive lung disease and mortality (Schuh et al., 2002).

CCL17 and CCL22 are strong chemoattractants for CCR4 expressing T_H2 and T_C2 cells and therefore play a role in an amplification loop which shifts an immune response towards a T_H2 response. For that reason it is not surprising that CCL17 and CCL22 are associated with T_H2-mediated allergic and asthmatic airway diseases (Hirata et al., 2003; Sekiya et al., 2000) and atopic dermatitis (Leung et al., 2003; Vestergaard et al., 2003).

1.7 Gene Targeting

Gene targeting is defined as the introduction of site-specific modifications into the murine genome by homologous recombination (Capecchi, 1989a; Capecchi, 1989b). It is carried out using pluripotent murine embryonic stem cell lines (ES cells). Although homologous recombination of foreign DNA with endogenous genomic sequences in mammalian cells is a rare event, this method is widely and successfully used to generate mutant animals with a specific gene disruption to study the function of this gene *in vivo*.

In the first step of the gene targeting procedure a replacement vector (targeting vector) is planned and generated which typically consist of two DNA regions which are homologous to the genomic target locus. If it is planned to identify the homologous recombinant ES cell clones (positive clones) or the mutant mice by PCR it is necessary to keep one of the homologous DNA sequences relatively short (1-2 kb). For that reason it is called the "short arm of homology" (SAH). The second homologous DNA sequences is longer and has a length between 3 and 9 kb which is called the "long arm of homology" (LAH). SAH and LAH are separated by a positive selection marker gene which usually is the bacterial aminoglycoside phosphotransferase (neo) gene that renders the homologous recombinant ES cell clones sensitive to the antibiotic G-418. The neo cassette usually replaces (deletes) the genomic DNA sequence which is located between the SAH and LAH which leads to disruption of the genes function (Torres and Kühn, 1997).

As the neo gene has a very strong promoter it is possible that it interferes with the expression of genes which are adjacent or coded on the opposite strand. It is also possible that the presence of the neo gene leads to alternative splicing of the affected exons and/or the potential generation of dominant negative molecules. Additionally, the expression and translation of the neo gene might have an influence on the mutant cells and animals which could interfere with analysis of the target genes function. For these reasons it is desirable to remove the neo gene after successful selection. In order to accomplish the removal of the neo cassette, two lox P sites are inserted into the targeting vector flanking the neo gene which has the same orientation (Torres and Kühn, 1997).

Lox P sites (locus of crossover (x) in P1) have a length of 34 bp which include two 13 bp inverted repeats which are seperated by an 8 bp palindromic sequence which defines the orientation of the overall sequence. LoxP sites are recognised by the P1 bacteriophage Cre-recombinase which mediates site specific recombination between the loxP sites. If the two loxP sites are placed in the same orientation and are separated by at least 82 bp, the Cre-mediated recombination results in deletion of the DNA sequence which is located between the recognition sites. In this process, one loxP site is left behind which will remain in the targeted locus (Sauer and Henderson, 1988; Sauer and Henderson, 1990). Alternatively, the Flp/FRT recombination system can be used. In this system, the yeast derived Flp recombinase recognises a sequence of 48 bp length which is called the FRT site. If the FRT sites are placed in the same orientation, Flp-mediated site specific recombination leads to deletion of the DNA sequence between the recognition sites in the same way as in the Cre/loxP system. But as the Flp/FRT recombination system is not as efficient as the Cre/loxP system it is not as frequently used (Branda and Dymecki, 2004; Dymecki, 1996a; Dymecki, 1996b). The deletion of the neo cassette can be carried out by transfection of the selected ES cells with an expression vector which express either Cre-recombinase or Flp-recombinase or alternatively, by crossing the mutant animals with either Cre- or Flp-deleter mice.

As random integration of foreign DNA with endogenous genomic sequences takes place more frequently than homologous recombination, it is helpful to additionally include a negative selection marker into the targeting vector. This negative selection marker is placed downstream of the LAH and will only be integrated into the endogenous genomic sequence if random integration takes place. A negative selection marker which is frequently used is a thymidine kinase gene, which is called TK cassette. If the targeting vector was randomly integrated into the genome, the target ES cell also integrated the TK cassette which makes the cell sensitive to the antiviral drug ganciclovir so that the cell will eventually die. The basic structure of a gene replacement (targeting) vector is schematically shown in Fig 1.3.

Fig 1.3: The Basic Structure of a Gene Replacement (Targeting) Vector
SAH: Short Arm of Homology; LAH: Short Arm of Homology; lox P: locus of crossover (x) in P1; neo: bacterial aminoglycoside phosphotransferase gene; TK: thymidine kinase gene

In the next step, the targeting construct is transfected into pluripotent murine embryonic stem cells (ES cells) which is usually accomplished by electroporation. The transfected ES cell clones undergo positive and negative selection. The positively selected clones have to be further tested for homologous recombination by PCR and/or Southern Blot. After identification of a homologous ES cell clone it is introduced into a pre-implantation embryo which leads to the generation of chimeric mice. If these mice are germline chimeras, they can transmit the mutation to their progeny which allows breeding of heterozygous animals. Crossing of these heterozygous animals finally leads to the generation of a homozygous mutant mouse strain which allows analysis of the function of the target gene *in vivo* (Torres and Kühn, 1997).

1.8 *Listeria Monocytogenes*

Listeria Monocytogenes (LM) is a Gram-positive intracellular pathogenic bacterium which is able to infect a broad range of hosts including humans, mice and other mammals (Murray et al., 1926; Pirie, 1927). Human individuals are usually infected by ingestion of contaminated food, mainly dairy products, meat and fish (Bibb et al., 1990; Pamer, 2004; Vazquez-Boland et al., 2001). Infection with LM results in gastroenteritis and can even lead to septicaemia and meningitis (Gellin and Broome, 1989; Vazquez-Boland et al., 2001). Immunocompromised individuals e.g. neonates, young children, elderly people and HIV patients are particularly susceptible and the course of infection can even be lethal. Pregnant women, especially those in their third trimester are particularly at risk, as they can additionally develop chorioamnionitis and infection of the fetus can result in septic abortion (Pamer, 2004; Vazquez-Boland et al., 2001). In

immunological research, LM infections are frequently used in mouse models as the bacterium is well characterised and can be nicely used to investigate the function of the mammalian immune system. Most laboratory studies use intravenous and intraperitoneal inoculation to initiate infection so that most *in vivo* studies examine the immune response to systemic infection.

Most natural infections with LM in humans occur after ingestion of contaminated food and the subsequent uptake of bacteria by intestinal epithelial cells. This process is mediated by the interaction of bacterial internalin A and E-cadherin (Gaillard et al., 1991). Mice are more resistant to intestinal infection with LM due to a single amino acid difference between human and murine E-cadherin (Lecuit et al., 2001). The bacteria are able to pass the epithelial layer, enter the bloodstream and are transported into other organs, mainly the spleen and the liver where they are internalised by resident macrophages. In the liver, the bacteria are able to enter hepatocytes in a process which is mediated by binding of bacterial internalin B to a hepatocyte growth factor receptor (Shen et al., 2000).

After the bacteria have been phagocytosed by resident macrophages, they are able to escape the phagosomes with the help of the bacterial protein listeriolysin O (LLO) which destroys the phagosomal cell membrane (Bielecki et al., 1990). The invasion of the cytosol triggers innate inflammatory responses and induces long-term immunity which is dependent on the presence of LLO (Berche et al., 1987). Once in the cytosol, the listeria show high mobility which is mediated by the bacterial protein actin-assembly-inducing protein (ActA) which also enables the listeria to infect neighbouring cells (Domann et al., 1992; Kocks et al., 1992).

Immediately after infection innate immune responses are activated which are essential to the survival of the host (Unanue, 1997). IFNy and TNF which are produced by NK cells and macrophages have been shown to be essential for the primary defense against LM (Buchmeier and Schreiber, 1985; Havell, 1989; Tripp et al., 1993). Neutrophils and macrophages are thought to be the principal mediators of the killing of the bacteria (Conlan and North, 1994; Rogers and Unanue, 1993) but the recruitment of monocytes is also important for the bacterial clearance (North, 1970).

Although the mechanism of bacterial killing by recruited neutrophils, monocytes and macrophages is not yet completely understood, it has been shown that the oxidative burst and the production of nitric oxide (NO) strongly contribute to the pathogen clearance *in vivo* (Endres et al., 1997; Shiloh et al., 1999). It has been assumed that macrophages are the main source of TNF and NO during an infection *in vivo* until the analysis of spleens from infected wt mice recently allowed the identification of a dendritic cells population, the so called TipDCs which are essential for the control of bacterial growth (Pamer, 2004; Serbina et al., 2003).

The adaptive immune system is also very important for the control of infection and elimination of the bacteria from the body. It has been demonstrated that humoral immunity provides a small contribution to protective immunity (Edelson and Unanue, 2001) while (δ T cells help to control the inflammatory response after infection (Egan and Carding, 2000). But the most important players in the adaptive immune response against *L. monocytogenes* are the αβ T cells as it has been shown that they are critically involved in bacterial clearance and long-term immunity (Pamer, 2004).

Analysis of the αβ T cell-mediated immune responses showed, that $CD8^+$ T cells provide a more substantial contribution to protective immunity than $CD4^+$ T cells (Ladel et al., 1994). It has been demonstrated that LLO (Villanueva et al., 1995) and p60 (Bubert et al., 1992) induce significant T cell responses Both bacterial proteins are rapidly degraded in the proteasome of the host cells which allows fast generation of bacterial peptides that can enter the MHC class I antigen-processing pathway. This in turn leads to fast priming of T cells (Vijh et al., 1998; Villanueva et al., 1995).

$CD8^+$ T cell populations which are specific for bacteria derived antigenic peptides differ in quantity and stability. However, these $CD8^+$ T cell populations clonally expand in a coordinated fashion (Badovinac et al., 2002; Badovinac et al., 2004; Busch et al., 2000; Mercado et al., 2000; Wong and Pamer, 2001). The magnitude of $CD8^+$ T cell responses is unaffected by increased presentation of antigen after a threshold quantity of antigen has been presented (Pamer, 2004).

It has also been shown that *in vivo* priming of $CD8^+$ T cells occurs with optimal efficiency 24 h after infection and that after 72 h naïve $CD8^+$ T cells were not primed any more despite the persistence of viable bacteria (Wong and Pamer, 2003). Therefore, $CD8^+$ T cell priming is independent of the quantity or duration of in vivo antigen presentation. Priming of $CD8^+$ T cells is mediated by $CD11c^+$ DCs (Lenz et al., 2000; Jung et al., 2002) which is probably done by cross-presentation of antigens that are derived from infected macrophages as DCs themselves are not a significant *in vivo* reservoir of listeria. Approximately 8 days after i.v. infection $CD8^+$ T cell responses reach peak frequencies (Busch et al., 1998).

Infection with *L. monocytogenes* induces memory T cells which are able to mediate protective immunity. It has been demonstrated that mice which lack $CD4^+$ T cells show normal primary $CD8^+$ T cell responses but impaired long term maintenance of memory $CD8^+$ T cells so that protective immunity against re-infection is gradually lost (Shedlock et al., 2003; Kursar et al., 2002; Sun et al., 2003). The exact mechanism that is used by $CD4^+$ T cells to maintain memory $CD8^+$ T cells is not yet known.

1.9 Aim of the Study

The main focus of my studies, which are presented in this work, was to examine the functional relevance of the chemokines CCL17 and CCL22 for dendritic cell biology. In order to accomplish this goal three different approaches were chosen.

As knock-out mice are a good system to study gene functions *in vivo* and a $CCL17^{-/-}$ mouse was already available in our lab, I aspired to generate a CCL17/CCL22-double-knock-out mouse to examine the combined effect of the lack of these chemokines on dendritic cell biology. The genomic loci of CCL17 and CCL22 are both located on murine chromosome 8 within a range of about 70 kb flanking the chemokine CX_3CL1.

The close vicinity of CCL17 and CCL22 has the consequence that the likelihood of a crossing over was extremely small so that it was impossible to gain a double knock out by crossing the already available CCL17$^{-/-}$ mouse with a newly generated CCL22-/- mouse. The 50A8 ES cell line, which has a targeted disruption of the CCL17 gene and had been used to generate the knock out mice, was also available in our lab. This ES cell line has been generated by I. Lieberam at the University of Cologne by replacing a part of exon 2 of the CCL17 gene with an eGFP cDNA. As a consequence, 50A8 ES cells express GFP in place of CCL17 under the control of the CCL17 promoter. A scheme of the gene targeting approach that was carried out by I. Lieberam is shown in Appendix A1. By successful targeting of the CCL22 locus with a suitable replacement vector using the 50A8 ES cell line it was therefore possible to generate CCL17/CCL22 double knock out mice if integration of the CCL22 vector construct had taken place on the same chromosome as the CCL17 integration. The chosen targeting strategy for the targeting of the CCL22 locus in 50A8 ES cells is described in section 3.1.

My second approach to study the biological function of CCL17 and CCL22 *in vivo* was to analyse the influence of these chemokines on Langerhans cell migration. CCL1 is the only confirmed ligand for the chemokine receptor CCR8 while CCL17 and CCL22 are the only verified ligands for CCR4. However, there is some experimental evidence that CCL17 might also bind to CCR8 and there is also some evidence that CCL22 might bind to an yet unidentified chemokine receptor (Imai et al., 1997; Imai et al., 1998; Struyf et al., 1998; Bernardini et al., 1998; Mantovani et al., 2000; Garlisi et al., 1999; Inngjerdingen et al., 2000).

CCR4 and CCR8 are both expressed on LC's which, as they also express the receptors ligands CCL1, CCL17 and CCL22, can lead to autrocrine stimulation which might induce Langerhans cell migration (Katou et al., 2001; G. Randolph, Mt. Sinai School of Medicine, New York, unpublished data). G. Randolph and her group were able to show, that treatment of wt mice with neutralising anti-CCL1 antibodies had no effect on LC migration while treatment of mice with a combination of anti-CCL17 and anti-CCL22 antibody had minor inhibitory effect on migration. Combined treatment of wt mice with anti-CCL17 and anti-CCL1 resulted in a strong reduction of Langerhans cell migration which was further enhanced by combined treatment with neutralising anti-CCL1, anti-CCL17 and anti-CCL22 antibodies (G. Randolph, personal communication).

They were also able to show that CCR8-/- mice showed a strong reduction of LC migration. Langerhans cell migration had not yet been studied in the $CCR4^{-/-}$ mouse and as this mouse is also available in our institute, I decided to investigate this. As CCL1 also seems to be involved, I also planned to study Langerhans cell migration in $CCR4^{-/-}$ which had been treated with neutralising anti-CCL1 antibody. The results of this project are shown in section 3.2.

In my third project I analysed the role of CCL17 and CCL22 during systemic infection with *Listeria Monocytogenes*. It had been previously shown by J. Alferink in our group that there was no difference in survival between $CCL17^{-/-}$ mice in C57Bl/6 background and $CCL17^{+/-}$ littermate controls after infection with high and medium doses ($10\times LD_{50}$ and $10\times LD_{50}$) of *L. monocytogenes* while $CCL17^{-/-}$ mice showed better survival than $CCL17^{+/-}$ littermate controls after infection with low doses ($0.1\times LD_{50}$) of *L. monocytogenes* (see Fig 1.4).

I therefore decided to further investigate the influence of CCL17-deficiency and, additionally, CCR4-deficiency on the outcome of listeria infection. First of all, I planned to further explore the effect of CCL17- and CCR4-deficiency on survival after systemic infection with *L. monocytogenes*. Additionally, I aspired to analyse the influence of CCL17- and CCR4-deficiency on innate and adaptive immune responses. To analyse innate immune responses, I intended to determine the bacterial clearance in $CCL17^{-/-}$ mice and $CCR4^{-/-}$ mice in comparison to C57Bl/6 wt mice. Finally, I aimed to investigate the effect of CCL17- and CCR4-deficiency on adaptive immune response by studying specific T cell responses against *L. monocytogenes* during systemic primary and recall infection in wild type and knock out mice.

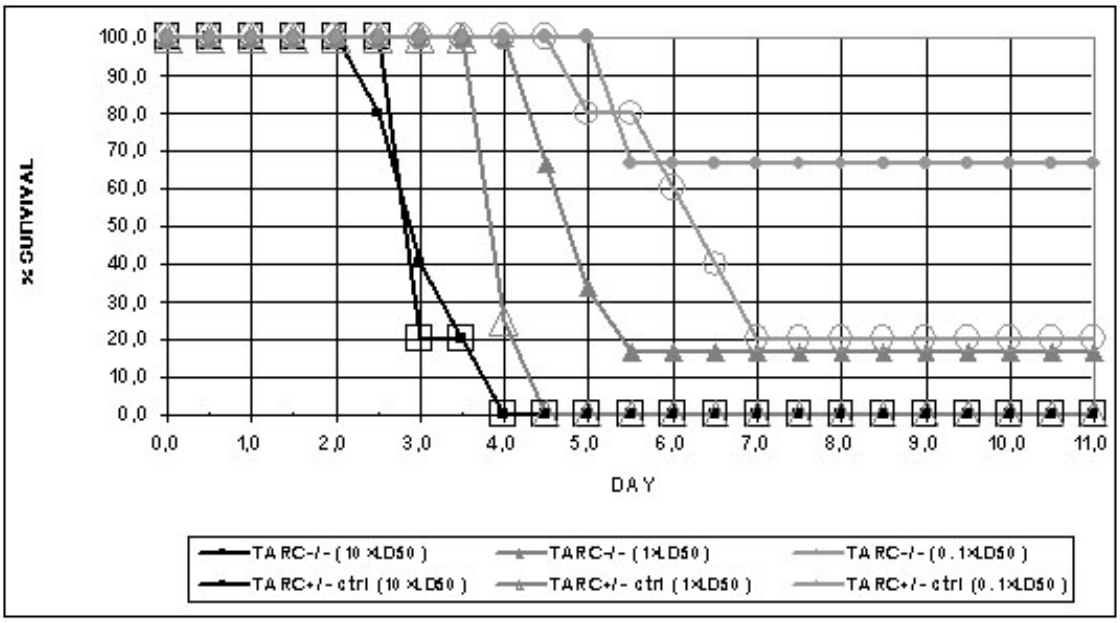

Fig. 1.4: Survival of CCL17$^{-/-}$ Mice and CCL17$^{+/-}$ Littermate Controls in C57Bl/6 Genetic Background after Infection with Low, Medium and High Doses of *L. Monocytogenes* (Data Generated by J. Alferink).

2 MATERIAL AND METHODS

2.1 List of Suppliers

2.1.1 Chemicals and Reagents

Chemical/Reagent	Supplier
λDNA/HindIII Fragments	Invitrogen, Karlsruhe
α[^{32}P]-dCTP (Redivue™)	Amersham, Braunschweig
β-Mercaptoethanol	Sigma, Taufkirchen
50 bp DNA Ladder Marker	Invitrogen, Karlsruhe
100 bp DNA Ladder Marker	Invitrogen, Karlsruhe
1 kb DNA Ladder Marker	Invitrogen, Karlsruhe
10×Herculase® Reaction Buffer	Stratagene, Netherlands
10×REACT® Buffers for restriction digest	Invitrogen, Karlsruhe
10×PBS	Biochrom, Berlin
1×PBS	Invitrogen, Karlsruhe
20×SSC	Invitrogen, Karlsruhe
10×TAE	Invitrogen, Karlsruhe
1×Trypsin/EDTA	Invitrogen, Karlsruhe
Acetone	Pharmacy, Klinikum rechts der Isar
Agarose	Invitrogen, Karlsruhe
Ampicillin	Sigma, Taufkirchen
BSA	Biomol, Hamburg
BSA (PCR)	Roche, Mannheim
Bromophenolblue	Sigma, Taufkirchen
Cytofix/Cytoperm	Pharmingen, Heidelberg
dNTP	Invitrogen, Karlsruhe
Dibutylphtalate	Sigma, Taufkirchen
DMEM Medium	Invitrogen, Karlsruhe
DMF	Sigma, Taufkirchen
DMSO	Sigma, Taufkirchen
EDTA	Sigma, Taufkirchen
EMA	Molecular Probes, USA
Ethanol pA	Merck, Darmstadt
Ethanol, 70%	Pharmacy, Klinikum rechts der Isar
Ethidiumbromide	Roth, Karlsruhe
Ficoll Type 400	Amersham, Braunschweig
Formaldehyde	Sigma, Taufkirchen
FCS	PAN Biotech, Aidenbach
FITC	Sigma, Taufkirchen
G418 (Geneticin)	Invitrogen, Karlsruhe
Gancyclovir (Cymeven®)	Syntex, Aachen
GolgiPlug	Pharmingen, Heidelberg
Hydrochloric Acid (HCl)	Roth, Karlsruhe
Isopropanol	Pharmacy, Klinikum rechts der Isar

L-Glutamine (ES culture)	Biochrom, Berlin
L-Glutamine	Invitrogen, Karlsruhe
2-Methylbutane	Sigma, Taufkirchen
Mineral Oil	Sigma, Taufkirchen
Mineral Oil, Embryo Tested	Sigma, Taufkirchen
Mitomycin C	Sigma, Taufkirchen
Olive Oil	Fluka, Switzerland
Paraformaldehyde	Sigma, Taufkirchen
Penicillin/Streptomycin (ES cells)	Biochrom, Berlin
Penicillin/Streptomycin	Invitrogen, Karlsruhe
PerfectHyb™Plus Hybridization Buffer	Sigma, Taufkirchen
PermWash	Pharmingen, Heidelberg
Peptides (SIINFEKL, $LLO_{188-201}$)	Affina GmbH, Berlin
Phenol/Chloroform	Roth, Karlsruhe
Sarcosyl	Sigma, Taufkirchen
SDS	Roth, Karlsruhe
Sodium Acetate	Merck, Darmstadt
Sodium Azide	Merck, Darmstadt
Sodium Chloride	Merck, Darmstadt
Sodium Hydroxide	Merck, Darmstadt
Sucrose	Sigma, Taufkirchen
Tissue Freeze Medium™	Leica, Heidelberg
Tris	Roth, Karlsruhe
TRITON-X 100	BioRad, München
Trypan Blue	Sigma, Taufkirchen
Vectashield® Mounting Medium	Linaris, Wertheim
Xylencyanol	Sigma, Taufkirchen

2.1.2 Laboratory Supplies

Article	Supplier
Cover Slips	Roth, Karlsruhe
Cryovials	Peske OHG, Aindling-Pichl
Cell strainer (70µm, 100µm)	Falcon, USA
Cryomold® Tissue Molds	Miles Inc, USA
Electoporation Cuvettes	BioRad, München
Inocculation Loop	Greiner, Austria
Microbank™ Vials	Ontario, Canada
Microscope Slides	Menzel GmbH und Co KG, Braunschweig
Nylon Mesh	ThermoLifeSciences, Egelsbach
Nylon Membrane “Gene Screen Plus”	Perkin-Elmer, Belgium
Plastics (Tissue Culture)	Falcon, USA
Whatman Paper	Schleicher & Schuell, Dassel

2.1.3 Kits

Kit	Supplier
MicroSpin™ S-200 HR Kit	Amersham, Braunschweig
QIAfilter Plasmid Maxi Kit	QIAGEN, Hilden
QIAprep Spin Miniprep Kit	QIAGEN, Hilden
QIAquick Gel Extraction Kit	QIAGEN, Hilden
QIAquick PCR Purification Kit	QIAGEN, Hilden
Rediprime™ II Random Prime Labelling Kit	Amersham, Braunschweig
TaKaRa Ligation Kit	TaKaRa Biomedicals Inc, Japan
TOPO-TA Cloning® Kit	Invitrogen, Karlsruhe

2.1.4 Enzymes

Enzyme	Supplier
Bacterial Alkaline Phosphatase	Invitrogen, Karlsruhe
Collagenase Type VIII	Sigma, Taufkirchen
DNAse I	Roche Diagnostics GmbH, Mannheim
Herculase®	Stratagene, Netherlands
Restriction Enzymes	Invitrogen, Karlsruhe
Proteinase K	Roche Diagnostics GmbH, Mannheim
Pronase E	Sigma, Taufkirchen
Taq Polymerase	Invitrogen, Karlsruhe

2.1.5 Antibodies and Secondary Reagents

All antibodies are monoclonal and directed against murine antigens unless stated otherwise.

Antibody/Sec.Reagent	Clone/Species	Application/Dilution	Supplier
CD4-PerCp	RM4-5/rat	FACS 1:50	BD Pharmingen
CD8α-PE	53-6.7/rat	FACS 1:100	BD Pharmingen
CD8α-APHC	5H10/rat	FACS 1:100	Caltag
CD11c-PE	HL3/arm. hamster	FACS 1:100	BD Pharmingen
CD11b-bio	M1/70/rat	FACS 1:100	BD Pharmingen
CD62L-APHC	MEL-14/rat	FACS 1:100	BD Pharmingen
CD62L-FITC	MEL-14/rat	FACS 1:50	BD Pharmingen
IAb-bio (MHC II)	25-9-17/mouse	FACS 1:100	BD Pharmingen
IgG1-FITC Isotype control ICS	R3-34/rat	FACS 1:500	BD Pharmingen
IFNγ-FITC	XMG1.2/rat	FACS 1:500	BD Pharmingen
TNFα-FITC	MD6-XT22/rat	FACS 1:500	BD Pharmingen
TCRβ-FITC	H57-597/ arm. hamster	FACS 1:100	BD Pharmingen
Fc block (CD16/32)	2.46G2/rat	FACS 1:50	BD Pharmingen
MHC I-Tetramer SIINFEKL-PE	----	FACS 1:50	Prof. D.H. Busch, Technical University Munich
SA-APHC	----	FACS 1:50	BD Pharmingen
TCA-3 (I-309)	polyclonal/goat	*In vivo* neutralisation 50µg/mouse	R&D Systems
Anti-goat IgG	polyclonal/donkey	Isotype Control 50µg/mouse	R&D Systems

2.1.6 Equipment

Equipment	Supplier
Centrifuges	Biofuge stratos, Heräus, Hanau
	Varifuge 3.ORS, Heräus, Hanau
	Biofuge pico, Heräus, Hanau
	Biofuge fresco, Heräus, Hanau
	Sepatech, Heräus, Hanau
Confocal Microscope	Axiovert 100 M, Zeiss, Jena
Cryotome	Jung CM 3000, Leica, Nussloch
Electrophoresis Equipment	BioRad, München
Electroporator	Gene Pulser, BioRad, München
Flow Cytometer	FACScalibur, Becton Dickinson, USA
Hybridisation Equipment	Biometra, Göttingen
Gel Documentation	Eagle EYE II, Stratagene, Heidelberg
	Gel Doc 2000, BioRad, München
Incubator for Cell Culture	Cytoperm 2
Incubator for Bacterial Culture	Mytron, Heilbad Heiligenstadt (S1)
	Incubator Model B, Memmert, Schwabach (S2 lab)
	Minitron, Infors AG, Switzerland (S2 lab)
Microscopes	Zeiss Axiolab, Zeiss, Jena
	Zeiss Axiovert 25, Zeiss, Jena
	LeicaMZ7_5 with KL1500_{LCD} unit, Leica, Nussloch
PCR Maschine	TRIO Thermoblock®, UNO Thermoblock®, Biometra, Göttingen
Phosphoimager	Storm 840, Molecular Dynamics, USA
	Molecular Imager®FX,BioRad, München
Safety Cabinet	HERAsafe, Heräus, Hanau
UV Crosslinker	UV Stratalinker®2400, Stratagene, Heidelberg
UV Photometer	Ultrospec3000pro, Amersham, Braunschweig
	NanoDrop®ND-1000, NanoDrop Technologies, USA
	BioPhotometer, Eppendorf, Hamburg

2.2 Stock Solutions and Buffers

Stock Solution/Buffer	Composition	
Ampicillin Stock	50 mg/ml	ampicillin in H_20dd
BSA Solution (PCR)	4 µg/ml	BSA in H_2Odd
Chloramphenicol Stock	25 mg/ml	chloramphenicol in EtOH
Collagenase Solution	1 mg/ml	collagenase type VIII
	200 U/ml	DNAse I
	1×	PBS
dNTP Mix	1 mM	dATP
	1 mM	dCTP
	1 mM	dGTP
	1 mM	dTTP
EMA	2 mg/ml	EMA in DMF
EMA Mix	1 ml	FACS buffer
	1 µl	EMA
	10 µl	Fc block
Erythrocyte Lysis Buffer	1.55 M	NH_4Cl
	100 mM	$KHCO_3$
	10 mM	EDTA
		pH 7.2
ES Cell Lysis Buffer I	1×	TNE buffer
	1%	SDS
	150 µg/ml	Proteinase K
	500 µg/ml	Pronase E
ES Cell Lysis Buffer II	100 mM	Tris-Cl; pH 8.5
	5 mM	EDTA
	200 mM	NaCl
	0.2%	SDS
	100 µg/ml	Proteinase K
ES Cell Lysis Buffer III	10 mM	Tris-Cl; pH 7.5
	10 mM	EDTA
	10 mM	NaCl
	0.5%	sarcosyl
	400 µg/ml	proteinase K
EtBr Stock Solution	10 mg/ml	EtBr

EtBr Staining Solution	50 µl	EtBr stock solution
	1 l	H_2Odd
		keep dark at RT
FACS Buffer	1×	PBS
	0.5% (w/v)	BSA
	0.1%	sodium azide
FITC Solution	1 :1	acetone/dibutyl phtalate
	0.5% (w/v)	FITC
Freezing Solution for Organs	33%	Tissue Freezing Medium™
	66%	20% sucrose solution
G-418 Stock Solution	100 mg/ml	active G-418 protein (according to supplier) in ES medium (see 2.3.2) adjust pH to physiological condition with 10 N NaOH
Gancyclovir Stock Solution	2 mg/ml	gancyclovir in sterile H_2Odd
Hybridisation Buffer	1 M	NaCl
	50 mM	Tris pH 7.5
	10% (w/v)	dextran sulfate (Na-salt)
	1% (w/v)	SDS
	0.5 mg/ml	salmon sperm DNA
Mitomycin C Stock Solution	10 µg/ml	Mitomycin C in EF medium (see 2.3.2)
Neutralisation Solution	0.5 M	Tris-Cl; pH 7.0
	1 M	NaCl
PBS (1×)	137 mM	NaCl
	2.7 mM	KCl
	80.9 mM	Na_2HPO_4
	1.5 mM	KH_2PO_4
		pH 7.4
Peptide Stock Solutions (SIINFEKL, $LLO_{188-201}$)	1 mg/ml	peptide solved in DMSO

Phenol/Chloroform/Isoamylalkohol	50% (v/v)	phenol pH 8.0
	48% (v/v)	chloroform
	2% (v/v)	isoamylalkohol
Proteinase K Solution	10 mg/ml	proteinase K
		solved in H_2Odd
		store at -20º
Pronase E Solution	10 mg/ml	pronase E
	10 mM	Tris-Cl; pH 8.0
	10 mM	NaCl
		digest 1h at 37º
		store at -20º
Splenocyte Lysis Buffer	0.1%	Triton-X 100 in H_2O
TE Buffer (pH 8.0)	10 mM	Tris-Cl; pH 8.0
	1 mM	EDTA; pH8.0
TNE Buffer	10 mM	Tris-Cl; pH 8.0
	100 mM	NaCl
	1 mM	EDTA; pH 8.0
Transfer Buffer	0.4 N	NaOH
(Southern Blot)	0.6 M	NaCl
Washing Buffer	2×	SSC
(Southern Blot)	0.1% (w/v)	SDS
10×PCR Buffer	500 mM	Tris-Cl; pH 8.3
(33 mM NEB)	33 mM	$MgCl_2$
	200 mM	$(NH4)_2SO4$
	75 mM	β-mercaptoethanol
20×SSC	3M	NaCl
	0.3M	trisodium citrate
10×TAE	0.4 M	Tris-Cl; pH 8.0
	0.01 M	EDTA
	0.2 M	glacial acid
6×DNA Loading Buffer	15%	Ficoll Type 400
	0.05%	bromophenol blue
	0.05%	xylene cyanol
6×Orange G Loading Buffer	1 mg/ml	orange G
	20 mM	Tris-Cl
	30% (v/v)	glycerol

4% PFA Solution	4% (w/v) PFA 1× PBS heat up to 65ºC until dissolved
5% Sucrose Solution	5% (w/v) sucrose 1× PBS
10% Sucrose Solution	10% (w/v) sucrose 1× PBS
20% Sucrose Solution	20% (w/v) sucrose 1× PBS

2.3 Media

2.3.1. Media for Bacterial Cultures

All media were autoclaved (121º/2 bar/20 min) for sterilisation. Bacteroagar was added before autoclaving procedure, antibiotics were added after autoclaving procedure.

Medium	Composition
LB Medium (E. coli)	10 g trypton 5 g yeast extract 10 g NaCl ad 1 l H_2Odd
LB/Ampicillin	1× LB Medium 100 µg/ml ampicillin
LB/Chloramphenicol	1× LB Medium 12.5 µg/ml chloramphenicol
LB Agar Plates with Ampicillin (E. coli)	1 l LB Medium 15 g bactoagar 100 µg/ml ampicillin
BHI Medium (L.m. and L.m.ova)	37 g/l Brain-Heart Infusion ad 1 l H_2Odd
BHI Agar Plates (L.m. and L.m.ova)	1 l BHI medium 15 g/l bactoagar

2.3.2 Media for Cell Cultures

Medium	Composition
EF Medium	1× DMEM
	5% FCS
	2 mM L-glutamine
	100 U/ml penicillin
	100 U/ml streptomycin
	0.05 mM β-mercaptoethanol
Mitomycin C Medium	1× EF Medium
	10 ng/µl Mitomycin C
ES Medium	1× DMEM
	15% FCS
	1% LIF stock
	2 mM L-glutamine
	100 U/ml penicillin
	100 U/ml streptomycin
	0.05 mM β-mercaptoethanol
Selection Medium I	1× ES medium
	200 µg/ml G-418
Selection Medium II	1× ES medium
	200 µg/ml G-418
	512 ng/ml (2 µM) gancyclovir
EF Cell Freezing Medium	7 ml EF medium (5% FCS)
	2 ml FCS (25% final conc)
	1 ml DMSO (10% final conc)
ES Cell Freezing Medium I	8 ml ES medium (15% FCS)
	1 ml FCS (25% final conc)
	1 ml DMSO (10% final conc)
ES Cell Freezing Medium II	8 ml FCS (80%)
	2 ml DMSO (20%)
Splenocyte Medium	1× RPMI 1640 w/o L-glu
	10% FCS
	2 mM L-glutamine
	100 U/ml penicillin
	100 U/ml streptomycin
	0.05 mM β-mercaptoethanol

2.4 Bacterial Strains, Cells and Laboratory Animals

2.4.1 Bacterial Strains

Bacterial Strain	Supplier	Genotype
E. coli TOP 10	Invitrogen, Karlsruhe	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *deoR* *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G
E.coli DH5α	Invitrogen, Karlsruhe	F^- Φ80*lacZ*ΔM15 Δ(*lacZ*YA-ar*g*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk^-, mk^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1
L. monocytogenes	Prof. K. Pfeffer Heinrich-Heine University, Düsseldorf	wt Listeria strain (ATCC 43251)
L. monocytogenes/ ova	Prof. D. Busch Technical University, Munich	ova-expressing Listeria strain

2.4.2 Cell Lines

Cells/Cell Line	Origin
EF cells	generated from CD1 embryos at embryonic day 14
E14.1 ES cell line	embryonic stem cell line generated from 129/ola mice Institute for Genetics, Cologne
50A8 ES cells	generated from E14.1 ES cell line by insertion of CCL17-eGFP reporter Gene Reference: I. Lieberam, doctoral thesis

2.4.3 Laboratory animals

All laboratory mice were bred and kept in a specific pathogen free (SPF) animal facility at the Institute for Medical Microbiology, Immunology and Hygiene (Technical University Munich) if not stated otherwise. For Listeria infection experiments mice were kept in a special quarantine facility at the Institute for Medical Microbiology, Immunology and Hygiene (Technical University Munich).

Strain	Genetic Background	Source
C57Bl/6	-----	Listeria Infection: Harlan/Winkelmann Migration Assay: own breeding
Balb/c	-----	Harlan/Winkelmann; own breeding
CD1	outbred	Harlan/Winkelmann
$CCR4^{-/-}$	backcrossed to C57Bl/6 (N8)	Chvatchko et al., 2000
TARC-eGFP KO/KO	backcrossed to C57Bl/6 and Balb/c (N8)	Alferink et al., 2003
TARC-eGFP KO/+	backcrossed to C57Bl/6 (N8)	Alferink et al., 2003

2.5 Primer

2.5.1 PCR Primers

All PCR primers were synthesized by TIB Molbiol, Metabion or ARK Scientific

Name/	Sequence (5'→ 3')	Application
MDC5	GAG CCT CAC AGA GTG ACA GCC CAG Forward Primer	Identification of Bac Fragments containing CCL22 Gene
MDC3	ACT TGT GCC GAT CCC AGG CAG GTC Reverse Primer	
MDC1	GGA TTA GCT CCC CTG TGC TTA TGA GAG GA Forward Primer	Identification of Bac Fragments containing CCL22 Gene
MDC2	CGT CAG GTG TCT AAT GAC CAG AGG GCA GGA AGG T Reverse Primer	
SAHII/FO	NNN NGC GGC CGC TGG GGA ACC GTA AAG CAG TG Forward Primer	Amplification of SAH for cloning into pEasyFlox vector
SAHII/REV	NNN NGG ATC CAT TGC CAT ATG GAA CTA CTC AGA GGG GAC TG Reverse Primer	
LAH/FO	NNN NGT CGA CTT GGT GAT GGG ACG CAA TGC Forward Primer	Amplification of LAH for cloning into pEasyFlox vector
LAH/REV	NNN NAA GCT TTC CAG GGA AGC AAG AAT GGG Reverse Primer	
SAH/II/FO	NNN NGC GGC CGC CTC TGG TCA TTA GAC ACC TGA C Forward Primer	Amplification of replacement SAH for cloning into targeting vector
SAH/II/REV	NNN NCA TAT GAA GCT TGA CAC TAT GGT GGG AAG GAG GG Reverse Primer	

GL/FO	GGG CCA TGT TCC AGG TTA C Forward Primer	PCR Screen
GL/REV	ATG ACC AGA GGG CAG GAA G Reverse Primer	
TV/FO	AAG CAG TGT GGC GTG ATC Reverse Primer	
TV/REV	GCA TGC TCC AGA CTG CCT TG Reverse Primer	
5'S/I/FO	GGC TGA GCC ATC TCT CTA GG Forward Primer	Probe for Southern Blot Screen
5'S/I/REV	GGT TCA CCA AGT TGG ACC TT Reverse Primer	
5'S/II/FO	CTG GAG CAC ACA GTC AGC CA Forward Primer	Probe for Southern Blot Screen
5'S/II/REV	CTA TGG CCT GAC CCT GAC TA Reverse Primer	
MDC/3'Sonde/ F1	GGT CCC AGG GGA AGG AAT AAA C Forward Primer	Probe for Southern Blot Screen
MDC/3'Sonde/ B1	GAA TCA AGG ACA CAA GAA AGG G Reverse Primer	

2.5.2 Sequencing Primers

All sequencing primers were synthesized by TIB Molbiol or GATC

Name/	Sequence (5'→ 3')	Application
T3	AAT AAC CCT CAC TAA AGG GA	sequencing of inserts cloned into pBSIIKS$^-$ vector
T7	TAA TAC GAC TCA CTA TAG GG	
M13-FP	TGT AAA ACG ACG GCC AGT	sequencing of PCR products cloned into pCRII® TOPO vector
M13-RP	CAG GAA ACA GCT ATG ACC	
3NEO F	TAT CGC CTT CTT GAC GAG TTC	sequencing of FRT site from pFRT2neo
5NEO R	CAT CTT GTT CAA TGG CCG ATC C	
TARC2M	ACT CTC AGG ACA CCT GCT TCC	sequencing of FRT site from pTARC-KI
TARC2M/II	AAC ACT CCA CTG AGG	
FragI/Primer I	CCC AGA GCC TGT ACC CTG	Sequencing of BAC clone fragment I
FragI/Primer II	CCA CAC ACA CCC ATA TTC C	
SAH/FRT/FO	TTA TGC TTC CGG CTC GTA TG	check for correct cloning of SAH and FRT site into pEasyFlox vector
SAH/FRT/REV	GGA TGT GGA ATG TGT GCA AG	
LAH/FO (5)	GCC TGA AGA ACG AGA TCA GC	check for correct cloning of LAH into pEasyFlox vector
LAH/REV (5)	ATT CGC CAA TGA CAA GAC GC	

2.6. Plasmid Vectors

Name	Characteristics	Source of Supply
pCR®2.1 -TOPO	TA cloning of PCR products AmpR, KanR, LacZα, f1 ori, pUC ori lac promoter size: 3.9 kb	Invitrogen, Karlsruhe
pBluescriptIIKS$^-$	AmpR, LacZα, f1 ori, pUC ori, lac promoter size: 3.0 kb	Stratagene, Netherlands
pEasyFlox	cassette vector for construction of CCL22 targeting vector; AmpR, neo cassette flanked by loxP sites, HSV-TK size: 6.8 kb	Institute for Genetics, University of Cologne
pFRT2neo	source of FRT site for targeting vector AmpR, KanR, neo cassette, HSV-TK, FRT site; size: ≈ 4,5kb	Institute for Genetics, University of Cologne
pBeloBac11	vector control, no insert single copy plasmid F-factor origin, chloramphenicolR size: 7,5 kb	Incyte Genomics
pBeloBac11 # 24 123	pBeloBac11 vector containing insert of approx.120kb size including CCL22 gene	
12 kB HindIII-Fragment	12 kb fragment generated by HindIII digestion of pBeloBac11 #24 123 in pBSIIKS$^-$	-----

Spe-Fragment I	6 kb fragment generated by SpeI digestion of 12 kb Hind III fragment in pBSIIKS^{-}	-----
Spe-Fragment II	2.5 kb fragment generated by SpeI digestion of 12 kb Hind III fragment in pBSIIKS^{-}	-----
Spe-Fragment III	3.5 kb fragment generated by SpeI digestion of 12 kb Hind III fragment in pBSIIKS^{-}	-----
Spe-Fragment IV	500 bp fragment generated by SpeI digestion of 12 kb Hind III fragment in pBSIIKS^{-}	-----
CCL22 Targeting Vector I	800 bp SAH, 200 bp FRT site, 3.75 kb LAH in pEasyFlox	-----
CCL22 Targeting Vector II	500 bp SAH, 200 bp FRT site, 3.75 kb LAH in pEasyFlox	-----
5' SI SB Probe	250bp Southern Blot probe in pCRII®TOPO	-----
5' SI SB Probe	250bp Southern Blot probe in pCRII®TOPO	-----
3' SI SB Probe	540 bp Southern Blot probe in pCRII®TOPO	-----

2.7 Molecular Biology Methods

2.7.1 Amplification and Isolation of plasmid DNA and BAC DNA

Amplification and Isolation of Plasmid DNA

Chemically competent DH5α E. coli were transformed with plasmid DNA according to manufacturer's protocol. Transformed E.coli colonies were expanded in LB medium containing an appropriate antibiotic. For isolation of high copy plasmid DNA from transformed E.coli two different kits supplied by QIAGEN were used depending on the needed amount of DNA. For isolation of up to 20 µg of DNA the QIAprep Spin Miniprep Kit was used according to manufacturer's protocol. For isolation of up to 500 µg of DNA the QIAfilter Plasmid Maxi Kit was used according to manufacturer's protocol.

Amplification and Isolation of BAC DNA:

BAC containing E.coli from a glycerol stock were plated on LB-agar plates containing chloramphenicol. Colonies were picked and expanded in LB medium containing chloramphenicol. For isolation of BAC DNA the QIAGEN Plasmid Maxi Kit was used according to manufacturer's protocol for low copy plasmids.

2.7.2 Isolation of genomic DNA

Large Scale Preparation of ES cell DNA:

ES cells were expanded to 10 cm or 15 cm tissue culture dishes w/o feeder cells according to standard procedure (see section 2.9.1) and allowed to grow to high density. ES cells were trypsinised according to standard protocol (see section 2.9.1) and centrifuged at 1200 rpm and 4ºC for 7 min (Varifuge3.ORS). ES cells were lysed o/n in ES Cell Lysis Buffer I at 56ºC. The next day, a phenol/ chloroform extraction was carried out according to standard procedure. The ES cell DNA was solved in TE and stored at 4ºC.

Small Scale Preparation of ES cell DNA:

ES cells were expanded in 12 well plates according to standard procedure (see section 2.9.1) and allowed to grow to high density. ES cells were lysed o/n at 37ºC using ES Cell Lysis Buffer II. The next day, a phenol/chloroform extraction was carried out according to standard procedure. ES cell DNA was solved in TE and stored at 4ºC.

2.7.3 Restriction Digests

For analytical purposes 0.5-2 µg of DNA were digested with 10-20 U of one or two restriction endonucleases in an appropriate 1×REACT® buffer. Restriction digests were carried out for 1-2 h at the appropriate temperature (usually 37ºC). For preparative purposes 10-50 µg of DNA were digested with 50-250 U of one or two restriction enzymes in an appropriate 1×REACT® buffer. Restriction digests were carried out for 2-4 h at the appropriate temperature (usually 37ºC). If two restriction endonucleases required different buffers, the digest was carried out sequentially. After digestion of the DNA with the first restriction endonuclease, the buffer of the reaction mixture was changed for the digestion with the second enzyme. Rebuffering procedure was carried out with the QIAquick PCR Purification Kit according to manufacturer's protocol.

2.7.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was employed for analytical and preparative purposes. For both applications 1-1.5% agarose gels were used depending on the size of the expected DNA fragments. Isolation of DNA from the gel was carried out with the QIAquick Gel Extraction Kit according to supplier's protocol.

2.7.5. Determination of DNA Concentration

Determination of DNA concentration was carried out using either photometry or agarose gel electrophoresis. Photometric determination of DNA concentration was mainly used for genomic DNA or circular plasmid DNA. Agarose gel electrophoresis was used to determine the concentration of linearised plasmid vector DNA, digested DNA fragments or PCR products.

2.7.6 Cloning Procedures

Cloning of PCR Products:

PCR products were purified by gel electrophoresis and isolated using the QIAquick Gel Extraction Kit as described in section 2.7.4. Purified PCR products were cloned into the pCR®II-TOPO® vector using the TOPO-TA Cloning® Kit according to manufacturers protocol.

Cloning of DNA Fragments after Restriction Digest:

To clone DNA fragments (inserts) into the MCS of plasmid vectors the plasmid vector and the foreign DNA were preparatively digested with one or two appropriate restriction endonucleases as described in section 2.7.3. If the plasmid vector DNA had been prepared by cleavage with one restriction endonuclease, spontaneous religation of the plasmid vector was possible. Self-religation was prevented by dephosphorylation of the vector DNA using a bacterial alkaline phosphatase according to supplier's protocol. After that, vector and insert were purified by agarose gel electrophoresis and isolated using the QIAquick Gel Extraction Kit as described in section 2.7.4. The concentration of the eluted vector and insert DNA was determined by agarose gel electrophoresis prior to ligation. For ligation procedure, the TaKaRa Ligation Kit was used according to manufacturer's protocol. Chemically competent DH5α E.coli were transformed with the products of the ligation procedure by heat shock according to manufacturer's protocol.

2.7.7 PCR Procedures

PCR Protocol for DNA Amplification:

The PCR method was used for amplification of specific DNA fragments from BAC clones or genomic DNA. If further subcloning of PCR products into plasmid vectors had to be carried out, the PCR primers were designed to introduce restriction endonuclease recognition sites to facilitate specific cloning. For this application it was vital to prevent introduction of point mutations. Therefore the enzyme Herculase® was used as this polymerase has a proofreading activity. For pure analytical purposes it was sufficient to use Taq polymerase which lacks proofreading activity. The composition of the PCR reaction mixes for both polymerases is shown below:

Taq Polymerase:	Herculase®:
x µl DNA	x µl DNA
5 µl 10×33 mM NEB Buffer	5 µl 10×Herculase Buffer
2 µl BSA Solution	----------
1 µl Forward Primer (10 pmol/µl)	1 µl Forward Primer (10 pmol/µl)
1 µl Backward Primer (10 pmol/µl)	1 µl Backward Primer (10 pmol/µl)
1µl dNTP mix (1 mM each)	1µl dNTP mix (1 mM each)
1 µl Taq Polymerase	1 µl Herculase
x µl H_2Odd	x µl H_2Odd
∑ 50µl	∑ 50µl

The PCR procedure was carried out as follows:

Function	Duration	Temperature	
DNA Denaturation	60 s	94ºC	35 cycles
Annealing	30 s	55-60ºC	
Elongation	90 s	72ºC	
End	∞	15ºC	

96 well PCR Screening Protocol:

After the 96 well plate had been thawed, ES cells were resuspended in 20 µl H_2Odd per well. ES cell suspension and control templates were transferred to a thermostable 96 well PCR plates. After that, the plate was incubated at 56ºC for 10 min (lid temperature 102ºC). In the next step, 5 µl/well of proteinase K solution (2 µg/µl) were added.

The plate was incubated for 60 min at 56ºC and for 10 min at 95ºC. After that, 17µl/well of PCR mix were added and the plate was incubated for 5 min at 95ºC and for 10 min at 80ºC.

PCR Mix:
1 µl dNTP stock solution
5 µl 10×Herculase® Reaction Buffer
1 µl Forward Primer (10 pmol/µl)
1 µl Backward Primer (10 pmol/µl)
9 µl H_2Odd
∑17 µl

After that, 5 µl of Herculase/Taq polymerase mix were added per well and the PCR screening program was immediately started.

Herculase/Taq polymerase mix:
0.5 µl Taq Polymerase
0.5 µl Herculase®
4 µl H_2Odd
∑5 µl

Screening Program:

DNA Denaturation	60 s	94ºC	} 35 cycles
Primer Hybrydisation	30 s	62ºC	
Elongation	90 s	72ºC	
End	∞	15ºC	

The PCR screening reactions were analysed by agarose gel electrophoresis (see section 2.7.4). The DNA was blotted to a GeneScreen plus® nylon membrane and hybridised with a radioactively labelled DNA probe containing the full length sequence of the SAH. The membrane was exposed to a phosphoimager screen for 12-24 h and analysed on a phosphoimager. All these procedures are described in section 2.7.8.

2.7.8 Southern Blot Screens

Southern Blot Screens of PCR positive clones:

A large scale preparation of ES cell DNA from PCR positive clones was carried out as described in section 2.7.2. The concentration was determined by photometry (see section 2.7.4) and 10 µg of DNA were digested o/n with 150 U of restriction endonuclease at 37ºC. As control, 10 µg of E14 ES cell DNA were digested in the same way. The next day 100 U of restriction enzyme were added and the digest was further incubated for several hours. After that, the restriction digests were applied to a 0.8% agarose gel which was allowed to run o/n at 35-40 V. The gel was stained in EtBr solution for 30-45 min and analysed with the documentation system Eagle EYE. The DNA was blotted o/n to a GeneScreen Plus® nylon membrane by the downward blotting method following standard procedure and under consideration of the supplier's recommendations. After blotting, the DNA was covalently linked to the membrane (crosslinking) by UV radiation using the UV Stratalinker. In the next step pre-hybridisation of the membrane was carried out o/n at 65ºC. A DNA probe was prepared by restriction digest, purified by agarose gel electrophoresis and extracted with the QIAquick Gel Extraction Kit as described in section 2.7. The concentration of the DNA probe was determined by agarose gel electrophoresis and the DNA probe was labeled with radioactive α[^{32}P]dCTP using the rediprime™II random prime labelling kit according to manufacturers protocol. One modification was applied: the reaction mix was incubated for 0.5-2 h (instead of 10 min). The labelled DNA was separated from free radioactive nucleotides with the MicroSpin™ S-200 HR Kit according to manufacturer's protocol. The membrane was hybridised o/n with the purified probe at 65ºC. The next day, the radioactive probe was removed and the blot was washed with Washing Buffer until background radioactivity was low. After that, the membrane was placed inside a phosphoimager cassette containing a phosphoimager screen. After sufficient exposure, the screen was analysed.

12 Well Plate Southern Blot Screen of ES Cell Clones:

A small scale preparation of DNA from selected ES cell clones was carried out as described in section 2.7.2. 30 µl of DNA solution were digested o/n with 75 U of restriction endonuclease at 37ºC. As control, 5 µg of E14 ES cell DNA were digested in the same way. The next day, the restriction digests were applied to a 0.8% agarose gel which was allowed to run o/n at 35-40 V. The gel was stained in EtBr solution for 30-45 min and analysed with the documentation system Eagle EYE. The gel was blotted o/n, crosslinked and pre-hybridised as described above. Labelling of the Southern Blot probe and hybridisation were carried out as described. After removal of the radioactive probe the blot was washed with Washing Buffer until background radioactivity was low and the membrane was placed on a phosphoimager screen. After sufficient exposure, the screen was analysed.

96 Well Plate Southern Blot Screen of ES Cell Clones:

The 96 well plate was thawed at RT and ES cells were lysed using ES Cell Lysis Buffer III. The plate was incubated o/n at 56ºC in a humidified chamber. The next day, the DNA was precipitated with 100% EtOH p.A. The plate was gently inverted and the wells were carefully drained on paper towels. The plate was washed three times with 70% EtOH. After each wash, the plate was gently inverted and the wells were carefully drained on paper towels. The DNA was allowed to air dry before a restriction endonuclease digestion was carried out. 35 µl of restriction master mix were added to each well and the plate was incubated o/n at 37ºC.

Restriction Master Mix (100 samples):
350 µl 10×REACT® Buffer
100 µl Restriction Endonuclease HC (50 U/ml)
3050 µl H_2Odd
∑ 3500 µl

As wt control, 5 µg of 50A8 ES cell DNA were digested in the same way. The restriction digests were applied to a 0.8% agarose gel which was allowed to run o/n at 35-40 V. The gel was stained in EtBr solution for 30-45 min and analysed with the documentation system Gel Doc 2000.

The gel was blotted o/n, crosslinked and pre-hybridised as described above. The DNA probe was labeled with radioactive α[^{32}P]dCTP as described. One modification was applied: the reaction mix was incubated for 14-18 h at 37°C. Hybridisation was carried out as described above. After removal of the radioactive probe the blot was washed as described and the membrane was placed on a phosphoimager screen. After sufficient exposure, the screen was analysed.

2.8 Cell Culture and Cell Biological Methods

All cell culture procedures were carried out under sterile conditions in a special safety cabinet (HERAsafe). Incubation of all cell cultures were carried out in a cell culture incubator (Cytoperm2) at 37ºC, 7.5% CO_2 and 95% humidity. To avoid contamination, sterile equipment was used at all times.

2.8.1 Culture of Murine Embryonic Fibroblasts (EF Cells)

Embryonic fibroblast cells were prepared and frozen as described in section 2.9.2. Cryovials with frozen EF cells were thawed in a 37ºC water bath. The cells were transferred to 15 ml tubes containing cold EF medium. After that, the cells were immediately centrifuged for 5 min at 1200 rpm and 4ºC (Variofuge 3.ORS) to remove the DMSO that had been added to the Freezing Medium. After centrifugation, the s/n was removed, the pellet was resuspended in EF Medium (RT) and the cells were transferred to a 15 cm cell culture dish. The cells were grown in a cell culture incubator at 37ºC for 2-3 days. Before splitting, EF cells were treated with mitomycin C. The cells were incubated at 37ºC for 2,5 h in 12 ml mitomycin C containing medium, washed three times with PBS and trypsinised for 10 min at 37ºC. EF medium was added; the cells were resuspended and split to 5 cm and/or 10 cm cell culture dishes depending on density of EF cells and needed amount and size of dishes. If possible, the EF cells were allowed to adhere o/n before addition of ES cells.

2.8.2 Culture of Murine Embryonic Stem Cells (ES Cells)

Murine embryonic stem cells are co-cultured with mitomycin C treated EF cells (feeder cells). EF cells produce growth factors that are needed by the ES cells. Additionally, EF cells produce Leucemia Inhibitory Factor (LIF) which prevents differentiation of ES cells. To keep the amount of differentiated ES cells as low as possible, additional exogenous LIF was added to the cells. When cultivating and expanding ES cells it is vital to keep them at an optimal density range. If density of the cells is too low, cells will die. If density is too high, cells will differentiate. As a consequence, ES cells have to be split very frequently (usually every two days). Undifferentiated ES cells form round colonies with a smooth surface and shiny edges which can be nicely seen with a regular microscope. In contrast, differentiating ES cells become flat and grey and develop pseudopode-like structures. As ES cells have a very active metabolism with high turnover rates, it is necessary to feed ES cells daily.

Thawing of ES Cells:

ES cells were thawed at 37ºC and transferred to 15 ml tubes containing cold (4ºC) ES medium. After that, the cells were immediately centrifuged for 5 min at 1200 rpm and 4ºC to remove the DMSO which had been added to the Freezing Medium. The cells were resuspended in ES Medium (RT) and transferred to a 5 cm cell culture dish containing an EF cell layer.

Expansion of ES Cells:

After thawing, ES cells were allowed to grow on 5 cm cell culture dishes until reaching optimal density (normally after 2 days). ES cells were split to 2-4 10 cm cell culture dishes as described below and allowed to grow to optimal density. In the next step, the cells were split to 2-4 15 cm cell culture dishes to be used for electroporation or frozen in 2-4 cryovials to be stored long term in liquid nitrogen. For electroporation procedure see section 2.8.3; for freezing procedure see below.

Splitting of ES Cells:

ES cells were washed once with PBS and trypsinised for 5-10 min at 37ºC. In the next steps, ES medium was added; ES cells were resuspended and distributed to the needed amount of suitably sized tissue culture dishes or well plates. In the last step, ES medium was added to a final volume depending on the size of the dish or the plate, respectively.

Freezing of ES Cells:

Upon reaching optimal density, ES cells were washed three times with PBS, trypsinised for 5-10 min at 37ºC and resuspended in ES medium. If ES cells had to be further expanded, it was important to transfer an aliquot of the cell suspension to a fresh 10 cm cell culture dish before proceeding with the freezing protocol. ES cells which were destined to be frozen were centrifuged for 7 min at 1200 rpm and 4ºC. The cells were resuspended in ice cold ES Cell Freezing Medium I and distributed to 2-4 cryovials (1 ml cell suspension per vial). The cells were incubated at –20ºC for 30 min before being transferred to a –80ºC freezer where they were kept for 1-3 days before they were transferred to the liquid nitrogen tank.

2.8.3 Transfection and Selection of ES cells

Transfection of ES cells:

One day prior to transfection of ES cells the targeting vector DNA was linearised by restriction endonuclease digest. For this purpose, 200 µg of vector DNA were digested o/n at 37ºC with 900 U of Not I in a total volume of 2 ml. The next day, linearisation of the vector DNA was confirmed by agarose gel electrophoresis and the linearised vector DNA was isolated by sodium acetate precipitation according to standard procedure. The vector DNA was solved in 1 ml PBS at 37ºC for 2 h. In the meantime, ES cells were prepared for transfection. For that purpose, ES cells which had been previously expanded to 4×15 cm tissue culture dishes (see section 2.8.2.) were washed with PBS and trypsinised for 10 min at 37ºC. The cells were resuspended in ES medium, pooled and counted in a Neubauer counting chamber and centrifuged at 1200 rpm and 4ºC for 7 min.

The cells were resuspended in a calculated volume of ES medium to give a concentration of 7.14×10^6 cells/ml.7 ml of cell suspension were transferred to a 15 ml tube to give a total of 50×10^6 cells which were needed for transfection. The cells were mixed with the previously linearised vector DNA to give a total volume of 8 ml cell/DNA suspension and transferred to 10 electroporation cuvettes (800 µl per cuvette). The cells were electroporated at 250 µF and 340 V and incubated on ice for 5 min. After that, 400 µl of cell suspension were transferred to a 10 cm cell culture dish giving a total of 20 cell culture dishes. The cells were allowed to expand for two days before start of selection.

Selection of ES Cells:

The selection process of ES cells was started on day 2 after electroporation by feeding the cells with Selection Medium I which includes G 418. All ES cell clones which did not integrate the targeting vector DNA into their genome did not carry the neo resistance gene and started to die. The next step of selection was started 4 days after electroporation by feeding the cells with Selection Medium II which contains G 418 and gancyclovir for the remaining duration of the selection process. All cells which had randomly integrated the targeting vector into their DNA also integrated the TK cassete included in the targeting vector construct resulting in sensitivity to gancyclovir treatment. The cells were continuously fed every two days until the surviving clones had gained the right size to be picked (around day 12).

2.8.4 Picking and Expansion of Transfected and Selected ES Cell Clones

When picking clones, it is vital to choose the right time point to ensure that the colonies have the suitable size. If colonies are allowed to grow too long, ES cells which are located close to the edges of the colony will start to differentiate. If clones are picked too early, the picked clones are too small to expand and will eventually die.

Protocol 1 (PCR Screening):

Prior to picking, a suspension of EF cells had to be prepared. EF cells were thawed, expanded and treated with mitomycin C as described in section 2.8.1. After that, EF cells were trypsinised as previously described and centrifuged for 7 min at 1200 rpm and 4ºC. The cell pellet was resuspended in 50 ml ES Medium per 15 cm dish and kept in a cell culture incubator until use. In the next step, a binocular microscope was transferred to a safety cabinet to allow picking under sterile conditions. Colonies with appropriate size were picked and transferred to 96 well plates. After that, cells were trypsinised with 50 µl/well for 10 min at 37ºC and resuspended in 100 µl ES medium. Finally, 50 µl EF cell suspension were added per well and ES cells were allowed to expand until the majority of ES cell clones reached optimal density (normally 2-3 days). After that, the cells on each 96 well plate were split to two 48 well plates and one 96 well plate. ES cells in the 48 well plates were allowed to grow to optimal density and frozen. Briefly, ES cells were washed three times with PBS, trypsinised for 10 min at 37ºC and resuspended in ES medium. After that, an equal volume of ice cold Freezing Medium II was added, the plates were incubated at -20ºC for 30 min before being transferred to a -80ºC freezer where the cells could be stored for a maximum of 8 weeks. ES cells in the 96 well plate were allowed to expand to optimal density and split to three 96 well plates which did not contain feeder cells. ES cells were allowed to grow to maximum density before being frozen. Briefly, ES cells were washed three times with PBS, trypsinised for 10 min at 37ºC and resuspended in ES medium. After that, ES cells were transferred to 96 well V bottom plates, centrifuged at RT and 500 rpm for 5 min (Biofuge stratos), washed with PBS and re-centrifuged. After that, the plate was stored at –20ºC until PCR screening was carried out as described in section 2.7.6.

Protocol 2 (Southern Blot Screen):

ES cell clones were picked as described in protocol 1. ES cell clones were allowed to expand until the majority had reached optimal density (normally 2-3 days). After that, the cells on each 96 well plate were split to two 48 well plates and one 96 well plate as described in protocol 1. ES cells in the 48 well plate were allowed to grow to optimal density and frozen as described in protocol 1. ES cells in the 96 well plate were allowed to grow to optimal density and split to 2×48 well plates. ES cell clones were allowed to grow to optimal density and split to 24 well plates. ES cells were expanded to optimal density and each split to 12 well plates which did not contain feeder cells.

ES cells were cultured until they were grown confluent and a low yield DNA preparation was carried out as described in section 2.7.2. After that, a southern blot screen was carried out as described in section 2.7.7.

Protocol 3 (96 Well Southern Blot Screening:

One day prior to picking a sufficient amount of 96 well plates with feeder cells was prepared. Briefly, EF cells were thawed, expanded and treated with mitomycin C as described in section 2.8.1. After that, EF cells were trypsinised as previously described and centrifuged at 1200 rpm and 4ºC for 7 min. The cells were resuspended in 50 ml EF medium per 15 cm dish and transferred to 96 well plates (50 µl/ well). The feeder cells were allowed to settle o/n in a cell culture incubator. The next day, a sufficient amount of 96 well plates containing 1×trypsin was prepared. In the next step, colonies with appropriate size were picked under sterile conditions as described and transferred to the previously prepared 96 well plates. After 20 min of picking the plate was incubated for 3 min at 37ºC. The cells were resuspended in ES medium and distributed to three previously prepared 96 well plates containing feeder cells. ES cells were allowed to expand until the majority of ES cell clones were almost too dense (normally 2-3 days) and frozen. Briefly, two 96 well plates of each set of triplicates were washed three times with PBS, trypsinised for 3 min at 37ºC and resuspended in ES medium. After that, an equal volume of ice cold Freeze Medium II was added and the cells were covered with embryo tested mineral oil. The plate was incubated at -20ºC for 30 min before being transferred to a -80ºC freezer where the cells could be stored for a maximum of 8 weeks. The third 96 well plate of each set of triplicates was trypsinised and distributed to three 96 well plates w/o feeder cells as described. ES cell clones were allowed to expand until they were grown fully confluent and frozen. Briefly, ES cells were washed three times with PBS and stored at –20ºC until southern blot screening was carried out as described in section 2.7.6.

Thawing of 96 Well and 48 Well Stock Plates:

One day prior to thawing, a suitable amount of 96 well plates/48 well plates containing EF cells had to be prepared as previously described. Stock plates with ES cell clones were placed inside a cell culture incubator until ES cells were almost thawed. ES cells of positively screened clones were removed from the well and quickly transferred to 15 ml tubes containing cold (4ºC) ES medium.

After that, the cells were immediately centrifuged at 1200 rpm and 4ºC for 5 min and transferred to previously prepared 96 well plates or 48 well plates, respectively.

Expanding and Freezing of PCR Positive Clones:

ES cells were expanded to 12 well plates as previously described. When reaching optimal density, cells were washed three times with PBS, trypsinised for 10 min at 37ºC and resuspended in ES medium. Cells were transferred to 5 cm tissue culture dishes with feeder cells and were allowed to expand to optimal density. At this stage, 1-2 cryovials were frozen as described in section 2.8.2.

2.9 Immunological Methods and Animal Experiments

2.9.1 Organ Removal

Animals were sacrificed by cervical dislocation. The peritoneal and chest region of the mice was disinfected with 70% EtOH. The fur was cut and separated from the skin. The skin was cut and the organs were removed under aseptic conditions.

2.9.2. Preparation of Embryonic Feeder Cells

Pregnant CD1 animals were sacrificed by cervical dislocation at day 14 of pregnancy after positive plug check. Mice were transferred to a safety cabinet to allow a preparation under sterile conditions. The peritoneal and chest region of the mice was disinfected with 70% EtOH and the fur and skin were cut and removed. The uterus was removed and transferred to a 10 cm tissue culture dish with EF medium. The embryos were removed and transferred to a 10 cm tissue culture dish containing EF medium. After all embryos had been removed, 10 cm cell culture dishes with EF medium were prepared and a 100 µm cell strainer was placed inside each dish. In the next step, head and liver of each embryo were removed and the remaining body was transferred to the previously prepared 100 µm cell strainer.

3-4 embryos were transferred to each strainer. The embryos were squeezed through the cell strainer with a sterile plunger. Each used cell strainer was washed with EF medium and the gained cell suspension was transferred to a 50 ml tube. The process was repeated until the cells of all embryos from one mouse were pooled in one tube.
In the next step, the cells were centrifuged at 1200 rpm and 4ºC for 7 min, the cells were resuspended in EF medium, counted and 5×10^6 cells were transferred to a 10 cm cell culture dish. The cells were allowed to expand until grown fully confluent (normally after 4-5 days). Each 10 cm dish was split to 4×15 cm tissue culture dishes and was allowed to grow fully confluent. 3-4 vials of EF cells per 15 cm cell culture dish were frozen. EF cells were washed three times with PBS, trypsinised for 10 min at 37ºC and resuspended in EF medium. The EF cell suspension was centrifuged at 1200 rpm and 4ºC for 7 min. EF cells were resuspended in ice cold EF Cell Freezing Medium, distributed to 3-4 cryovials (1 ml cell suspension per vial) and incubated at –20ºC for 30 min. After that, the cells were transferred to a –80ºC freezer where they were kept for 1-3 days before being transferred to the liquid nitrogen tank for long term storage.

2.8.3 Treatment of Mice with Neutralising Antibody

100 µg of antibody were solved in 500 µl PBS. 250 µl of antibody solution (≡ 50 µg) were injected into the peritoneal cavity of each mouse 24 h prior to experiment.

2.8.4 Langerhans Cell Migration Assay

FACS Analysis:

Mice were anaesthetised and shaven at chest and abdomen. 100 µl of FITC Solution were applied to the shaven region and allowed to dry. After 18-20 h the mice were sacrificed by cervical dislocation. The draining lymph nodes, in this case inguinal, axillary and brachial LNs, were carefully removed as described in section 2.9.1. Additionally, the mesenteric LN was removed as an internal negative control.

After removal, a collagenase digestion of the LNs was carried out according to standard procedure. Briefly, Collagenase Solution was injected into the lymph nodes and incubated for 30 s. After that, the LNs were reduced to small pieces, Collagenase Solution was added to give a final volume of 10 ml and the LNs were incubated for 20 min at 37°C. After washing with PBS, the cells were counted in a Neubauer counting chamber. For each cell surface staining 2×10^6 cells were transferred to 1.5 ml reaction tubes and centrifuged for 3 min at 3000 rpm and 4ºC. The cell pellet was resuspended in FACS buffer containing Fc block (CD16/32; 1:50 dilution). The tubes were incubated on ice in darkness for 20 min. After that, the cells were washed with FACS buffer and resuspended in FACS buffer containing the indicated surface staining antibodies in appropriate dilution. The tubes were incubated on ice in darkness for 20 min and washed with FACS buffer. After that, the cells were resuspended in FACS buffer containing indicated streptavidin conjugate in appropriate dilution, incubated on ice in darkness for 20 min and washed with FACS buffer. Finally, the cells were resuspended in 400 µl of FACS buffer and flow cytometry was carried out.

Histology:

Mice were treated with FITC Solution as described above. After 18-20 h mice were sacrificed by cervical dislocation and lymph nodes were removed as described above. After that, LNs were fixed for 3 h in 4% PFA, incubated for 1 h in 5% sucrose solution at RT and for 1 h in 10% sucrose solution at RT. Finally, LNs were incubated o/n in 20% sucrose solution at 4ºC. The next day, LNs were transferred to tissue molds and coated with Freezing Solution. LNs were snap frozen in 2-methylbutane which was cooled by liquid nitrogen. After that, the organs could be stored long term at -80ºC. For histology, LN were cut into 7 µm cryosections which were allowed to air-dry at RT before being embedded in Vectashield® Mounting Medium. Cryosections were analysed with a confocal microscope.

2.9.5. Infection with *Listeria Monocytogenes*

As *L. monocytogenes* is a pathogenic bacterium, all work had to be carried out either in the S2 lab or the S2 animal facility.

Survival Measurement Assay:

In the first step, Microbank™ stock vials of *Listeria Monocytogenes* were prepared according to manufacturer's protocol. The Microbank™ stock was stored at –80ºC until use. One day prior to infection of mice, listeria were expanded in culture. One inocculation ring with attached listeria from a Microbank™ stock vial was transferred to a glass tube containing 6 ml of BHI medium.

The tube was incubated o/n at 37ºC in a bacterial incubator. The next day, the amount of grown bacteria was determined by photometry measuring the absorption at a wavelength of 600 nm (OD_{600}). The concentration of bacteria was calculated as follows: OD_{600} 0.1 ≈ 1×10^8 bacteria/ml. In the next step, the bacterial culture was diluted in BHI to give three different doses: $10\times LD_{50}$, $1\times LD_{50}$ and $0.1\times LD_{50}$. The amount of bacteria that had to be injected for these different doses depended on the genetic background of the animals as described below. The listeria were injected i.p in a volume of 350 µl.

C57Bl/6:

Dose	Amount of Listeria
$10\times LD_{50}$	5.0×10^6 bacteria
$1.0\times LD_{50}$	5.0×10^5 bacteria
$0.1\times LD_{50}$	5.0×10^4 bacteria

Balb/c:

Dose	Amount of Listeria
$10\times LD_{50}$	1.0×10^5 bacteria
$1.0\times LD_{50}$	1.0×10^4 bacteria
$0.1\times LD_{50}$	1.0×10^3 bacteria

For survival measurement, mice were checked twice a day and dead mice were registered and removed until the end of experiment (10-14d).

Determination of bacterial organ load:

In the first step, ova-expressing *Listeria monocytogenes* were expanded in culture. For this purpose, 20 µl of bacterial glycerol stock were transferred to a glass tube containing 6 ml BHI medium. The bacteria were allowed to grow at 37ºC in a bacterial incubator until reaching an OD_{600} of 0.05-0.1. The bacterial concentration was determined as described above and listeria were diluted in PBS. For primary infection of mice with C57Bl/6 genetic background, either 2000 or 20 000 L.m.ova were injected i.v. in a volume of 200 µl. For recall infection of mice with C57Bl/6 genetic background, 200 000 L.m.ova were injected. Three and, if designated, 7 days after primary infection or two and 5 days after recall infection the animals were sacrificed by cervical dislocation. After primary infection, spleen and liver were removed. After recall infection, only the spleen was removed. Each organ was minced in a petri dish using a 70 µm cell strainer and a sterile plunger and resuspended in 5 ml PBS. 100 µl of cell suspension were mixed with 900 µl of 0.1% Triton-X-100/H_2Odd solution and vortexed (Suspension 1). Suspension 1 was diluted 1:10 (Suspension 2) and 1:100 (Suspension 3). Each suspension was plated on BHI plates in triplicates using 10 µl of each suspension per plate. The BHI plates were incubated o/n at 37ºC in a bacterial incubator. The next day, all grown colonies were counted and the bacterial titer of each organ was calculated.

Tetramer Staining:

L.m.ova bacteria were expanded and prepared and mice were infected as described above. Five days after primary infection or seven days after recall infection mice were sacrificed by cervical dislocation to remove the spleen. Each organ was minced in a petri dish using a 70 µm cell strainer and a sterile plunger, resuspended in PBS and transferred to a 15 ml tube. The petri dishes and cell strainer were washed with Splenocyte Medium. The cell suspension was centrifuged at 1500 rpm at RT for 7 min, the pellet was resuspended in Erythrocyte Lysis Buffer and the lysate was incubated for 7 min at RT. After incubation, lysis was stopped by addition of Splenocyte Medium; the cells were centrifuged and resuspended in Splenocyte Medium. The cell suspension was transferred to a 15 ml tube through 70 µm nylon net and counted. After that, 3×10^7 cells of each splenocyte cell suspension were transferred to a 15 ml tube, centrifuged at 1500 rpm at RT for 7 min and the cells were resuspended in EMA Mix. The tubes were placed on ice underneath a light source and incubated for 20 min. In the next step, two aliquots of each cell suspension were transferred to a 96 well plate.

The first aliquot served as negative control while the second aliquot was stained with the indicated tetramer. The plate was centrifuged at 1500 rpm and 4ºC for 2 min and the cells were either resuspended in pure FACS buffer (negative control) or FACS buffer containing the indicated tetramer in appropriate dilution. The cells were incubated on ice in darkness for 20 min. FACS buffer containing indicated surface staining antibodies in appropriate dilution was added to the cells which were incubated on ice in darkness for further 25 min. Finally, the cells were washed three times with FACS buffer, fixed with 1% PFA and stored in darkness at 4ºC until flow cytometry was carried out.

Intracellular Cytokine Staining:

L.m.ova bacteria were expanded and prepared and mice were infected by i.v. injection as previously described. Five days after primary infection or seven days after recall infection mice were sacrificed by cervical dislocation, splenocyte cell suspension were prepared and the cells were counted as described. After that, 6×10^7 cells were transferred to a 15 ml tube and three aliquots of each cell suspension were transferred to a 24 well plate. The cells of the first and second aliquot were stimulated with 2 µg of SIINFEKL or $LLO_{188\text{-}201}$ respectively. As the peptides had been solved in DMSO, the third aliquot was treated with an equal volume of DMSO. The cells were incubated for 2 h at 37ºC, Golgi Plug was added according to manufacturer's recommendation and the cells were incubated for further 3 h at 37ºC. After that, the cells were transferred to 15 ml tubes, centrifuged at 1500 rpm and 4ºC for 7 min and resuspended in EMA Mix. The tubes were placed on ice below a light source and incubated for 20 min. Three aliquots of each cell suspension were transferred to 96 well plates, the plate was centrifuged at 1500 rpm and 4ºC for 2 min and the cells were resuspended in FACS buffer containing the indicated surface staining antibodies in appropriate dilution. The cells were incubated on ice in darkness for 20 min and washed three times with FACS buffer. The cells were resuspended in Cytofix/Cytoperm, incubated on ice in darkness for 20 min and washed three times with PermWash. The cells were resuspended in FACS buffer containing indicated antibodies for intracellular staining or isotype control in appropriate dilution. The cells were incubated on ice in darkness for 30 min, washed three times with PermWash and once with FACS buffer. The cells were fixed with 1% PFA and stored in darkness at 4ºC until flow cytometry was carried out.

3 RESULTS

3.1 Part I: Generation of a CCL17/CCL22-Double Knock Out Mouse

3.1.1 Characterisation of the Genomic Locus of Murine CCL22

Screening of a BAC Clone Library:

In the first step of the project, it was necessary to identify a BAC clone which contained the full length DNA sequence of the genomic locus of murine CCL22. This task was carried out by the company Incyte Genomics (USA). A BAC library derived from 129/SV mice was screened using a cDNA probe and one positive clone, pBeloBac11 # 24 123, was identified.

Cloning of a 12 kb HindIII BAC Clone Fragment:

As pBeloBAC11 vectors hold very large inserts with an average size of 120 kb it was necessary to identify a smaller DNA fragment containing the full length sequence of CCL22. For that purpose, a Southern Blot analysis of pBeloBac11 # 24 123 was carried out which allowed identification of a 12 kb HindIII fragment (data not shown). For cloning of this fragment, pBeloBac11 # 24 123 was preparatively digested with HindIII and applied to an agarose gel (see section 2.7.3 and 2.7.4). The target 12 kb band was extracted from the gel and cloned into the pBSIIKS$^-$ vector (see section 2.7.6). In the next step it was necessary to confirm that the CCL22 gene was included in the cloned 12 kb HindIII-Fragment. This was done by PCR using two different primer pairs. The first primer pair (MDC5/MDC3) was designed with the help of the known cDNA sequence (Schaniel et al. 1998; EMBL/GenBank DDBJ accession no. AF052505) to give rise to a 200 bp PCR product as is shown in Figure 3.1. The second primer pair (MDC1/MDC2) had been shown to bind specifically to the genomic locus of CCL22 giving rise to a 1.1 kb PCR product (Schaniel et al, 1999). All 20 clones which were tested by PCR using both primer pairs gave rise to the expected PCR products (see section 2.7.7.) confirming that the genomic locus of CCL22 was included in the 12 kb Hind III fragment (data not shown).

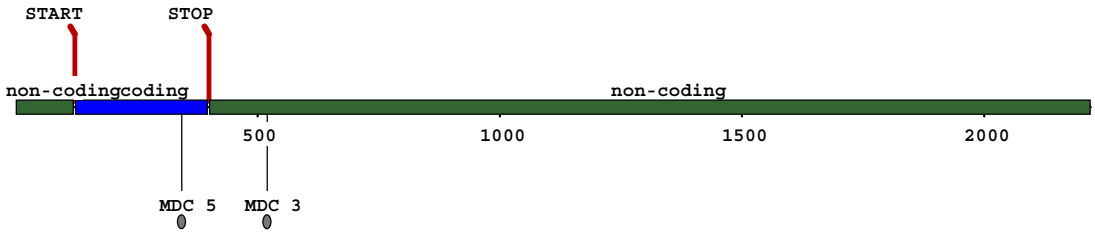

Fig. 3.1: cDNA Map of Murine CCL22 Including Position of the Primer Pair MDC5/MDC3

Sequencing of the 12 kb HindIII BAC Clone Fragment:

As the complete genomic sequence of CCL22 was not yet accessible in the gene bank it was necessary to sequence the gene prior to planning of a targeting strategy. Sequencing was also performed to confirm that the full length genomic sequence of CCL22 was included in the 12 kb HindIII-Fragment of pBeloBac11 # 24 123. As sequencing of long DNA fragments is difficult and expensive and the location of the genomic locus of CCL22 on the fragment was totally unknown, it was necessary to further map the 12 kb HindIII-Fragment. For this purpose, the fragment was analytically digested with different restriction enzymes (data not shown). The restriction patterns were compared to each other and to the cDNA restriction map which led to the identification of four differently sized SpeI fragments covering the complete genomic sequence of CCL22. The position of the identified SpeI Fragments on the 12kb HindIII-Fragment is shown in Figure 3.2.

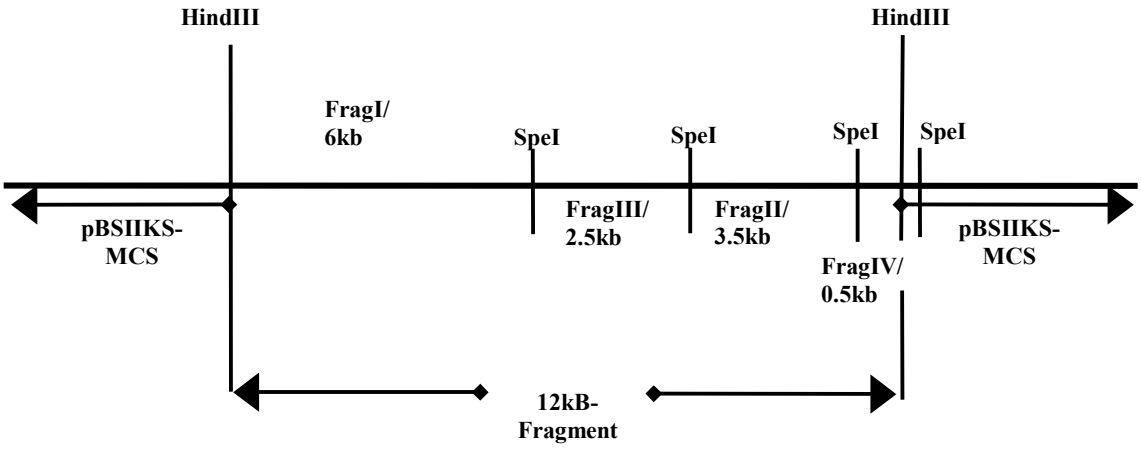

Fig. 3.2: Schematics of the subcloned 12 kb HindIII-Fragment from pBeloBac11 # 24 123 including position of SpeI-Fragments I-IV

The 12 kb HindIII-Fragment was preparatively digested and the four different SpeI Fragments were isolated as described in sections 2.7.3 and 2.7.4. The four fragments were subcloned into the pBSIIKS$^-$ vector as described in section 2.7.6. SpeI-Fragments II and III were sequenced by the company MWG Biotech AG using the primer walking method. As starting points, the standard sequencing primers T3 and T7 which bind to the T3 and T7 promoter regions flanking the MCS of the pBSIIKS$^-$ vector were used. SpeI-Fragments I and IV were sequenced by the company GATC Biotech AG using the primers T3 and T7 for standard sequencing so that a maximum of 900 bp of either end (5' and 3') of these fragments were sequenced. All sequences of the four different SpeI fragments were combined and analysed by comparison to the cDNA sequence. As expected, the SpeI-Fragments I-IV contain the full length genomic sequence in the predicted alignment. The schematic structure of the genomic locus including the position of SpeI- Fragments I-IV is shown in Figure 3.3.

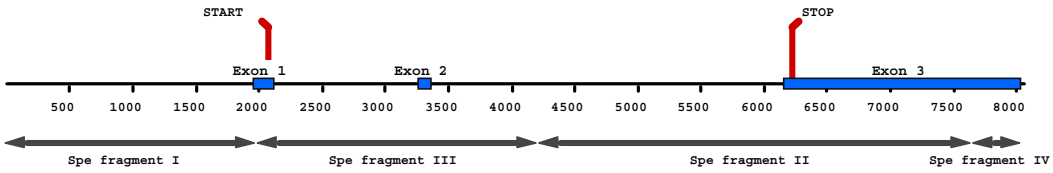

Fig 3.3: Map of the Genomic Locus of CCL22 Including the Position of SpeI-Fragments I-IV from pBeloBac11 # 24 123

Analysis of the full length genomic sequence showed that the genomic locus of CCL22 has a three exon structure which is the typical structure of CC chemokines. Approximately 60% of exon 1 and 95% of exon 3 are non-coding which is in accordance with the cDNA structure. The amino acid sequence of the translated protein matches with the known amino acid sequence. Three of the four highly conserved cystein residues which are crucial for the secondary structure of the mature CCL22 protein are located on exon 2. The fourth cystein residue is located on exon 3. The full length sequence of the genomic locus of CCL22 including the amino acid sequence of the translated protein is shown in Figure 3.4.

Fig. 3.4: Full Length Sequence of the Genomic Locus of CCL22 Including Amino Acid Sequence of the Translated Protein. Exon sequences are underlined, the translated protein sequence is written in italics, the conserved cystein residues are written in bold italics, the start and stop codons are written in bold letters.

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        10         20         30         40         50         60         70
AGGACATGAATGTCAGGCTTGGTAGTGACTAAGAGGTGCCNNAACTAGTGGGTGGAGAGAGCGTTTAAAT
TCCTGTACTTACAGTCCGAACCATCACTGATTCTCCACGGNNTTGATCACCCACCTCTCTCGCAAATTTA

         80         90        100        110        120        130        140
ATCAGACCTTCCTGCCCTCTGGTCATTAGACACCTGACGAGGACACATAACATC***ATG***GCTACCCTGCGTG
TAGTCTGGAAGGACGGGAGACCAGTAATCTGTGGACTGCTCCTGTGTATTGTAG***TAC***CGATGGGACGCAC
                                                        *M  A  T  L  R>*

        150        160        170        180        190        200        210
TCCCACTCCTGGTGGCTCTCGTCCTTCTTGCTGTGGCAATTCAGACCTCTGATGCAGGTGAGGCTGGGGA
AGGGTGAGGACCACCGAGAGCAGGAAGAACGACACCGTTAAGTCTGGAGACTACGTCCACTCCGACCCCT
V  P  L  L  V  A  L  V  L  L  A  V  A  I  Q  T  S  D  A G>

        220        230        240        250        260        270        280
GTAGGAGGGTGGAGACCAGAGAATCTGGAAAGAGTCCAAGAGTGAGTCTGCCACTGGACTCACTTTGGCT
CATCCTCCCACCTCTGGTCTCTTAGACCTTTCTCAGGTTCTCACTCAGACGGTGACCTGAGTGAAACCGA

        290        300        310        320        330        340        350
TTGGACTAACTCTGGCACTTCCATGGGCCAGCTCTATTACTTCTCAAAACCCAGATATACACATTTATAA
AACCTGATTGAGACCGTGAAGGTACCCGGTCGAGATAATGAAGAGTTTTGGGTCTATATGTGTAAATATT

        360        370        380        390        400        410        420
ACTGGGTTGCTAGGAAGGCTTGGCTTTTAGGATCTGCCAATTGGTTGGTGGTATCTTGCTGGGGATCTTG
TGACCCAACGATCCTTCCGAACCGAAAATCCTAGACGGTTAACCAACCACCATAGAACGACCCCTAGAAC

        430        440        450        460        470        480        490
CTGCCCAGATAGCTCAAATATAGGGTCCCTGGGAGTGGATAGAGAGCCTGCAAGGTACCAGGCTTGAGTC
GACGGGTCTATCGAGTTTATATCCCAGGGACCCTCACCTATCTCTCGGACGTTCCATGGTCCGAACTCAG

        500        510        520        530        540        550        560
AGGGAAGGTAGAGCCTCTCTTAAAAGCTGCCCTCCCCCAACACCCAAGGTTTGCTCTGGAGCCTGTGGTG
TCCCTTCCATCTCGGAGAGAATTTTCGACGGGAGGGGGTTGTGGGTTCCAAACGAGACCTCGGACACCAC

        570        580        590        600        610        620        630
GATACAGGGTCCAGACCCCTCCTTCCCACCATAGTGTCAACACTTCTGCTTTGAGTTGGGCAGGAGAGGG
CTATGTCCCAGGTCTGGGGAGGAAGGGTGGTATCACAGTTGTGAAGACGAAACTCAACCCGTCCTCTCCC

        640        650        660        670        680        690        700
GTCACGGTGAGGGGTGAGGCAACTACAACCAACCAATCAAGTGTCTGGAGAGCCTCCGTGTAGCCAAGCC
CAGTGCCACTCCCCACTCCGTTGATGTTGGTTGGTTAGTTCACAGACCTCTCGGAGGCACATCGGTTCGG

        710        720        730        740        750        760        770
CCTACGGAACTGGTATTCCTCCACGGGCCTTGGAGATGACAGATGACTAGGGTCAGTCAGGGACAGAGTG
GGATGCCTTGACCATAAGGAGGTGCCCGGAACCTCTACTGTCTACTGATCCCAGTCAGTCCCTGTCTCAC

        780        790        800        810        820        830        840
GGACTTGAACCAGGTCAGTGTCATGACTGTCACCTGTCTGAGTCTTGATTCTGACTCTGCTACAGTCTCA
CCTGAACTTGGTCCAGTCACAGTACTGACAGTGGACAGACTCAGAACTAAGACTGAGACGATGTCAGAGT

        850        860        870        880        890        900        910
CCATGACCTTGGATGCTCGCCTTCGCTCGGCTTCTCTGATGTAGCCTCACCAAGGCGCTGAGAAGAGGAA
GGTACTGGAACCTACGAGCGGAAGCGAGCCGAAGAGACTACATCGGAGTGGTTCCGCGACTCTTCTCCTT

        920        930        940        950        960        970        980
GATGCACCTCTAATTCTGTGTTGTATACATGGGGGTAGGTGGGTCTGCCGTAGGGCTGAGCATTCCACAA
CTACGTGGAGATTAAGACACAACATATGTACCCCCATCCACCCAGACGGCATCCCGACTCGTAAGGTGTT

       990       1000       1010       1020       1030       1040       1050
GATCCTGGGAAGCTGGTGCTATTGTCCACAGACTGACCCAAGGTGAGCATGAGGCTCTTCCTGGCTCAGA
CTAGGACCCTTCGACCACGATAACAGGTGTCTGACTGGGTTCCACTCGTACTCCGAGAAGGACCGAGTCT

      1060       1070       1080       1090       1100       1110       1120
CAGAGGTGCAGGACCCATTTAGAAGCCTGTTGATATTTAATCTTTCTGAATCCACTTCCTGGAGCTGACT
GTCTCCACGTCCTGGGTAAATCTTCGGACAACTATAAATTAGAAAGACTTAGGTGAAGGACCTCGACTGA
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      1130       1140       1150       1160       1170       1180       1190
GTCCCCAGAGCGTAGACATTGGACCTTTAGTGGCATCTCAGAGACTCCCCAGGCTAGTGTGCATGAGCCC
CAGGGGTCTCGCATCTGTAACCTGGAAATCACCGTAGAGTCTCTGAGGGGTCCGATCACACGTACTCGGG

      1200       1210       1220       1230       1240       1250       1260
TAGAAGATATCCATCAGTGTGGAAGAGAATTTTACTCCTATAGGTCAGCCTGTGATCAGGGGTAAGGCTG
ATCTTCTATAGGTAGTCACACCTTCTCTTAAAATGAGGATATCCAGTCGGACACTAGTCCCCATTCCGAC

      1200       1210       1220       1230       1240       1250       1260
TAGAAGATATCCATCAGTGTGGAAGAGAATTTTACTCCTATAGGTCAGCCTGTGATCAGGGGTAAGGCTG
ATCTTCTATAGGTAGTCACACCTTCTCTTAAAATGAGGATATCCAGTCGGACACTAGTCCCCATTCCGAC

      1270       1280       1290       1300       1310       1320       1330
AAGCTGGCCTCTCTGTGCTCCAGCTAACGTCCCCTGGGTACCCTTCCTCCAGGTCCCTATGGTGCCAATG
TTCGACCGGAGAGACACGAGGTCGATTGCAGGGGACCCATGGGAAGGAGGTCCAGGGATACCACGGTTAC
                                                        P  Y  G  A  N>

      1340       1350       1360       1370       1380       1390       1400
TGGAAGACAGTATCTGCTGCCAGGACTACATCCGTCACCCTCTGCCATCACGTTTAGTGAAGGAGTTCTT
ACCTTCTGTCATAGACGACGGTCCTGATGTAGGCAGTGGGAGACGGTAGTGCAAATCACTTCCTCAAGAA
V  E  D  S  I  C  C  Q  D  Y  I  R  H  P  L  P  S  R  L  V  K  E  F F>

      1410       1420       1430       1440       1450       1460       1470
CTGGACCTCAAAATCCTGCCGCAAGCCTGGCGTTGTGTGAGTAGGGAGCTGGGGCTTCAGAACCTTGGAG
GACCTGGAGTTTTAGGACGGCGTTCGGACCGCAACACACTCATCCCTCGACCCCGAAGTCTTGGAACCTC
  W  T  S  K  S  C  R  K  P  G  V  V>

      1480       1490       1500       1510       1520       1530       1540
GAGGGGCCTGACAAGTGTGGCCCGAGGGTAGGCAGGATGTATCGAACTCCAACTCAAGAAGGCTTGGCTG
CTCCCCGGACTGTTCACACCGGGCTCCCATCCGTCCTACATAGCTTGAGGTTGAGTTCTTCCGAACCGAC

      1550       1560       1570       1580       1590       1600       1610
GATGGGATGGCTCAGCCAGGCTTGGGTTTTCTGAATCCCAGAATGATGGTATTATTCCATCGATATTGTT
CTACCCTACCGAGTCGGTCCGAACCCAAAAGACTTAGGGTCTTACTACCATAATAAGGTAGCTATAACAA

      1620       1630       1640       1650       1660       1670       1680
GAACATCTGTGACATGACAGAGCTGAGGATGATGGCTGAATAAATAGGTACCACCCCAGCATGACATGTA
CTTGTAGACACTGTACTGTCTCGACTCCTACTACCGACTTATTTATCCATGGTGGGGTCGTACTGTACAT

      1690       1700       1710       1720       1730       1740       1750
TCTGTGGGCAGCATAGCCACAGGCTATTGGTGATGGGACGCAATGCTAGTTGTAGGGGTACAAACAGCAT
AGACACCCGTCGTATCGGTGTCCGATAACCACTACCCTGCGTTACGATCAACATCCCCATGTTTGTCGTA

      1760       1770       1780       1790       1800       1810       1820
TCCTAGAGCCAAACCTGCCAAGACATTCTGGGTCCTCCCAGTGCTCTGGAATTCTTCAAGGTAGCCATTT
AGGATCTCGGTTTGGACGGTTCTGTAAGACCCAGGAGGGTCACGAGACCTTAAGAAGTTCCATCGGTAAA

      1830       1840       1850       1860       1870       1880       1890
CAATCAGAGACATACTATTAAGAGCTGATACCCATAGATGGTTGCCCATGTGCCCAGGCTAATTTGAATA
GTTAGTCTCTGTATGATAATTCTCGACTATGGGTATCTACCAACGGGTACACGGGTCCGATTAAACTTAT

      1900       1910       1920       1930       1940       1950       1960
CTACCCTTGTGATTCATTCAATCTTTAAAGCAATTCTACAAGGAGATTAACCCACGTCGTAGGTGAGACC
GATGGGAACACTAAGTAAGTTAGAAATTTCGTTAAGATGTTCCTCTAATTGGGTGCAGCATCCACTCTGG

      1970       1980       1990       2000       2010       2020       2030
CAGGCTCAGCAAAGGGAAGAGAACCTAGACTTAGCAAAGGGAAGGGGGATCCAGGCTCAGCAAAGAAAAA
GTCCGAGTCGTTTCCCTTCTCTTGGATCTGAATCGTTTCCCTTCCCCCTAGGTCCGAGTCGTTTCTTTTT

      2040       2050       2060       2070       2080       2090       2100
GGGACCCAGGTTCAGGAGAGTTTCCTTTTTCCCTTTTTCCAGGACTCAGGCTTAGCAAAGGAATCCCAAC
CCCTGGGTCCAAGTCCTCTCAAAGGAAAAAGGGAAAAAGGTCCTGAGTCCGAATCGTTTCCTTAGGGTTG

      2110       2120       2130       2140       2150       2160       2170
CTAGGCTCCAGCAAGGGGCAAGCAGTCATAGTCTGTCTGGTTTCCTTGCACACTGAGGAGGTGGCATGAG
GATCCGAGGTCGTTCCCCGTTCGTCAGTATCAGACAGACCAAAGGAACGTGTGACTCCTCCACCGTACTC

      2180       2190       2200       2210       2220       2230       2240
CTTAAGGGTCTCTTAAGCTCACAGGGCAGCTACAGCAAGTGGAAGTCACAGAGCTCATGGCCTTTTACTC
GAATTCCCAGAGAATTCGAGTGTCCCGTCGATGTCGTTCACCTTCAGTGTCTCGAGTACCGGAAAATGAG

      2250       2260       2270       2280       2290       2300       2310
AGGAGATGCTCCGTGAAGGACACTAGTGATCCATTGACTCTGAGCCCAAAGGGACTCTAGAAAGAATATG
TCCTCTACGAGGCACTTCCTGTGATCACTAGGTAACTGAGACTCGGGTTTCCCTGAGATCTTTCTTATAC
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      2320       2330       2340       2350       2360       2370       2380
GCTTGACAGATGGAAAGACAGATGCACAGGGTGGGACCAGCCTTGGGTAAACTGCCCTAAGAAGTCAAAC
CGAACTGTCTACCTTTCTGTCTACGTGTCCCACCCTGGTCGGAACCCATTTGACGGGATTCTTCAGTTTG

      2390       2400       2410       2420       2430       2440       2450
TCAGGCAGGCCATGGTAGCCCACACCTTTAATCCCAGGACTCAGGAGGCAGAGGCAGATGACTCTCCATG
AGTCCGTCCGGTACCATCGGGTGTGGAAATTAGGGTCCTGAGTCCTCCGTCTCCGTCTACTGAGAGGTAC

      2460       2470       2480       2490       2500       2510       2520
AGTTCAAGGCCAACCTAGTCTACATAGTGAGTTAGCCAGGTCTGCATGGTGACACCTTGTCTCAAACAAA
TCAAGTTCCGGTTGGATCAGATGTATCACTCAATCGGTCCAGACGTACCACTGTGGAACAGAGTTTGTTT

      2530       2540       2550       2560       2570       2580       2590
AACAAACAAACAAACAAAACAAAACAAAACAAAACAAAAGACAGGCTCAGGTTGCTATTGCCCTTGGGCT
TTGTTTGTTTGTTTGTTTTGTTTTGTTTTGTTTTGTTTTCTGTCCGAGTCCAACGATAACGGGAACCCGA

      2600       2610       2620       2630       2640       2650       2660
CTGCTCAGTAGTCAGTGATGCGAGGACAGGTTAGGTATGTGGGGAGCTATGCGAGGACAGGTTAGGTATG
GACGAGTCATCAGTCACTACGCTCCTGTCCAATCCATACACCCCTCGATACGCTCCTGTCCAATCCATAC


      2670       2680       2690       2700       2710       2720       2730
TGGAGAGCCATGCGAGGACAGGTTAGGTATGTGGGGAGCCAGCAGCTCTCAGCTGTATGGCTGGACTCTT
ACCTCTCGGTACGCTCCTGTCCAATCCATACACCCCTCGGTCGTCGAGAGTCGACATACCGACCTGAGAA

      2740       2750       2760       2770       2780       2790       2800
AGGAAGCAACAATGGAATGGCAGCAGAGACCTCACCACCAGCTGGCCTGTACCCACCTTGCTAACTTCAG
TCCTTCGTTGTTACCTTACCGTCGTCTCTGGAGTGGTGGTCGACCGGACATGGGTGGAACGATTGAAGTC

      2810       2820       2830       2840       2850       2860       2870
CTTTAGTGCCCATAGCTGGCCCCACTTGCTTCAGGTTTCCCTTGGGTCACACTTTGTTGGGTGTCTTAGT
GAAATCACGGGTATCGACCGGGGTGAACGAAGTCCAAAGGGAACCCAGTGTGAAACAACCCACAGAATCA

      2880       2890       2900       2910       2920       2930       2940
TTCTTTTCTAATTGCTGTGCCAGATAACTGAATTCTGGCAAGAATGACTCAGCGGAGAAAGAGAGTGTTT
AAGAAAAGATTAACGACACGGTCTATTGACTTAAGACCGTTCTTACTGAGTCGCCTCTTTCTCTCACAAA

      2950       2960       2970       2980       2990       3000       3010
CAGCACACAGTTCAAGGTGCAGGGCATCCCAGCAGGGAAGTTGAGGCACTGGGAACTGAAAGCAGCTCGT
GTCGTGTGTCAAGTTCCACGTCCCGTAGGGTCGTCCCTTCAACTCCGTGACCCTTGACTTTCGTCGAGCA

      3020       3030       3040       3050       3060       3070       3080
CACAGTCATCCAAGCTCAGGAAACAGAGGGATGGATGCAAATGCTCAGCTTGCTCTCTCTACTTTATACC
GTGTCAGTAGGTTCGAGTCCTTTGTCTCCCTACCTACGTTTACGAGTCGAACGAGAGAGATGAAATATGG

      3090       3100       3110       3120       3130       3140       3150
ATCCAGGATCTACCGCCCAGGGAATAGCCCCACCACAATTAAGATGGGTTTTCTCTCATCACTTAAAATA
TAGGTCCTAGATGGCGGGTCCCTTATCGGGGTGGTGTTAATTCTACCCAAAAGAGAGTAGTGAATTTTAT

      3020       3030       3040       3050       3060       3070       3080
CACAGTCATCCAAGCTCAGGAAACAGAGGGATGGATGCAAATGCTCAGCTTGCTCTCTCTACTTTATACC
GTGTCAGTAGGTTCGAGTCCTTTGTCTCCCTACCTACGTTTACGAGTCGAACGAGAGAGATGAAATATGG

      3090       3100       3110       3120       3130       3140       3150
ATCCAGGATCTACCGCCCAGGGAATAGCCCCACCACAATTAAGATGGGTTTTCTCTCATCACTTAAAATA
TAGGTCCTAGATGGCGGGTCCCTTATCGGGGTGGTGTTAATTCTACCCAAAAGAGAGTAGTGAATTTTAT

      3160       3170       3180       3190       3200       3210       3220
AGATAATCCCCACAGGGACGCCCAGAGGTCTCCCTAAGGGATCCTAGATACTGCCAATTGGCAGTTACCA
TCTATTAGGGGTGTCCCTGCGGGTCTCCAGAGGGATTCCCTAGGATCTATGACGGTTAACCGTCAATGGT

      3230       3240       3250       3260       3270       3280       3290
CTACCATCACGGGCAAACCTATTTCTCCACCAGATGCTCTGGGACTCTCAAAGAGGCCTGGGAGGGATCT
GATGGTAGTGCCCGTTTGGATAAAGAGGTGGTCTACGAGACCCTGAGAGTTTCTCCGGACCCTCCCTAGA

      3300       3310       3320       3330       3340       3350       3360
CTACAAAGTCACAGTCCAGCCCCGACCCTTGACATATTTTACTCCTTCTTTTATTCAGCCAAATTTCTTC
GATGTTTCAGTGTCAGGTCGGGGCTGGGAACTGTATAAAATGAGGAAGAAAATAAGTCGGTTTAAAGAAG

      3370       3380       3390       3400       3410       3420       3430
ACATCTGTGGGTAGAACAGGCGTGCAGGGACAGGGAGCACAGGGGCATGGGACACCAGAGTGGTTGCAAG
TGTAGACACCCATCTTGTCCGCACGTCCCTGTCCCTCGTGTCCCCGTACCCTGTGGTCTCACCAACGTTC
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      3440       3450       3460       3470       3480       3490       3500
AATTAGAGATGGGGATGCGAGACTGCTTAGTCAGTAAAATGTTTGCTGAGCAAGCCTGAGGATCCAAGTC
TTAATCTCTACCCCTACGCTCTGACGAATCAGTCATTTTACAAACGACTCGTTCGGACTCCTAGGTTCAG

      3510       3520       3530       3540       3550       3560       3570
GGATCCTTTGCTCTCGTGCAAAACAAACAAACAAAAACAAACAAACAAACAAAACAGATGTGGCATCATG
CCTAGGAAACGAGAGCACGTTTTGTTTGTTTGTTTTTGTTTGTTTGTTTGTTTTGTCTACACCGTAGTAC

      3580       3590       3600       3610       3620       3630       3640
TGTCTATACTCCCCAGTCCTGAGGAAACAAAGATAGGAAGATTTCTCAGCCTTGCTGGCCAGCCAGTCTG
ACAGATATGAGGGGTCAGGACTCCTTTGTTTCTATCCTTCTAAAGAGTCGGAACGACCGGTCGGTCAGAC

      3650       3660       3670       3680       3690       3700       3710
GCTGAACTGGAGAGCTTCAAGTTCAAGGAACTGGGGTTTTTTTTGTTGGTTTTTTTTTTTTTAAAGTAAGA
CGACTTGACCTCTCGAAGTTCAAGTTCCTTGACCCCAAAAAAAACAACCAAAAAAAAAAAATTTCATTCT

      3720       3730       3740       3750       3760       3770       3780
TGGATAACAAATGAGGGAGACACTCTATGTTGACCTCTGGTCTACATGCATGTGTACATTTCTCTCTCTC
ACCTATTGTTTACTCCCTCTGTGAGATACAACTGGAGACCAGATGTACGTACACATGTAAAGAGAGAGAG

      3790       3800       3810       3820       3830       3840       3850
TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCACACACACACACACACACACACACACACACAC
AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGTGTGTGTGTGTGTGTGTGTGTGTGTGTG

      3860       3870       3880       3890       3900       3910       3920
ACACACACACACACACACACACACAGAGGAGAAGAAAAAGAAAGCAATGTAAAACGTTAGGGTCATCTTC
TGTGTGTGTGTGTGTGTGTGTGTGTCTCCTCTTCTTTTTCTTTCGTTACATTTTGCAATCCCAGTAGAAG

      3930       3940       3950       3960       3970       3980       3990
TGACTCAATTATATACAGCACCTGCTATGTGCTGTAGCCCTTAGTAGTGTCTGCTTTCTTGAGCAGATCG
ACTGAGTTAATATATGTCGTGGACGATACACGACATCGGGAATCATCACAGACGAAAGAACTCGTCTAGC

      4000       4010       4020       4030       4040       4050       4060
GTATGTATCTAGACGGCTTCATCAAAATATCACGCAGCTGGGCAGGGCCGTCAGCCCCATTTTACAGATA
CATACATAGATCTGCCGAAGTAGTTTTATAGTGCGTCGACCCGTCCCGGCAGTCGGGGTAAAATGTCTAT

      4070       4080       4090       4100       4110       4120       4130
TAGAAACTAGGGCCTAGGGAAGTTAGCTGACTTGCTAACAAGGAGCAAAACTAGAAAGCAATCCAGATAG
ATCTTTGATCCCGGATCCCTTCAATCGACTGAACGATTGTTCCTCGTTTTGATCTTTCGTTAGGTCTATC

      4140       4150       4160       4170       4180       4190       4200
GTAGGCTGGAAGGTGGGTCCATACTTTCAGCAGCAGGCCTTCTAATGCTACAACTTTCTTCTTTACACCT
CATCCGACCTTCCACCCAGGTATGAAAGTCGTCGTCCGGAAGATTACGATGTTGAAAGAAGAAATGTGGA
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      4210       4220       4230       4240       4250       4260       4270
CTTCCAGTTTGATAACCGTCAAGAACCGAGATATCTGTGCCGATCCCAGGCAGGTCTGGGTGAAGAAGCT
GAAGGTCAAACTATTGGCAGTTCTTGGCTCTATAGACACGGCTAGGGTCCGTCCAGACCCACTTCTTCGA
         L  I  T  V  K  N  R  D  I  C  A  D  P  R  Q  V  W  V  K  K L>

      4280       4290       4300       4310       4320       4330       4340
ACTCCATAAACTGTCCTAGGGAGGAGGACCTGATGACCATGGGTCTGGTGTGGTCCAGGGAGGCTCAGCA
TGAGGTATTTGACAGGATCCCTCCTCCTGGACTACTGGTACCCAGACCACACCAGGTCCCTCCGAGTCGT
  L  H  K  L  S>

      4350       4360       4370       4380       4390       4400       4410
AGCCCTATTCTTCTGCCATTCCAGCAAGAGCCTTGCCAACGACGCCACCTTTACTCACCTCCATCCCCTG
TCGGGATAAGAAGACGGTAAGGTCGTTCTCGGAACGGTTGCTGCGGTGGAAATGAGTGGAGGTAGGGGAC

      4420       4430       4440       4450       4460       4470       4480
GGCTGTCACTCTGTGAGGCTCTGGTCCCTCTACCTCCCCTCTATCCCTTCCAGCTTATCCCCCTTCAATG
CCGACAGTGAGACACTCCGAGACCAGGGAGATGGAGGGGAGATAGGGAAGGTCGAATAGGGGGAAGTTAC

      4490       4500       4510       4520       4530       4540       4550
TGGCAGCTGGGAAACACATTCAGGCCAGCCTTACCCAATGCCTACTCCCCACTGCTTTAGATGAGACCAG
ACCGTCGACCCTTTGTGTAAGTCCGGTCGGAATGGGTTACGGATGAGGGGTGACGAAATCTACTCTGGTC

      4560       4570       4580       4590       4600       4610       4620
CGTCCTTGTTTTGATGCCCTGATCCTATGATGCCTTCCCCATCCCCAGCCTTGGCCCCCTTCTCTTCTTG
GCAGGAACAAAACTACGGGACTAGGATACTACGGAAGGGGTAGGGGTCGGAACCGGGGGAAGAGAAGAAC

      4630       4640       4650       4660       4670       4680       4690
CATGTAGGGAAGGCCCATAGGTTTCAAATATGTGCTACCTAGTTCCCTTTCTGGGGGGTTCTAATACCCA
GTACATCCCTTCCGGGTATCCAAAGTTTATACACGATGGATCAAGGGAAAGACCCCCCAAGATTATGGGT

      4700       4710       4720       4730       4740       4750       4760
GCATGTTTTTCCTGCTGCAGGCACCTATCCAGTGCCACACACCTCCCAAGTTTCTATCAGTCCCAGTGGG
CGTACAAAAAGGACGACGTCCGTGGATAGGTCACGGTGTGTGGAGGGTTCAAAGATAGTCAGGGTCACCC

      4770       4780       4790       4800       4810       4820       4830
CATCCACCAAGCCCCAAACTTCAGACTTCCTTGGCCTCCACCTACTCTCAGTAGAATTCTGGGAGTTTCA
GTAGGTGGTTCGGGGTTTGAAGTCTGAAGGAACCGGAGGTGGATGAGAGTCATCTTAAGACCCTCAAAGT

      4840       4850       4860       4870       4880       4890       4900
GGCTGGTCCACCAGGCCCCCCAGGGTTAGGCCAAGGTCCCCACCAGAGCTCCTCCTGTTTCTTGGTCTGC
CCGACCAGGTGGTCCGGGGGGTCCCAATCCGGTTCCAGGGGTGGTCTCGAGGAGGACAAAGAACCAGACG

      4910       4920       4930       4940       4950       4960       4970
AGCACGGGGCAGGGAGCAAGGAGCAGGCTCAGAATCAGATTTCTTAAAGGAGCTGCAGACTCCATCAGTA
TCGTGCCCCGTCCCTCGTTCCTCGTCCGAGTCTTAGTCTAAAGAATTTCCTCGACGTCTGAGGTAGTCAT

      4980       4990       5000       5010       5020       5030       5040
AAAGGAATCTTTCTCCCATCCCTGAATATAAGGCAGTTTTCTGTCAACACAGAGACTCAGGTTGTTAGAA
TTTCCTTAGAAAGAGGGTAGGGACTTATATTCCGTCAAAAGACAGTTGTGTCTCTGAGTCCAACAATCTT

      5050       5060       5070       5080       5090       5100       5110
ATGGCCACATAGATCAACTGTGAAACCCTAAATTTACCAAGAATCAACTTCCACCCCTCTTCAACCACAT
TACCGGTGTATCTAGTTGACACTTTGGGATTTAAATGGTTCTTAGTTGAAGGTGGGGAGAAGTTGGTGTA

      5120       5130       5140       5150       5160       5170       5180
GCTAGGGTCTTTTACTTTCTCTGCCCCACACCTTTGACTCCTTGCCTGTGTAGCTGATAGTCGAAGTTAT
CGATCCCAGAAAATGAAAGAGACGGGGTGTGGAAACTGAGGAACGGACACATCGACTATCAGCTTCAATA

      5190       5200       5210       5220       5230       5240       5250
GCTATGGTGTCAGTGACTGCCACAGTTTGTTTGGTATTATAAGCTATAGTTATATTTATATAGGAAAGAG
CGATACCACAGTCACTGACGGTGTCAAACAAACCATAATATTCGATATCAATATAAATATATCCTTTCTC

      5260       5270       5280       5290       5300       5310       5320
GATAAATATATGTGAGGCCAAATAGACGAACTGGAGAGTTTTAGGATCTGGGGGCAGGAAGGGCCATACA
CTATTTATATACACTCCGGTTTATCTGCTTGACCTCTCAAAATCCTAGACCCCCGTCCTTCCCGGTATGT

      5330       5340       5350       5360       5370       5380       5390
AAGTGATACCTCAGAAAATAGATGGTTGTGGGAGCTGCTGCCAGTGGCAGAGTTAACTTAAAGAACTTAA
TTCACTATGGAGTCTTTTATCTACCAACACCCTCGACGACGGTCACCGTCTCAATTGAATTTCTTGAATT

      5400       5410       5420       5430       5440       5450       5460
TTGAAATTATTCTTGAGTGGCTGAGGCCAAGACAAGAATATAGAACCCATTCTTGCTTCCCTGGAGACAA
AACTTTAATAAGAACTCACCGACTCCGGTTCTGTTCTTATATCTTGGGTAAGAACGAAGGGACCTCTGTT
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      5470       5480       5490       5500       5510       5520       5530
CAGTGGTCCCAGGGGAAGGAATAAACCTTCTTGCTCCTCTGGAGGGAGCATGGCCTGGCTTAGCCGAGTG
GTCACCAGGGTCCCCTTCCTTATTTGGAAGAACGAGGAGACCTCCCTCGTACCGGACCGAATCGGCTCAC

      5540       5550       5560       5570       5580       5590       5600
ACTGGACTGTGTGAGATTGGGGGCATCGCTTTTCCTCTCTGAGCCTCAGCTGACAGCATATGGGACCACA
TGACCTGACACACTCTAACCCCCGTAGCGAAAAGGAGAGACTCGGAGTCGACTGTCGTATACCCTGGTGT

      5610       5620       5630       5640       5650       5660       5670
AAGGGCTTGATCCAAACCACAGGGATTGACAGTGCCAGCCACAGCTGTGTCCAGGGCTCGTGTTCTGCCA
TTCCCGAACTAGGTTTGGTGTCCCTAACTGTCACGGTCGGTGTCGACACAGGTCCCGAGCACAAGACGGT

      5680       5690       5700       5710       5720       5730       5740
GAAGGAGCACCTGGACGACCAGGGCCACCACTAGTGCTACTTTGCTCACTGCCCATGCATGTCCTGAAGG
CTTCCTCGTGGACCTGCTGGTCCCGGTGGTGATCACGATGAAACGAGTGACGGGTACGTACAGGACTTCC

      5750       5760       5770       5780       5790       5800       5810
TCCCTCCCCCTCCTCTCCTACTTCTGGGAAAATAAATGCTCGCCAATAATACCTGACTTGGGTCCTTGTC
AGGGAGGGGGAGGAGAGGATGAAGACCCTTTTATTTACGAGCGGTTATTATGGACTGAACCCAGGAACAG

      5820       5830       5840       5850       5860       5870       5880
CTCTGTGTTGCTGTGAAATAGAACGGGGCCTTCCTTTACCCAGACCTGCCCTCCTCCAGGGTCTGACCCC
GAGACACAACGACACTTTATCTTGCCCCGGAAGGAAATGGGTCTGGACGGGAGGAGGTCCCAGACTGGGG

      5890       5900       5910       5920       5930       5940       5950
TCCATGGAAAAGGAAGGTTTGTGGGATGGTGGGAAATGGGATGTGCACCTATTGTGCCATGTGTCATTGG
AGGTACCTTTTCCTTCCAAACACCCTACCACCCTTTACCCTACACGTGGATAACACGGTACACAGTAACC

      5960       5970       5980       5990       6000       6010       6020
TGGCTTTCCTTAGTGGCTGGGGGAGATTTTATAATCCCTTTCTTGTGTCCTTGATTCTAAAGCTAGAACC
ACCGAAAGGAATCACCGACCCCCTCTAAAATATTAGGGAAAGAACACAGGAACTAAGATTTCGATCTTGG

      6030       6040       6050       6060       6070       6080       6090
ATGTGCCTGGAGCTGCCAAGTTCTGCTGCTTGTTGGGGCTGGAACACGGCCCCCTCATTCAAATATATAT
TACACGGACCTCGACGGTTCAAGACGACGAACAACCCCGACCTTGTGCCGGGGGAGTAAGTTTATATATA

      6100
AACCAACTTTCT
TTGGTTGAAAGA
```

3.1.2 Gene Targeting

3.1.2.1 First Approach

With the knowledge of the full length sequence and the structure of the genomic locus of CCL22 it was possible to design the overall targeting strategy including a targeting vector construct and a screening strategy which is shown in Appendix A2. First of all it had to be decided which part of the gene had to be deleted to ensure that no functional CCL22 could be produced by targeted cells. Exon 1 and 2 code for approximately 60% of the protein sequence including three of the four conserved cysteine residues. I decided to completely delete both exons. Additionally, this strategy includes deletion of the START codon in exon 1 so that transcription of CCL22 was prevented. Therefore, the short arm of homology (SAH) was placed upstream of exon 1 in the promoter region with a length of 750 bp. The long arm of homology (LAH) was positioned downstream of exon 2 spanning large parts of intron 2 and exon 3. The size of the LAH was 3.75 kb. Exon 1 and 2 should be replaced by a floxed neo cassette and an additional FRT site. It was therefore necessary to generate a targeting vector with the SAH at the 5'end followed by the FRT site, the floxed neo cassette, the LAH and a TK cassette (see section 1.4). The FRT site which is located between the SAH and the 5' loxP site can be used after successful CCL22-targeting for the Flp-mediated deletion of the CX_3CL1 locus using the FRT site which remained in the locus of CCL17 after Flp-mediated deletion of the neo cassete after successful targeting (see sections 1.4 and 1.6 and appendix A1).

For PCR screening, a KO primer pair, GL/FO and TV/REV, was designed which only gives rise to a specific PCR product after homologous recombination. The forward primer, GL/FO, binds to a region upstream of the SAH. The backward primer, TV/REV, binds to a sequence inside the neo cassette which is only present in the locus of CCL22 after recombination. The position of the primer pair in the targeted locus is shown in Appendix A1. Additionally, two control primer pairs were designed. The first primer pair, GL/FO and GL/REV, serves as wt control primer pair. The forward primer GL/FO binds to a region upstream of the SAH. The backward primer, GL/REV, binds to a region in exon 2 which is only present in the wt locus. The position of the primer pair in the wt locus is shown in A1.

The second control primer pair, TV/FO and TV/REV, serves as positive control (vector) primer pair which gives rise to a PCR product if the targeting vector is used as template. The forward primer, TV/FO binds to a region on the targeting vector which is located upstream of the SAH cloning site. The reverse primer, TV/REV, binds to the neo cassette.

To plan a Southern Blot screening strategy, the restriction sites of the wt genomic sequence and the targeted locus sequence were analysed with the help of the MacVector™ program. A restriction digest of the targeted locus with the restriction endonuclease XbaI gives rise to a DNA fragment which is approximately 2.2 kb smaller than the corresponding wt DNA fragment. This change is due to the introduction of an additional XbaI restriction site located in the FRT site. The differently sized DNA fragments can be detected with the help of two Southern Blot probes, 5'SI and 5'SII, which are located upstream of the SAH. The positions of the XbaI restriction sites and the southern blot probes 5'SI and 5'SII are shown in Appendix A1.

For generation of the targeting vector construct, the pEasyFlox vector was used as this vector already has a floxed neo cassette and a downstream TK cassette. A map of the cassette vector pEasyFlox is shown in Fig. 3.5. The SAH and LAH were amplified by PCR as described in section 2.7.7 and subcloned into the pCR®II-TOPO® vector as described in section 2.7.6. In the next step, the SAH was cut out of the pCR®II-TOPO® vector with the restriction enzymes NotI and BamHI. Both restriction sites had been added to the SAH with the help of the PCR primer pair together with an NdeI restriction site upstream of the BamHI restriction site. The pEasyFlox vector was digested with the same restriction enzymes to allow subcloning of the SAH into the vector upstream of the floxed neo cassette (see section 2.7.6) as shown in Fig. 3.5. In the next step, the pFRT2neo vector was preparatively digested with the restriction enzymes NdeI and BamHI to isolate a 190 bp fragment containing the full length FRT sequence (vector map not shown). The pEasyFlox vector was digested with the same restriction enzymes and the FRT site was cloned into the vector in 5'→ 3' orientation between the SAH and the floxed neo cassette as shown in Fig. 3.5.

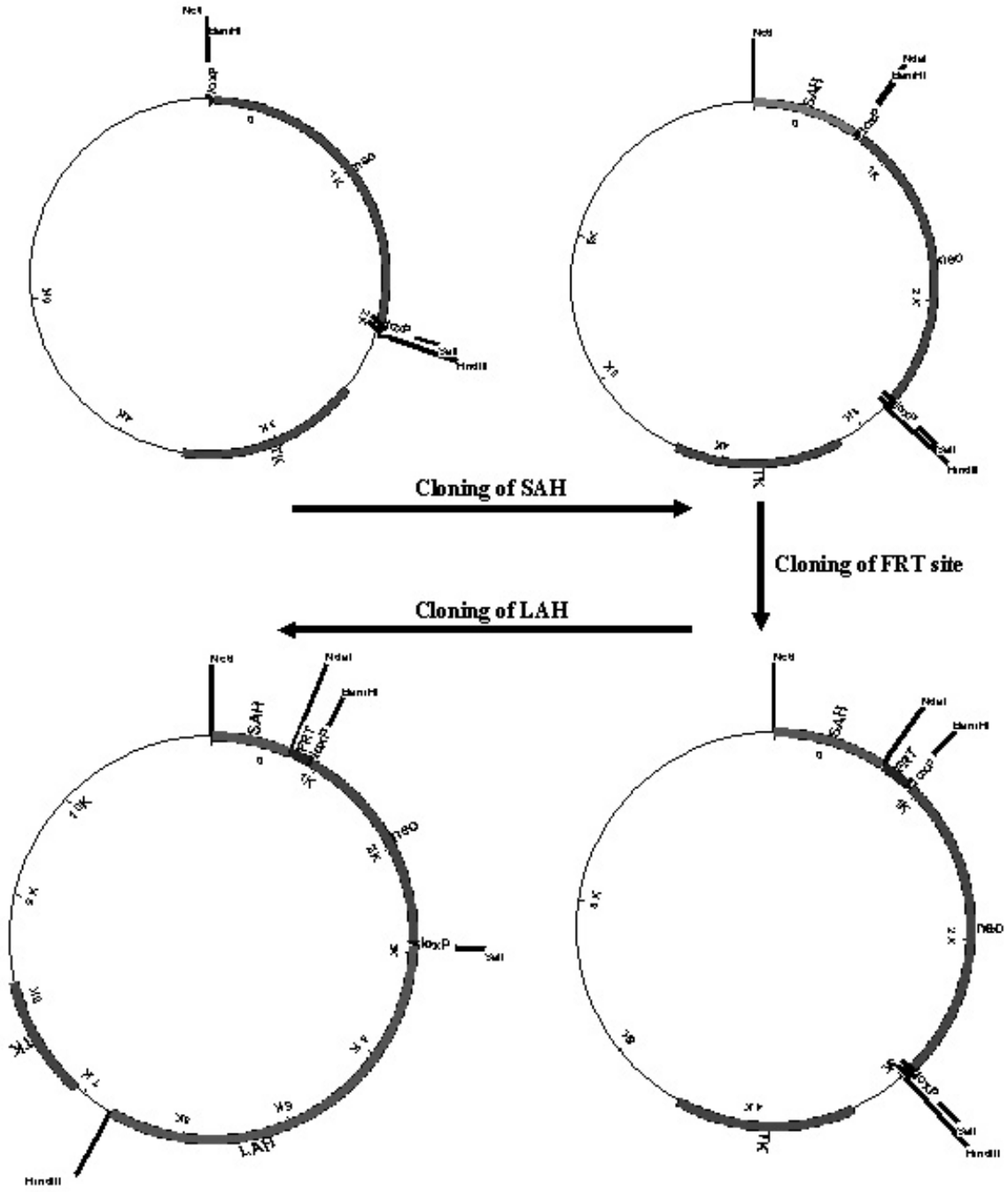


Fig 3.5: Generation of the CCL22 Targeting Vector. The vector map of the pEasyFlox cassette vector is shown. The strategy for cloning the SAH, FRT site and LAH is shown schematically.

In the last step of the procedure, the LAH was cloned into the pEasyFlox vector to complete the targeting vector construct. For this purpose, the LAH was cut out of the pCR®II-TOPO® vector with the restriction enzymes SalI and HindIII. Both restriction sites had been added to the LAH using the PCR primer pair. The pEasyFlox vector was digested with the same restriction enzymes to allow subcloning of the LAH into this vector downstream of the floxed neo cassette under the loss of the third lox P site as shown in Fig. 3.5. The successful generation of the vector construct was confirmed by sequencing.

The final targeting vector construct, which will be referred to as CCL22/TV-I, was used for targeting of 50A8 ES cells. For this purpose, 50A8 ES cells were thawed, expanded, transfected and selected as described in section 2.8.3. After selection, 178 ES cell clones were picked as described in Protocol 1 (see section 2.8.4). The selected ES cell clones were expanded and 48 well stock plates were frozen as described in section 2.8.4. PCR screening of the ES cell clones was carried out as described in section 2.7.7. 19 clones showed a signal of the expected size of 1.1 kb: K16, 18, 19, 28, 30, 32, 39, 57, 65, 66, 67, 72, 74, 77, 87, 123, 124, 133 and 148 One typical PCR Screen Blot which shows the results for the clones 1-90 is shown in Figure 3.6.

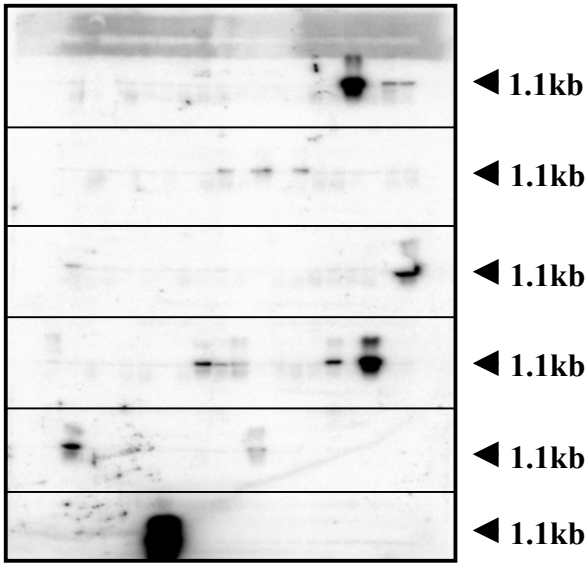


Fig 3.6: PCR Screen of Picked ES Cell Clones. In this blot the screening results for the clones 1-90 are shown (clones: lanes 1-5, positive and negative controls: lane 6). 15 ES cell clones (K 16, 18, 19, 28, 30, 32, 39, 57, 65, 66, 67, 72, 74, 77, and 87) showed a signal of the expected size of 1.1 kb.

All 19 positive ES cell clones that were identified with the help of the PCR screen were further tested for homologous recombination by Southern Blot. For that purpose, PCR positive clones were thawed and expanded until a sufficient amount of vials had been frozen and stored in liquid nitrogen as described in section 2.8.4. After that, the DNA of the PCR positive clones was isolated as described in section 2.7.2 and a Southern Blot screen was carried out as described in section 2.7.8. Restriction digests were carried out with the restriction endonuclease XbaI. Hybridisation was carried out with the radioactively labelled Southern Blot probe 5'SII.

All 19 PCR positive clones showed a wt band of the same size as the E14 control at 5.6 kb. None of the 19 PCR positive clones showed a KO band of an expected size of 3.3 kb. This leads to the conclusion that all 19 PCR positive clones did not integrate the targeting vector construct in a homologuous way and therefore still have a functional gene for CCL22. One example of the Southern Blot Screen is shown in Figure 3.7.

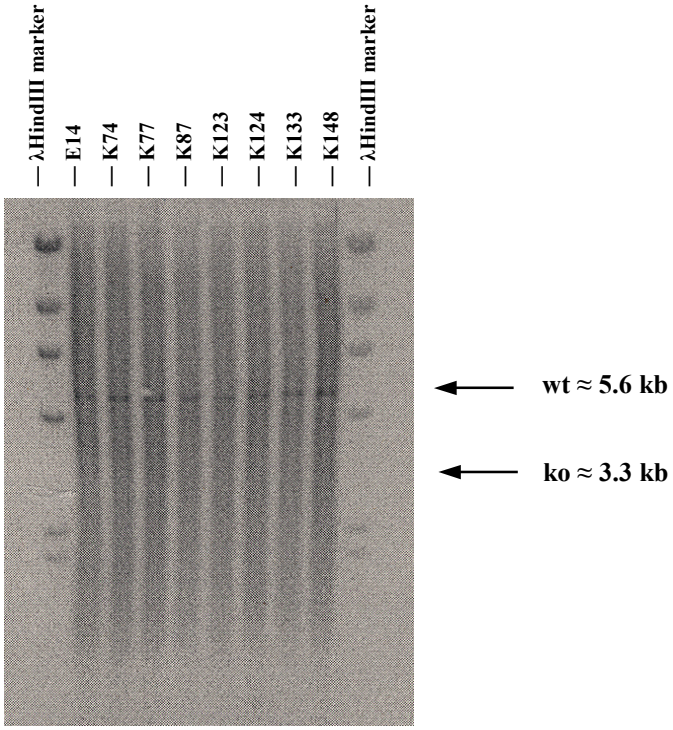


Fig 3.7: Southern Blot Screen of PCR Positive ES Cell Clones. Restriction digest of genomic DNA was carried out with XbaI. Hybridisation was carried out with the radioactively labelled Southern Blot probe 5'SII. In this blot the screening results for the clones K 74, 77, 87, 123, 124, 133, and 148 are shown. All 7 clones show the same sized wt band as the E14 control but no KO band.

Repetition of the PCR screens of clones 1-178 showed that the PCR results were not reproducible leading to the conclusion that the PCR Screen was not reliable (data not shown). It was therefore decided to directly screen picked ES cell clones from future transfections by Southern Blot.

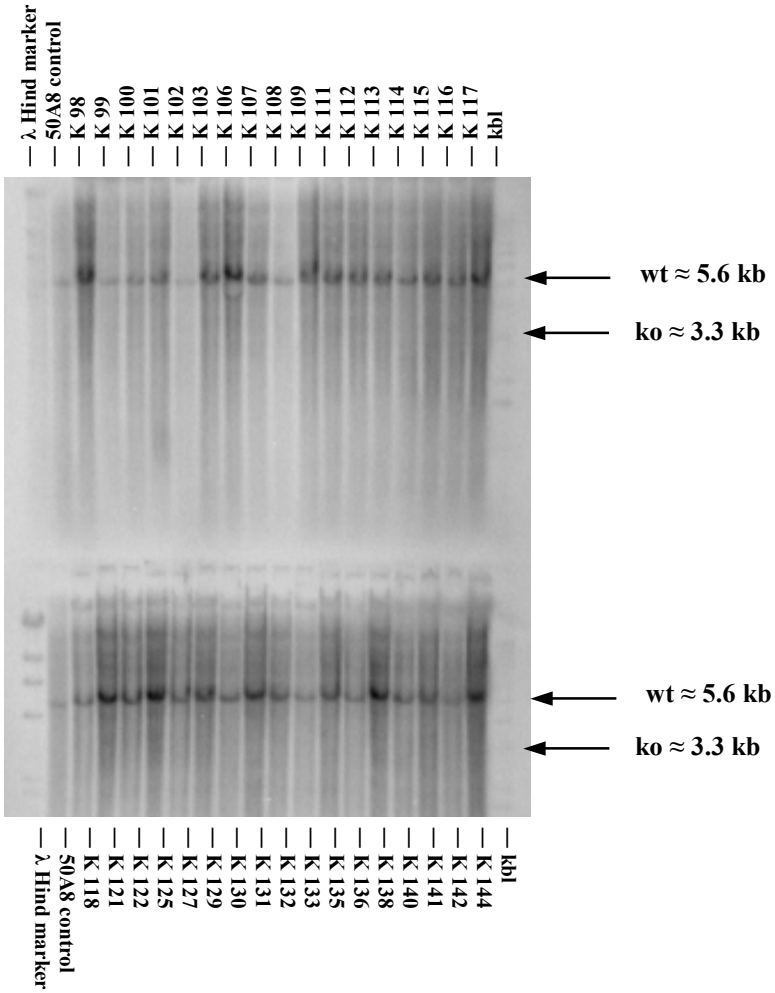


Fig 3.8: Southern Blot Screen of Picked ES Cell Clones. Restriction digest of genomic DNA was carried out with XbaI. Hybridisation was carried out with the radioactively labelled Southern Blot probes 5'SI and 5'SII. In this blot the screening results for the clones K 98-103, 106-109, 111-118, 121, 122, 125, 127, 129-133, 135, 136, 138, 140-142 and 144 are shown. All clones show the same sized wt band as the 50A8 control but no KO band.

As the first transfection of 50A8 ES cells had been unsuccessful, the procedure had to be repeated. For this purpose, a new aliquot of 50A8 ES cells was thawed, expanded, transfected and selected as described. After selection, 300 ES cell clones were picked as described. The selected ES cell clones were expanded, 48 well stock plates were frozen and aliquots of ES cell clones were expanded to 12 well plates as described in Protocol 2 (section 2.8.4). In the next step, the genomic DNA of the ES cell clones was isolated as described in section 2.7.2 and Southern Blot screens were carried out as described in section 2.7.8. All 300 ES cell clones showed a wt band of the same size as the E14 control at 5.6 kb but no clone showed a KO band of the expected size of 3.3 kb. Thus all 300 clones did not integrate the targeting vector construct in a homologuous way and therefore still had a functional gene for CCL22. One example of the screening Southern Blot is shown in Figure 3.8.

3.1.2.2 Second Approach

So far, a total of 478 ES cell clones, which had been transfected with CCL22/TV-I, had been screened for homologous recombination. Unfortunately, none of the tested clones showed homologous recombination indicating that the targeting procedure was unsuccessful. A possible reason for failure of recombination was that the SAH was positioned in the promoter region of the CCL22 gene which might contain repetitive sequences. I therefore decided to change the targeting vector construct by replacing the SAH with a new SAH (SAH II) which was positioned further downstream as shown in Appendix A2. The new SAH was designed to have a size of 500 bp and to span the full length of exon 1 as well as part of intron 1. This has the consequence that exon 1 which includes the START codon remains in the targeted locus. But since exon 2, which will be replaced by the floxed neo cassette and the FRT site, codes for three of the four conserved cystein residues it was highly unlikely that a functional CCL22 protein was translated even if exon 1 remained in the locus.

For screening of the transfected ES cell clones, a new Southern Blot Strategy was designed which is shown in Appendix A2. It was decided to introduce a new HindIII restriction site in the primer pairs that were used for the amplification of SAH II. Restriction digests of the wt DNA gives rise to a 12 kb DNA fragment (see section 3.1.1) while restriction digest of the KO DNA gives rise to a DNA fragment which is 6 kb smaller. The differently sized DNA fragments can be detected with the help of a Southern Blot probe, 3'SI, which is located at the 3' end of exon 3. The positions of the HindIII restriction sites and the probe 3'SI are shown in A2.

To generate the new targeting vector construct, SAH II was amplified by PCR as described in section 2.7.7 and subcloned into the pCR®II-TOPO® vector as described in section 2.7.6. In the next step, the SAH II was cut out of the pCR®II-TOPO® vector with the restriction enzymes NotI and NdeI. Both restriction sites had been added to the SAH II sequence with the help of the PCR primers together with a HindIII restriction site upstream of the NdeI restriction site. The targeting vector construct was digested with the same restriction enzymes leading to the removal of the previous SAH. After that, SAH II was subcloned into the vector upstream of the floxed neo cassette. The success of the subcloning procedure was confirmed by sequencing.

The final second targeting vector construct, which will be referred to as CCL22/TV-II, was used for targeting of 50A8 ES cells as previously described. Two independent transfections were carried out as described in section 2.8.3 and a total of 1250 selected ES cell clones were picked as described in Protocol 3 (see section 2.8.4). The selected ES cell clones were expanded and 96 well stock plates were frozen as described in section 2.8.4. Southern Blot screening of the ES cell clones from 96 well plates was carried out as described in section 2.7.7. To be able to analyse all 96 clones from one plate in one southern blot I used two special combs which each possess two rows of pockets which are arranged in a zick-zack pattern (see Figure 3.13).

Every selected ES cell clone showed a wt signal at the expected size of 12 kb. Unfortunately, no clone showed a KO signal at the expected size of 6 kb. One typical blot is shown in Figure 3.13. Taken together, a total of 1728 clones had been screened. No positive clone could be identified leading to the conclusion further transfections will not be successful either. It was therefore decided that the ko approach would not be further proceeded.

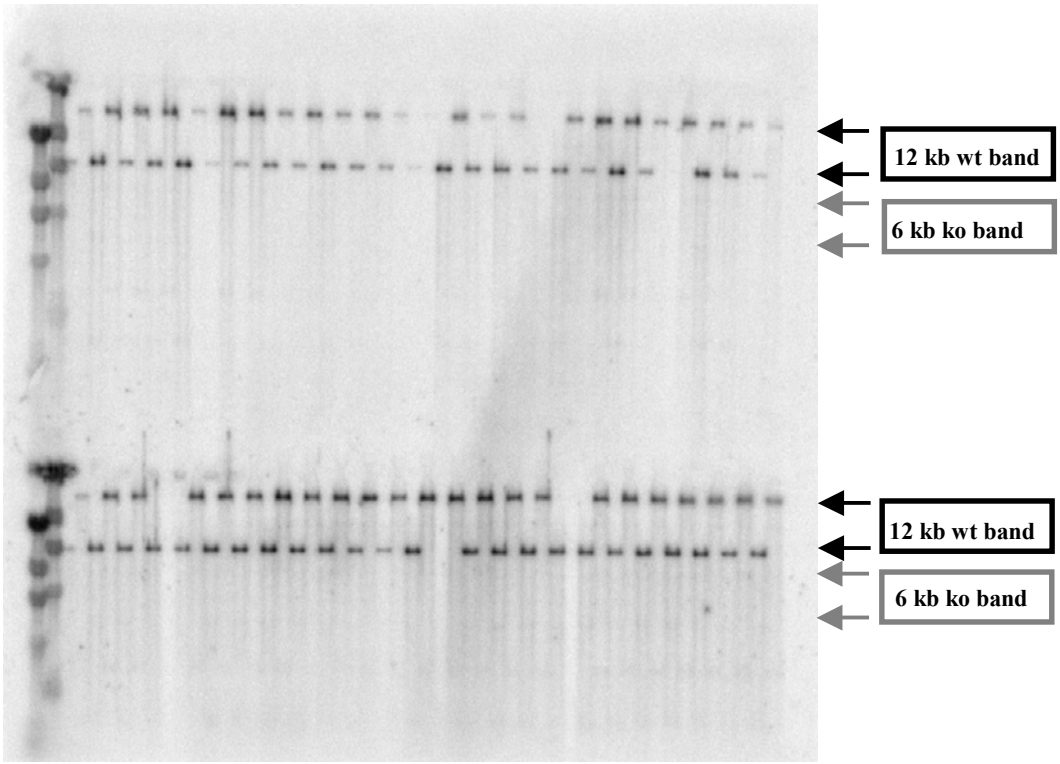


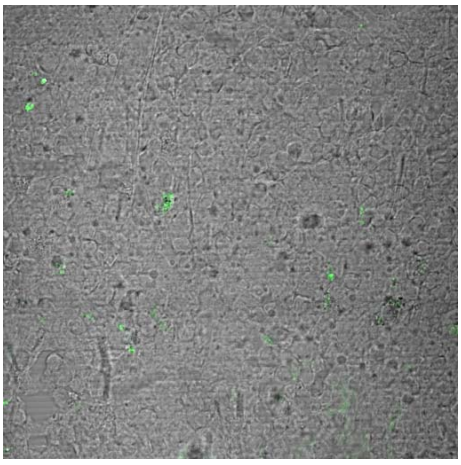
Fig 3.9: Southern Blot Screen of Picked ES Cell Clones. Restriction digest of genomic DNA was carried out with HindIII. Hybridisation was carried out with the radioactively labelled Southern Blot probe 3'SI . In this blot the screening results for the clones 769-864 are shown. All clones show the same sized wt signal as the 50A8 controls, which are located on lane 2 of each row, but no additional ko signal.

3.2 Part II: Influence of the CCL17/CCL22-CCR4 and CCL1-CCR8 Chemokine Receptor-Ligand Pairs on Langerhans Cell Migration

3.2.1 Langerhans Cell Migration in CCR4$^{-/-}$ Mice

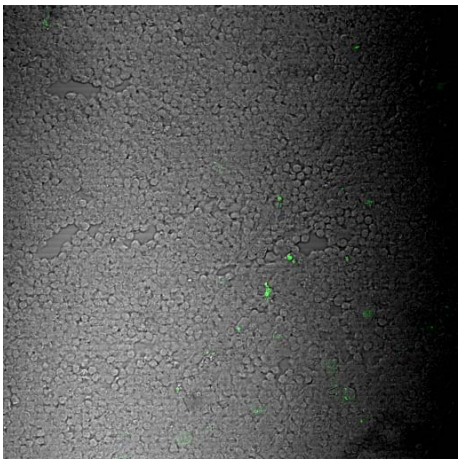
The aim of the first part of the project was to study the influence of the CCL17/CCL22-CCR4 chemokine receptor-ligand pairs on Langerhans cell migration. As the generation of CCL17/CCL22 KO mice had been unsuccessful, the CCR4 KO mice were used as a good alternative as CCR4 is the only confirmed receptor for these chemokines (see also section 1.3.2). Migration of Langerhans was analysed *in vivo* in 6-10 weeks old female CCR4$^{-/-}$ mice as described in section 2.8.4 and compared to age matched C56Bl/6 control mice. In the first step of the project, it had to be shown that the applied FITC dye was able to penetrate the skin and stain resident cells. Further it had to be shown that the FITC dye was not passively transported into the draining LNs (axillary, brachial, inguinal) by lymph but actively transported by stained migrating cells. For that purpose cryosections of draining lymph nodes of FITC painted mice were analysed by confocal microscopy and compared to cryosections of mesenteric LNs from the same mice, which served as internal controls. To determine background fluorescence cryosections of draining LNs of unpainted control C57Bl/6 mice were analysed as well. Results are shown in Fig. 3.10.

a)



draining LNs, C57Bl/6
(56×magnification)
unpainted control

b)



mesenteric LNs, C57Bl/6
(28×magnification)
FITC painted, internal control

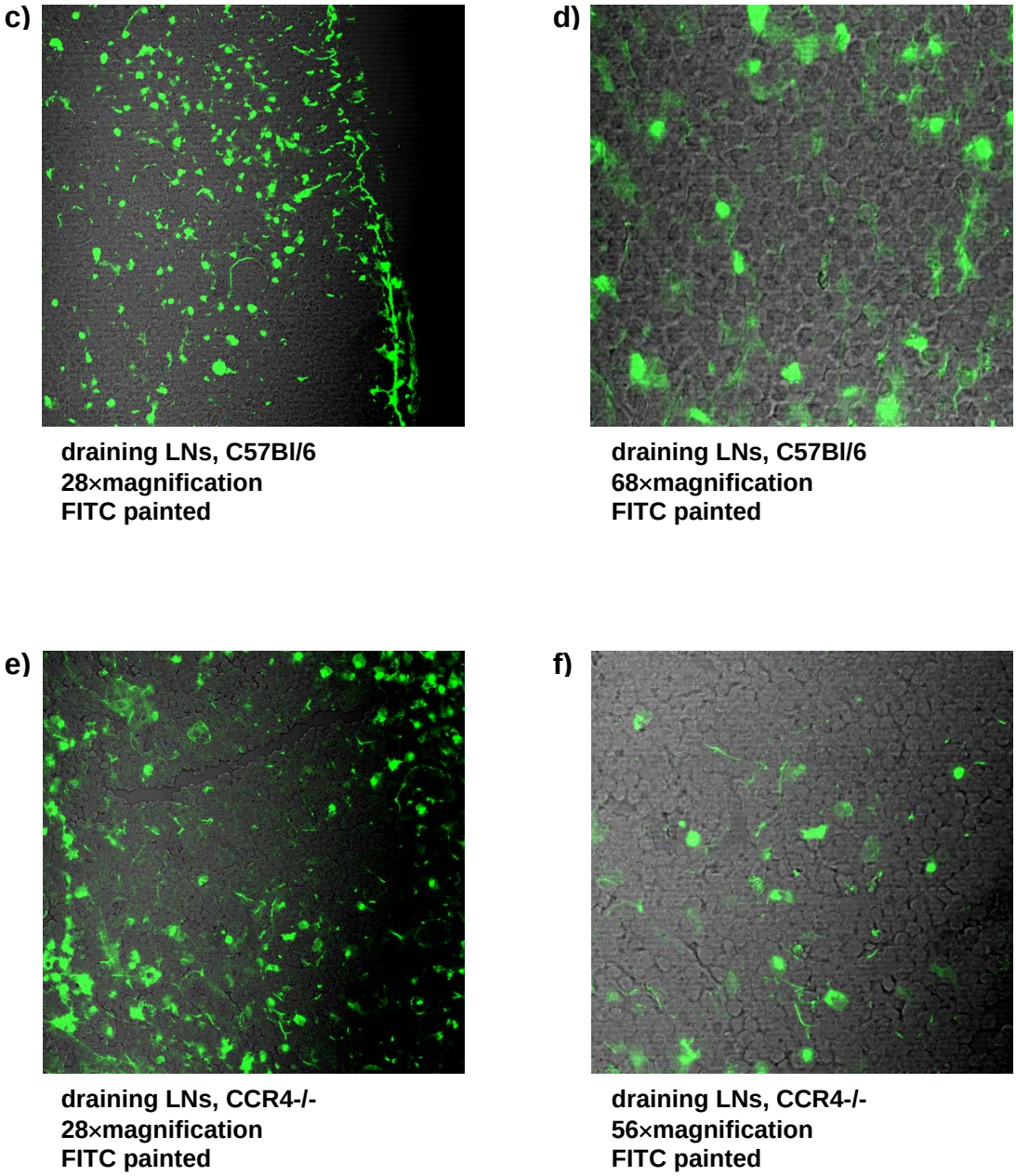


Fig. 3.10: Analysis of Migration of Resident Langerhans Cells from the Skin into the Draining Lymph Nodes after Application of FITC Dye to the Skin.

Cryosections of the draining LNs of unpainted C57Bl/6 control mice (a), the mesenteric LNs of FITC painted C57Bl/6 mice (b) the draining LNs of FITC painted C57Bl/6 mice (c, d) and the draining LNs of FITC painted $CCR4^{-/-}$ mice (e, f)

Analysis of cryosections of the draining LNs of unpainted C57Bl/6 control mice showed that the background fluorescence is negligible (Fig 3.14a). Analysis of the mesenteric LNs of FITC painted C57Bl/6 mice demonstrated the same low background fluorescence as the unpainted control (fig 3.14b). The results therefore confirmed that the FITC dye was not unspecifically transported through the lymphatic system and that the mesenteric LNs were a suitable internal control. Analysis of the draining LNs of FITC painted C57Bl/6 and $CCR4^{-/-}$ mice demonstrated that the FITC dye had been able to penetrate the skin, stain resident cells and was actively transported into the draining LNs by migrating Langerhans cells (Fig 3.14c-f). Taken together it had been proven that the experimental set up was suitable to examine migration of resident Langerhans cells from the skin to the draining LNs.

To determine Langerhans cell migration in a quantitative way, FACS analysis was carried out. $CCR4^{-/-}$ mice and C57Bl/6 control mice were painted with FITC dye and cell suspensions of the draining LNs and the mesenteric LNs were prepared as described in section 2.8.4. The cells were either stained with anti-CD11c and anti-IA^b (MHC II) antibodies or with anti-CD11c and anti-CD11b antibodies. FACS analysis showed that an average of 0.46 +/- 0.11% of the total cell population in the draining LNs of C57Bl/6 controls and an average of 0.27 +/- 0.07% of the total cell population in the draining LNs of $CCR4^{-/-}$ mice were $FITC^+$ (data not shown). The $CD11c^+$ population which includes Langerhans cells was analysed for FITC uptake and MHC II expression or CD11b expression respectively. FACS blots of one representative experiment for each staining are shown in Figure 3.11 and 3.12. For each staining three independent experiments with groups of two mice each were carried out. The LN cell suspensions of each group were pooled before analysis. The results of all experiments are summarised in Figure 3.13.

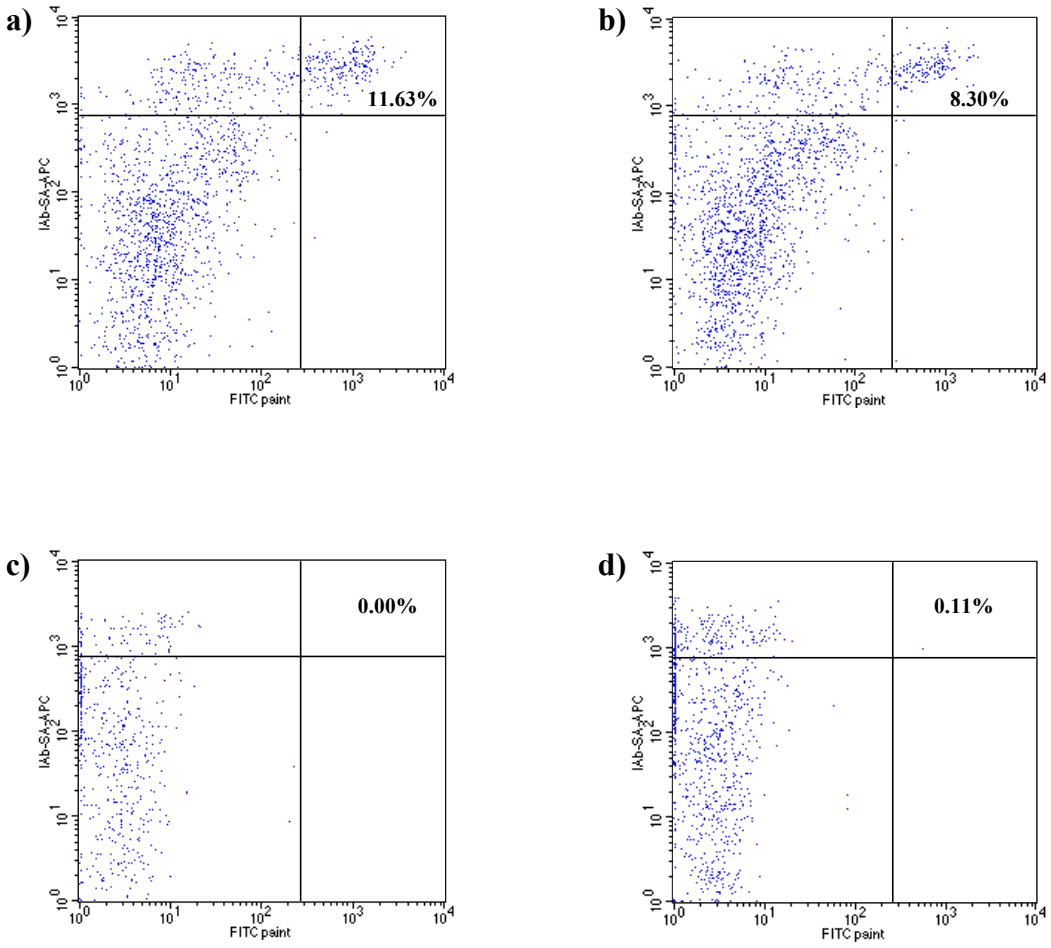


Fig. 3.11: Representative FACS Blots for Analysis of the CD11c$^+$ Population for MHC II Expression and FITC Uptake. FACS analysis of the CD11c$^+$ population of pooled inguinal, axillary and brachial LNs (dLNs) (a, b) and mesenteric LNs (internal control) (c, d) 18-20 h after FITC application to the skin of the peritoneal and chest region. CD11c$^+$ cells from C57Bl/6 control mice and CCR4$^{-/-}$ mice are analysed for MHCII expression (IA^b) and FITC uptake. Fig 3.15a) dLNs of C57Bl/6 control mice; Fig 3.15b) dLNs of CCR4$^{-/-}$ mice; Fig 3.15c) mLNs of C57Bl/6 control mice; Fig 3.15d) mLNs of CCR4$^{-/-}$ mice.

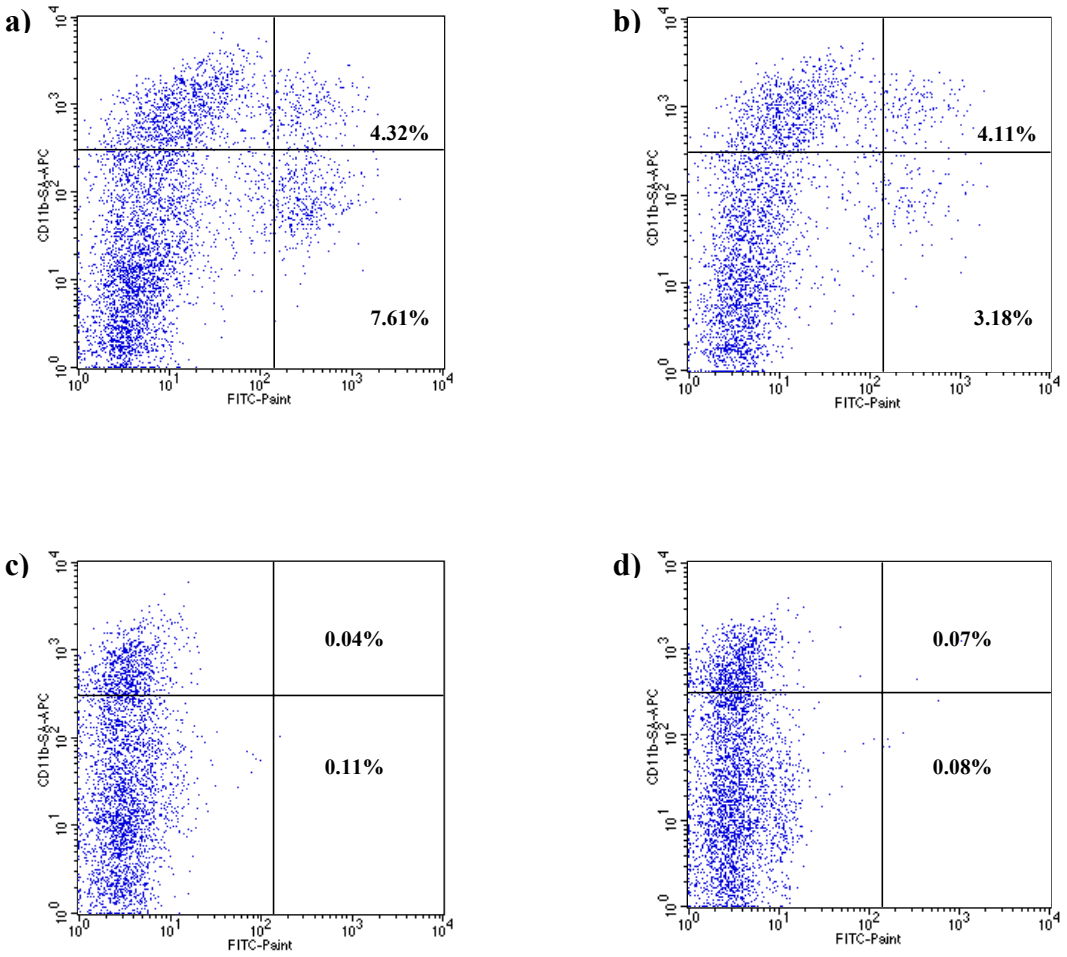


Fig. 3.12: Representative FACS Blots for Analysis of the CD11c$^+$ Population for CD11b Expression and FITC Uptake. FACS analysis of the CD11c$^+$ population of pooled inguinal, axillary and brachial LNs (dLNs) and mesenteric LNs (internal control) 18-20 h after FITC application to skin of the abdominal and chest region. CD11c$^+$ cells from C57Bl/6 control mice and CCR4$^{-/-}$ mice are analysed for CD11b expression and FITC uptake. Fig 3.16a) dLNs of C57Bl/6 control mice; Fig 3.16b) dLNs of CCR4$^{-/-}$ mice; Fig 3.16c) mLNs of C57Bl/6 control mice; Fig 3.16d) mLNs of CCR4$^{-/-}$ mice.

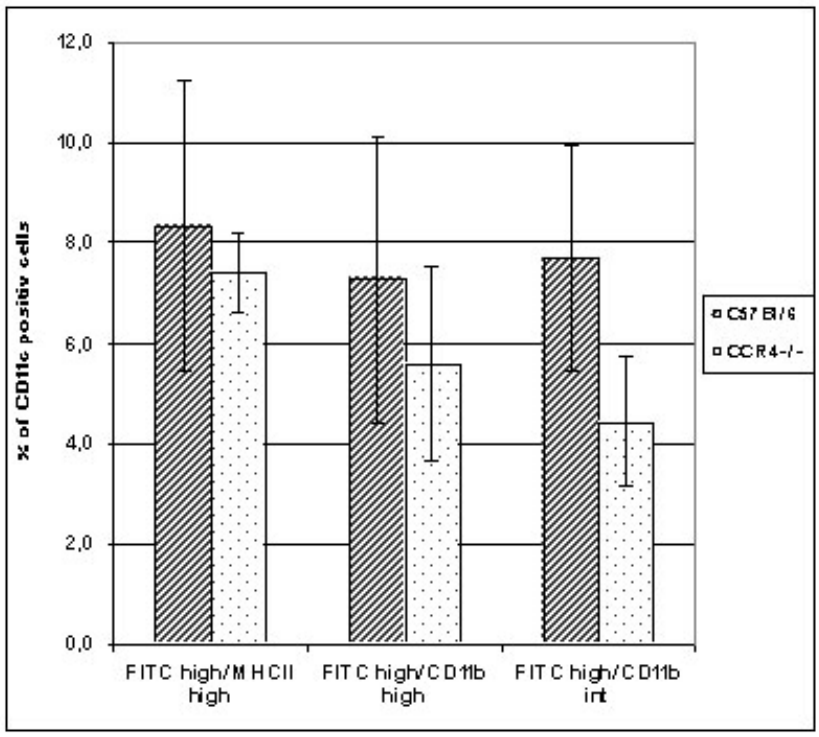


Fig. 3.13: Summary of FACS Analysis of the CD11c$^+$ Population

The CD11c$^+$ population of pooled inguinal, axillary and brachial LNs (dLNs) was analysed 18-20 h after FITC application to skin of the abdominal and chest region. CD11c$^+$ cells from C57Bl/6 control mice and CCR4$^{-/-}$ mice were analysed for FITC uptake and MHCII expression or CD11b expression, respectively. Three independent migration experiments with groups of two mice each were carried out and analysed. The average and standard deviation of each analysed population was calculated and displayed.

Analysis of all three experiments showed that an average of 8.34 +/- 2.90% of the CD11c$^+$ population in the draining LNs from C57Bl/6 mice was FITC high/MHCII high while an average of 7.41 +/- 0.79% of the CD11c$^+$ population in the draining LNs from CCR4$^{-/-}$ mice was FITC high/MHCII high. The results therefore showed that the migration of skin resident CD11c$^+$ cells which had taken up high amounts of FITC and strongly up-regulated MHC II expression was comparable between B/6 control mice and CCR4$^{-/-}$ mice. Evaluation of the FACS blots additionally revealed a CD11c$^+$/FITC intermediate/MHCII high population which was demonstrated to be an experimental artefact by a control experiment using the blue dye BODIPY®. It was verified by confocal microscopy that this dye is not able to induce migration of stained skin resident cells to the draining lymph nodes (data not shown).

However, FACS analysis of BODIPY® painting experiments display the same $CD11c^+$/ BODIPY® intermediate/MHCII high cell population that is observed in the FITC painting assay (data not shown).

Further evaluation of the experimental data demonstrated that an average of 7.28 +/- 2.86% of the $CD11c^+$ population of the draining LNs from C57Bl/6 mice was FITC high/CD11b high and an average of 5.60 +/- 1.94% of the $CD11c^+$ population of the draining LNs from $CCR4^{-/-}$ mice was FITC high/CD11b high. Examination further indicated that an average of 7.71 +/- 2.26% of the $CD11c^+$ population of the draining LNs from C57Bl/6 mice was FITC high/CD11b intermediate while an average of 4.44 +/- 1.29% of the $CD11c^+$ population of the draining LNs from $CCR4^{-/-}$ mice was FITC high/CD11b intermediate. The CD11c high population represents the migrated Langerhans cells while the CD11b intermediate population probably corresponds to migrated dermal DCs (Henri et al, 2001 ; O'Keeffe et al., 2002 : Heath et al., 2004). Taken together the experimental data showed that the migration of skin resident $CD11c^+$ cells expressing high or intermediate amounts of CD11b which had taken up high amounts of FITC was only slightly decreased in $CCR4^{-/-}$ mice. Taken together, the results reveal that the CCL17/CCL22-CCR4 receptor ligand pairs have an insubstantial effect on Langerhans cell migration.

3.2.2 Langerhans Cell Migration in $CCR4^{-/-}$ Mice after Injection of Neutralising Anti-CCL1 Antibody

The aim of the second part of the project was to analyse the combined influence of the CCL17/CCL22-CCR4 and CCL1-CCR8 Chemokine Receptor-Ligand Pairs on Langerhans cell migration. For this purpose migration of Langerhans cells from the skin to the draining LNs was analysed in $CCR4^{-/-}$ mice which had been treated with neutralising anti-CCL1 antibody and compared to C56Bl/6 control mice which had been treated with isotype control antibodies. Application of the indicated antibodies was carried out as described in section 2.9.3. Langerhans cell migration was analysed *in vivo* in 6-10 weeks old female mice as described in section 2.8.4.

It had already been shown by confocal microscopy that the experimental set up was suitable to examine migration of resident cells from the skin to the draining LN (see section 3.2.1 and Fig. 3.14). Langerhans cell migration was analysed in a quantative way by FACS analysis as described in section 3.2.1. Briefly, antibody treated $CCR4^{-/-}$ mice and C57Bl/6 control mice were painted with FITC dye, cell suspensions of the draining and mesenteric LNs were prepared and cells were stained with either anti-CD11c and anti-IA^b (MHC II) or anti-CD11c and anti-CD11b antibodies, respectively.

FACS analysis showed that an average of 0.61 +/- 0.35% of the total cell population in the draining LNs of antibody treated C57Bl/6 controls and an average of 0.70 +/- 0.21% of the total cell population in the draining LN of antibody treated $CCR4^{-/-}$ mice were $FITC^+$ (data not shown). The $CD11c^+$ population was analysed for FITC uptake and MHC II expression or CD11b expression, respectively. FACS blots of one representative experiment for each staining are shown in Figure 3.14 and 3.15. Two independent experiments with groups of two mice each were carried out. The LN cell suspensions of each group were pooled and analysed. The results of all experiments are summarised in Figure 3.16.

Evaluation of both experiments showed that an average of 11.33 +/- 5.35% of the $CD11c^+$ population in the draining LN from C57Bl/6 control mice was FITC high/MHCII high while an average of 12.67 +/- 1.59% of the $CD11c^+$ population in the draining LN from $CCR4^{-/-}$ mice which had been treated with neutralising anti-CCL1 antibody was FITC high/MHCII high. These results showed that the migration of skin resident $CD11c^+$ cells which had taken up high amounts of FITC and strongly upregulated MHC II expression was marginally increased in antibody treated $CCR4^{-/-}$ mice compared to isotype treated C57Bl/6 mice.

Examination of the experimental data indicated that an average of 6.76 +/- 1.71% of the $CD11c^+$ population of the draining LNs from C57Bl/6 control mice was FITC high/CD11b high while an average of 7.61 +/- 0.35% of the $CD11c^+$ population of the draining LN from $CCR4^{-/-}$ mice which had been treated with neutralising anti-CCL1 antibody was FITC high/CD11b high.

Analysis demonstrated as well that an average of 9.04 +/- 2.52% of the $CD11c^+$ population of the draining LNs from C57Bl/6 control mice was FITC high/CD11b intermediate while an average of 9.63 +/- 0.85% of the $CD11c^+$ population of the draining LNs from antibody treated $CCR4^{-/-}$ mice was FITC high/CD11b intermediate. These results showed that the migration of skin resident $CD11c^+$ cells expressing high or intermediate amounts of CD11b which had taken up high amounts of FITC was fractionally increased in antibody treated CCR4 ko mice compared to isotype treated C57Bl/6 controls. Taken together, the experimental data demonstrate that the combined influence of the CCL17/CCL22-CCR4 receptor ligand pairs and the CCL1-CCR8 receptor ligand pair on Langerhans cell migration is negligible.

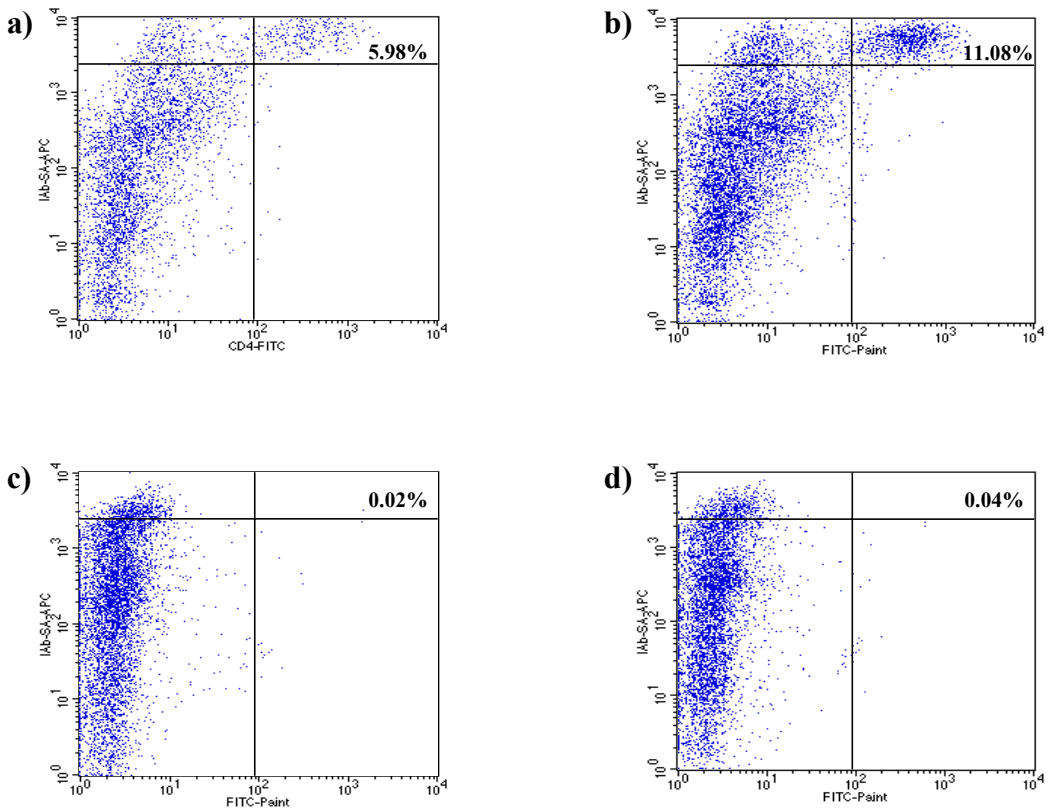


Fig. 3.14: Representative FACS Blots for Analysis of the $CD11c^+$ Population for MHC II Expression and FITC Uptake. FACS analysis of the $CD11c^+$ population of pooled inguinal, axillary and brachial LNs (dLNs) and mesenteric LNs 18-20 h after FITC application to the skin of the peritoneal and chest region. $CD11c^+$ cells from C57Bl/6 control mice and $CCR4^{-/-}$ mice which had been treated with neutralising anti-CCL1 antibody 24 h prior to FITC painting are analysed for MHCII expression (IA^b) and FITC uptake. Fig 3.15a) dLNs of C57Bl/6 control mice; Fig 3.15b) dLNs of $CCR4^{-/-}$ mice; Fig 3.15c) mLNs of C57Bl/6 control mice (internal control); Fig 3.15d) mLNs of $CCR4^{-/-}$ mice (internal control).

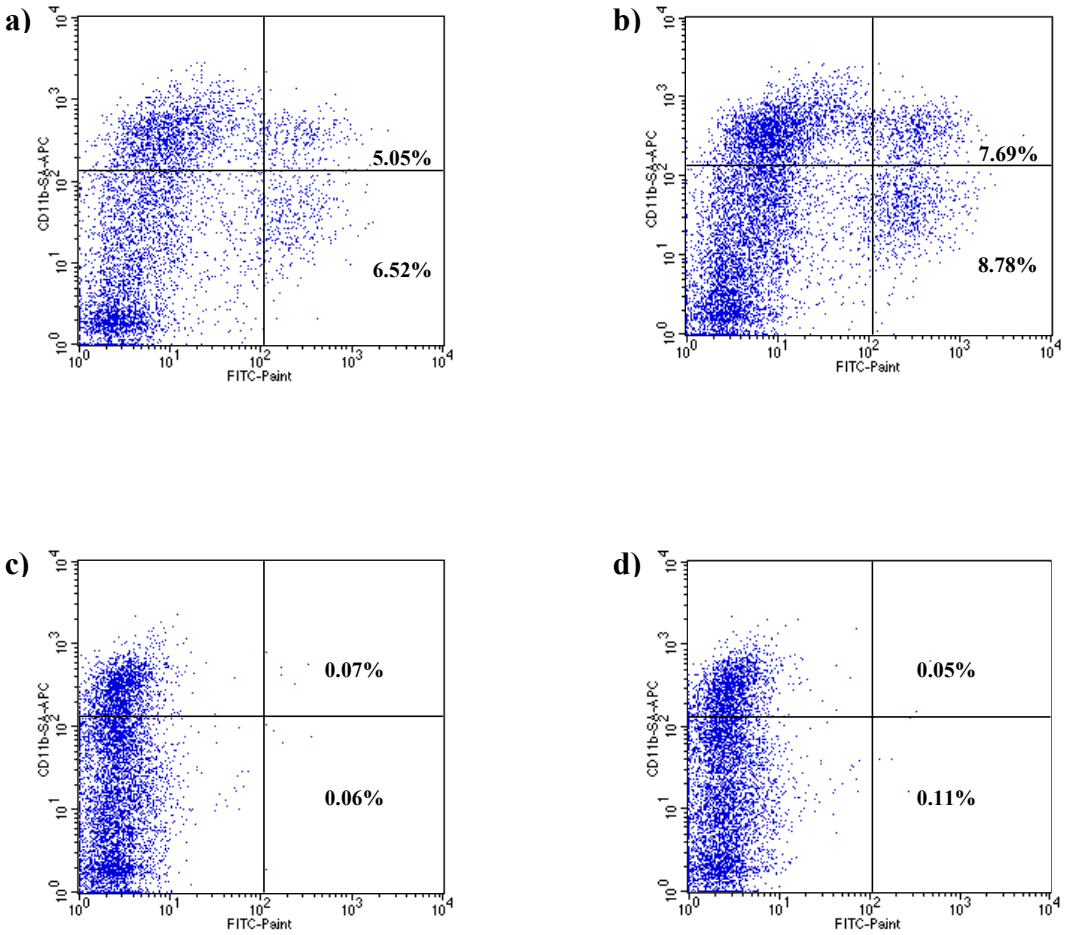


Fig. 3.15: Representative FACS Blots for Analysis of the CD11c^{+} Population for CD11b Expression and FITC Uptake. FACS analysis of the CD11c^{+} population of pooled inguinal, axillary and brachial LNs (dLNs) and mesenteric LNs 18-20 h after FITC application to skin of the peritoneal and chest region. CD11c^{+} cells from C57Bl/6 control mice and CCR4$^{-/-}$ mice which had been treated with neutralising anti-CCL1 antibody 24 h prior to FITC painting are analysed for CD11b expression and FITC uptake. Fig 3.16a) dLNs of C57Bl/6 control mice; Fig 3.16b) dLNs of CCR4$^{-/-}$ mice; Fig 3.16c) mLNs of C57Bl/6 control mice (internal control); Fig 3.16d) mLNs of CCR4$^{-/-}$ mice (internal control).

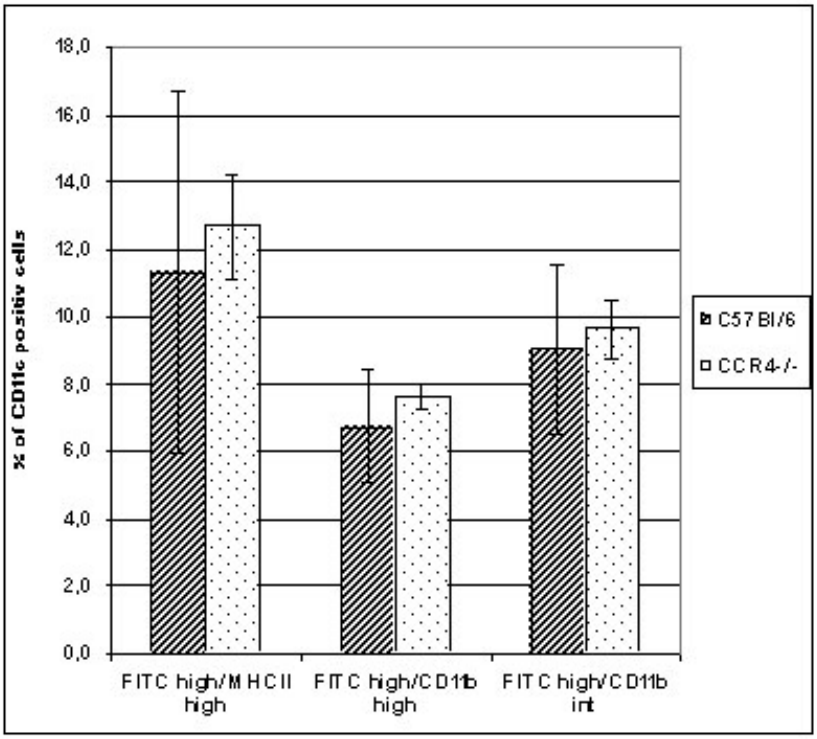


Fig. 3.16: Summary of FACS Analysis of the CD11c$^+$ Population

Summary of FACS analysis of the CD11c$^+$ population of pooled inguinal, axillary and brachial LNs (dLNs) 18-20 h after FITC application to skin of the peritoneal and chest region. CCR4$^{-/-}$ mice had been treated with neutralising anti-CCL1 antibody 24 h prior to FITC application while C57Bl/6 control mice had been treated with isotype control antibody. CD11c$^+$ cells from antibody treated mice were analysed for FITC uptake and MHCII expression or CD11b expression, respectively. Two independent migration experiments with groups of two mice each were carried out and analysed. The average and standard deviation of each analysed population was calculated and displayed.

3.3 Part III: Role of the CCL17/CCL22-CCR4 Chemokine Receptor-Ligand Pair During Systemic Infection with *Listeria Monocytogenes*

The aim of this project was to analyse the role of CCL17/CCL22-CCR4 in systemic infections *in vivo*. For this purpose mice were infected with the intracellular pathogen *Listeria monocytogenes* which is a well established and characterised *in vivo* infection model in the murine system.

3.3.1 Effect of CCL17- or CCR4-Deficiency on Survival after Infection with *Listeria Monocytogenes*

First, it was analysed if CCL17-deficiency has a significant effect on survival of mice after systemic infection with *L. monocytogenes*. In an initial experiment J. Alferink found that there was no difference in survival between $CCL17^{-/-}$ mice in C57Bl/6 background and $CCL17^{+/-}$ littermate controls after infection with high and medium doses ($10\times LD_{50}$ and $10\times LD_{50}$) of *L. monocytogenes* while $CCL17^{-/-}$ mice showed better survival than $CCL17^{+/-}$ littermate controls after infection with low doses ($0.1\times LD_{50}$) of *L. monocytogenes* (unpublished data, see section 1.9). Therefore, I repeated this experiment using medium and low doses ($1\times LD_{50}$ and $0.1\times LD_{50}$) of *L. monocytogenes* for infection. For each dose of listeria age and weight matched groups of 5-6 female mice were infected as described. However, there was no difference between $CCL17^{-/-}$ mice and $CCL17^{+/-}$ littermate controls in both dose groups as shown in Figure 3.17.

Mice with C57Bl/6 genetic background are much more resistant to listeria infection than mice on Balb/c background. It was therefore possible that a minor effect of CCL17-deficiency on survival of mice after infection could not be shown in C57Bl/6 background. To exclude this possibility, I analysed the resistance of $CCL17^{-/-}$ mice on Balb/c background in comparison to Balb/c wt control mice after infection with high, medium and low doses ($10\times LD_{50}$, $1\times LD_{50}$, $0.1\times LD_{50}$) of *L. monocytogenes*. For each dose of listeria age and weight matched groups of 4 female mice were infected as described. The results of the experiment are shown in Figure 3.18.

Evaluation of the experimental data showed that there was no difference in survival between CCL17$^{-/-}$ mice and control mice in all three dose groups. Taken together the results indicate that CCL17-deficiency has no significant influence on survival of mice after systemic infection with high, medium and low doses of *L. monocytogenes*.

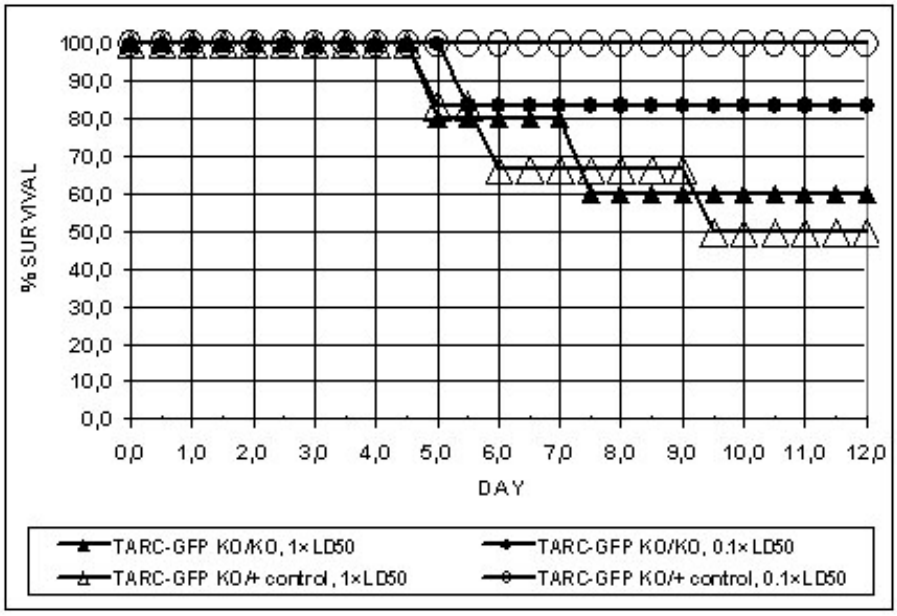


Fig. 3.17: Survival of CCL17$^{-/-}$ Mice and CCL17$^{+/-}$ Littermate Controls in C57Bl/6 Genetic Background after Infection with Low and Medium Doses of *L. Monocytogenes*.

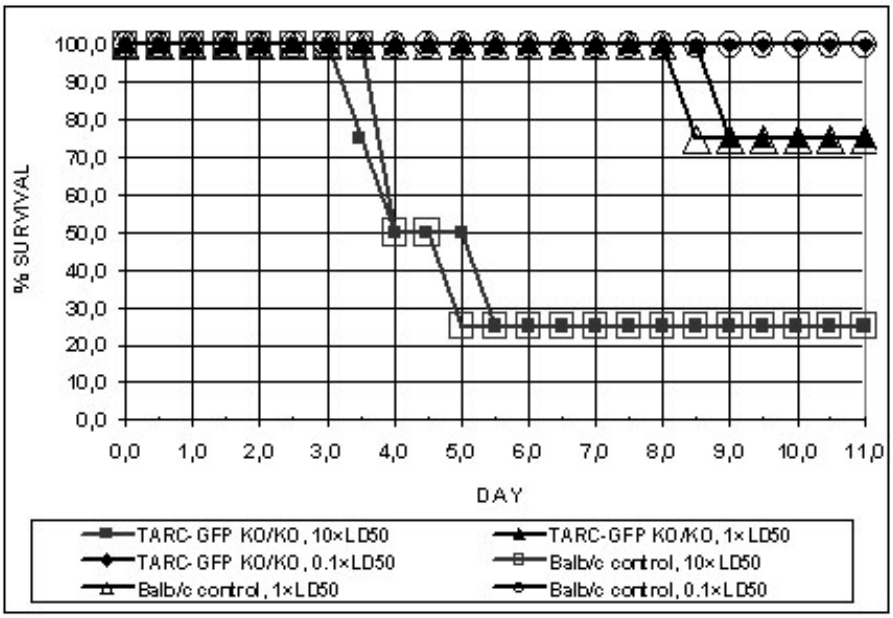


Fig. 3.18: Survival of CCL17$^{-/-}$ Mice in Balb/c Genetic Background and Balb/c Wild Type Controls after Infection with High, Medium and Low Doses of *L. Monocytogenes*.

Since CCL22, which also binds to CCR4, is still present in CCL17$^{-/-}$ mice and these two chemokines have redundant functions it was possible that CCL22 was able to compensate for the lack of CCL17. In the second part of the project it was therefore analysed if CCR4-deficiency had a stronger effect on survival of mice after systemic infection with *L. monocytogenes*. To investigate this question, CCR4$^{-/-}$ mice on C57Bl/6 background and wt control mice were infected with high, medium and low doses ($10\times LD_{50,}$ $1\times LD_{50}$ and $0.1\times LD_{50}$) of *L. monocytogenes*. For each dose of listeria age and weight matched groups of 4 female mice were infected as described. Examination of the data showed that there was no difference between CCR4-/- mice and C57Bl/6 control mice in the medium and low dose groups as shown in Fig. 3.19. Analysis further demonstrated a marginally prolonged survival of CCR4$^{-/-}$ mice in the high dose group in comparison to control mice. Therefore, CCR4-deficiency only has a negligible, if any, influence on survival of mice after systemic infection with high, medium and low doses of *L. monocytogenes*. As CCR4$^{-/-}$ mice are only available in C57Bl/6 genetic background it was not possible to repeat the experiment on Balb/c background.

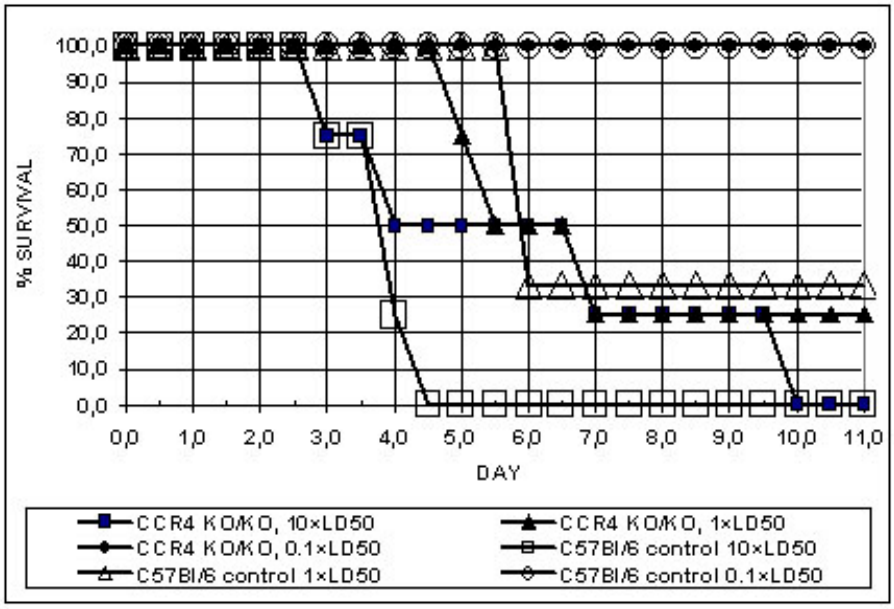


Fig. 3.19: Survival of CCR4$^{-/-}$ Mice in C57Bl/6 Genetic Background and Wild Type Control Mice after Infection with High, Medium and Low Doses of *L. Monocytogenes*.

3.3.3 Effect of CCL17- and CCR4-Deficiency on Bacterial Clearance after Infection with *Listeria Monocytogenes*

Although CCL17-deficiency has no effect on survival of mice after listeria infection, it could not be excluded that it has an effect on innate immune responses which are essential for the host to clear infection. It has been previously demonstrated that IFNy and TNFα which are produced by NK cells and macrophages are essential for the primary defence against LM (Tripp et al., 1993). It is also known that macrophages are important for the killing of the bacteria (Rogers and Unanue, 1993; Zuprynski et al., 1994) and that the recruitment of monocytes is vital for bacterial clearance (North, 1970). As CCR4, the corresponding receptor for CCL17, is expressed on macrophages, monocytes and NK cells it is feasible that lack of CCL17 weakens the innate immune response and, as a consequence, has an effect on bacterial clearance in spleen and liver after systemic infection with *L. monocytogenes*. In order to investigate this question, the bacterial load of these organs after primary infection with listeria was determined.

Age matched groups of three female CCL17$^{-/-}$ mice and three female C57Bl/6 control mice were infected i.v. with 20 000 CFU of the mutant ovalbumin-expressing *L. monocytogenes* strain L.m.ova. Three days after primary infection the bacterial organ load was determined as described in section 2.9.5. Evaluation of the results, which are depicted in Fig. 3.20, showed that the bacterial clearance in the liver is more efficient in CCL17$^{-/-}$ mice than in wt control mice while the bacterial clearance in the spleen is marginally less effective in the control mice compared to KO mice.

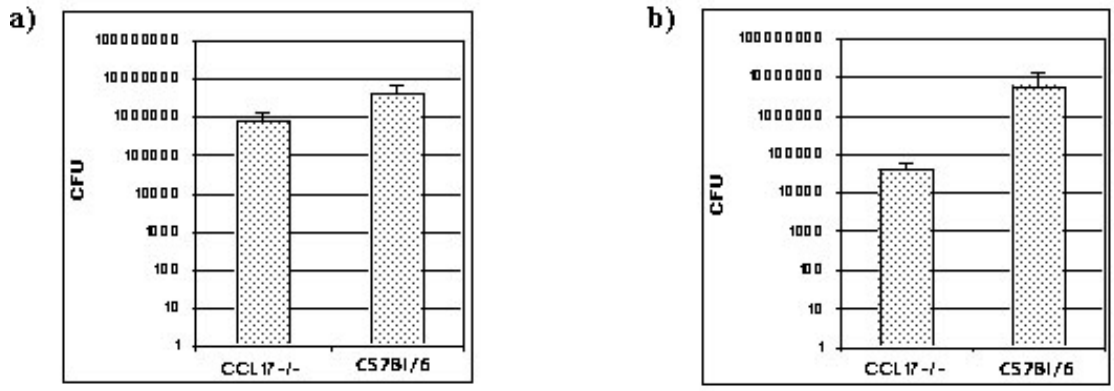


Fig 3.20: Determination of the Bacterial Organ Load after Primary Infection of CCL17$^{-/-}$ Mice and C57Bl/6 Controls with 20 000CFU *Listeria Monocytogenes*.
a) bacterial load of the spleen 3 days after infection
b) bacterial load of the liver 3 days after infection

Next, the bacterial clearance in spleen and liver was analysed after primary infection with a lower dose of listeria by infecting age matched groups of six female CCL17$^{-/-}$ mice and six female C57Bl/6 control mice i.v. with 2000 CFU of L.m.ova. Three and seven days after primary infection the bacterial organ load of three mice of each group was determined as described. Analysis of the obtained data which are displayed in Fig 3.21 confirmed the results of the previous experiment. Bacterial clearance in spleen and liver is slightly more efficient in CCL17$^{-/-}$ mice than in wt controls although the difference is difficult to quantify due to the low numbers of colony forming units.

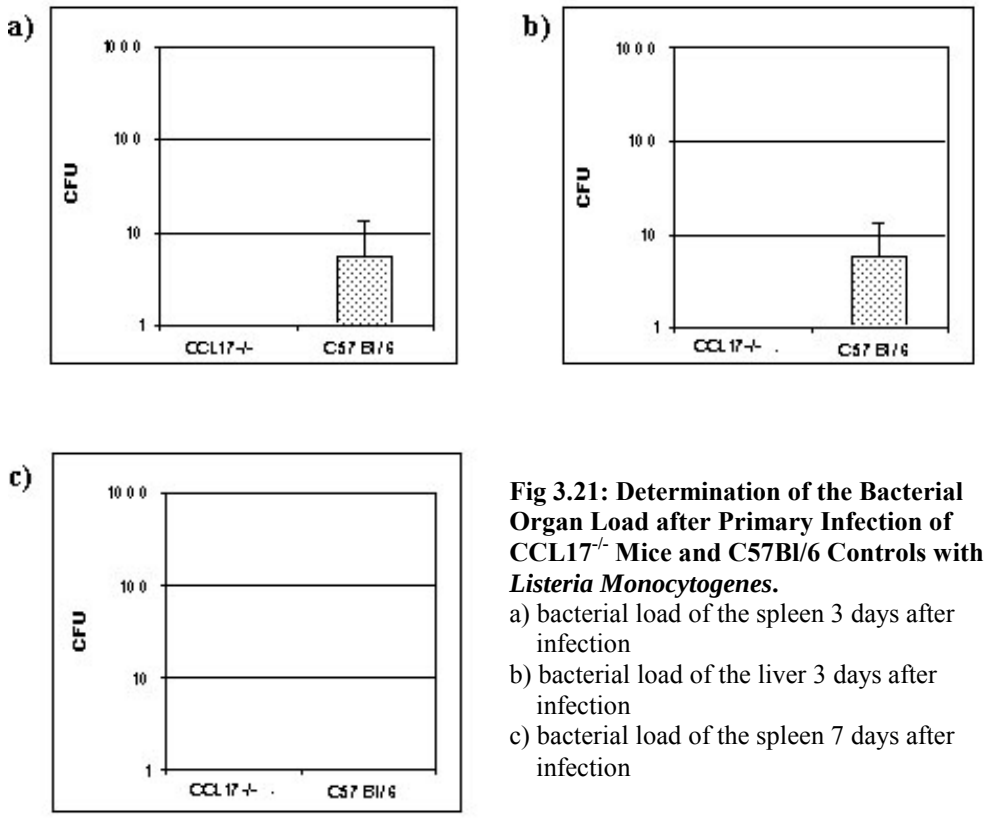


Fig 3.21: Determination of the Bacterial Organ Load after Primary Infection of CCL17$^{-/-}$ Mice and C57Bl/6 Controls with *Listeria Monocytogenes*.
a) bacterial load of the spleen 3 days after infection
b) bacterial load of the liver 3 days after infection
c) bacterial load of the spleen 7 days after infection

As the results had shown a small difference in bacterial clearance during primary infection between CCL17$^{-/-}$ mice and wt controls the bacterial clearance in spleen and liver after recall infection was investigated next. For this purpose, mice were primarily infected with 2000 CFU as described above and, after five weeks, mice were re-infected i.v. with 200 000 CFU of L.m.ova. Two and five days after recall infection the bacterial organ load of three mice of each group was determined as described. Two independent experiments were carried out and the results obtained are presented in Fig 3.22. and 3.23. In contrast to primary infection, the bacterial clearance in liver and spleen was slightly less efficient in CCL17$^{-/-}$ mice than in wt control mice after recall infection although, again, the difference was difficult to quantify because of the low numbers of CFU detected.

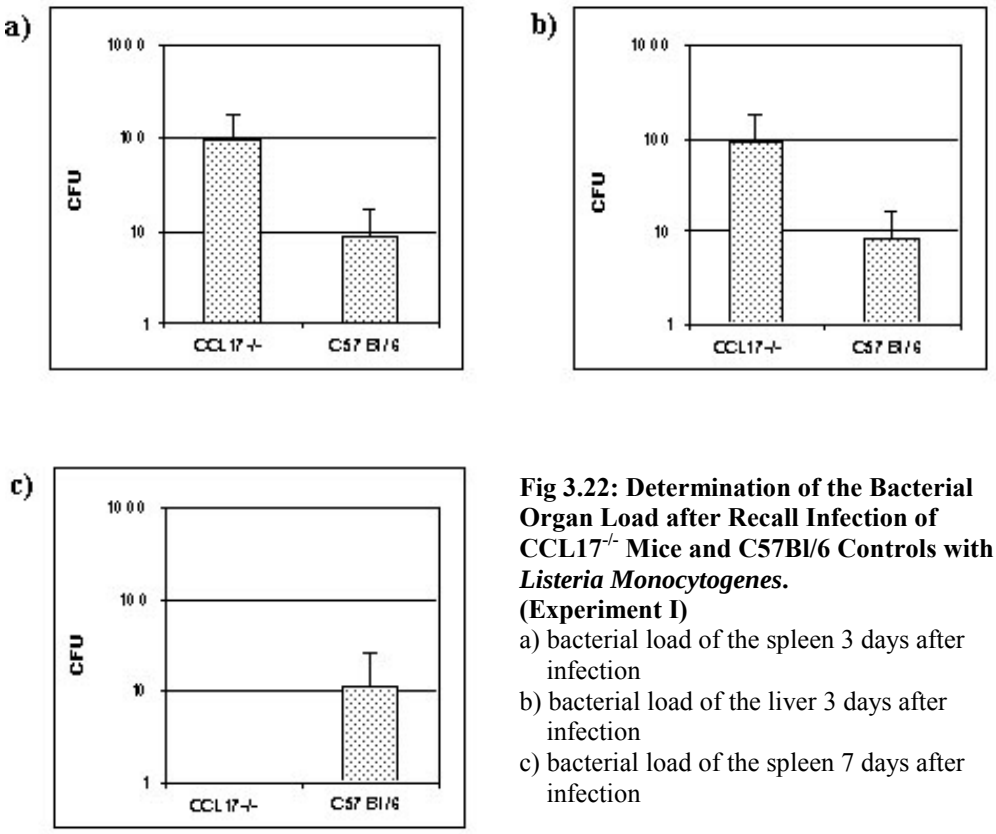


Fig 3.22: Determination of the Bacterial Organ Load after Recall Infection of CCL17$^{-/-}$ Mice and C57Bl/6 Controls with *Listeria Monocytogenes*.
(Experiment I)
a) bacterial load of the spleen 3 days after infection
b) bacterial load of the liver 3 days after infection
c) bacterial load of the spleen 7 days after infection

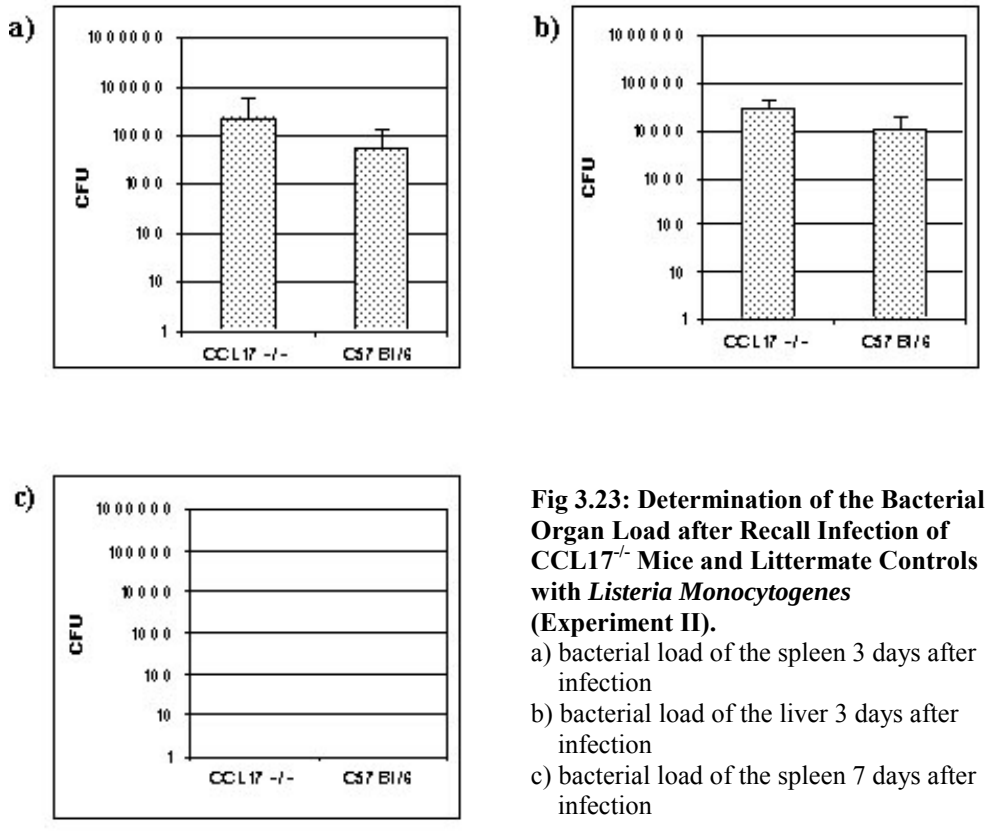


Fig 3.23: Determination of the Bacterial Organ Load after Recall Infection of CCL17$^{-/-}$ Mice and Littermate Controls with *Listeria Monocytogenes* (Experiment II).
a) bacterial load of the spleen 3 days after infection
b) bacterial load of the liver 3 days after infection
c) bacterial load of the spleen 7 days after infection

It has been shown that CCL17 and CCL22, which both bind to the chemokine receptor CCR4, have redundant functions. As CCL22 is still produced in CCL17$^{-/-}$ mice it could not be excluded that CCL22 was able to compensate for the lack of CCL17 during infection with *L. monocytogenes*. Therefore, I investigated whether CCR4-deficiency had a stronger effect on bacterial clearance than CCL17-deficiency alone after primary and recall infection with listeria. Infection and analysis were carried out as described above and the obtained results are presented in Fig 3.24 and 3.25.

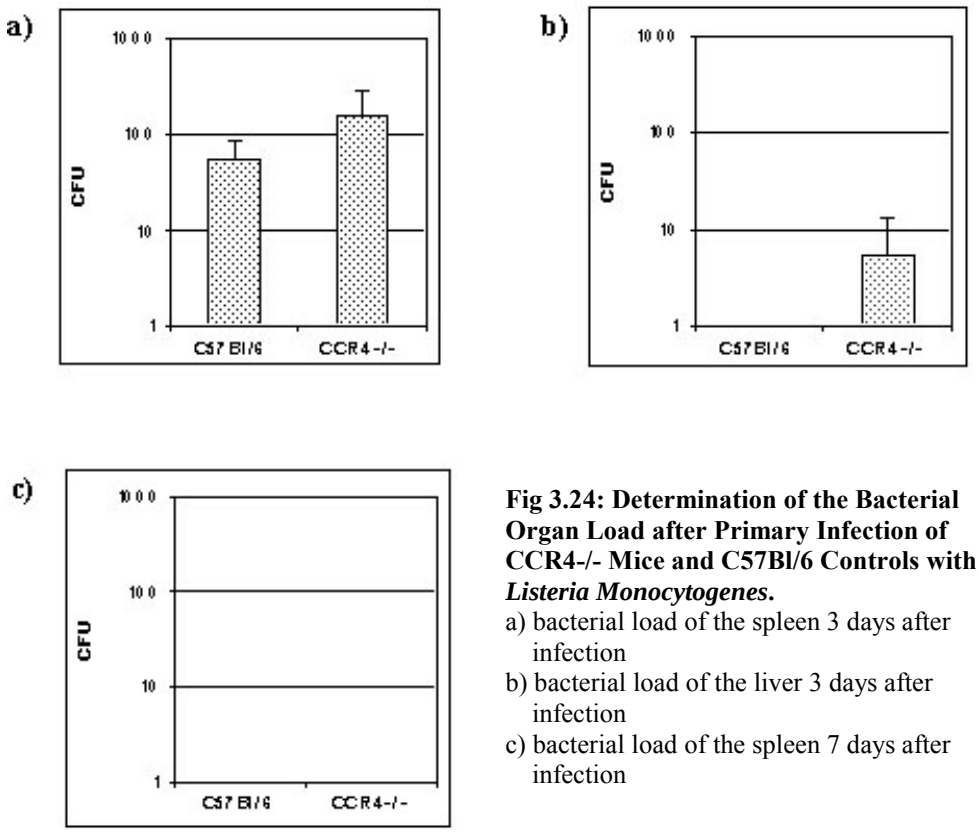


Fig 3.24: Determination of the Bacterial Organ Load after Primary Infection of CCR4-/- Mice and C57Bl/6 Controls with *Listeria Monocytogenes*.
a) bacterial load of the spleen 3 days after infection
b) bacterial load of the liver 3 days after infection
c) bacterial load of the spleen 7 days after infection

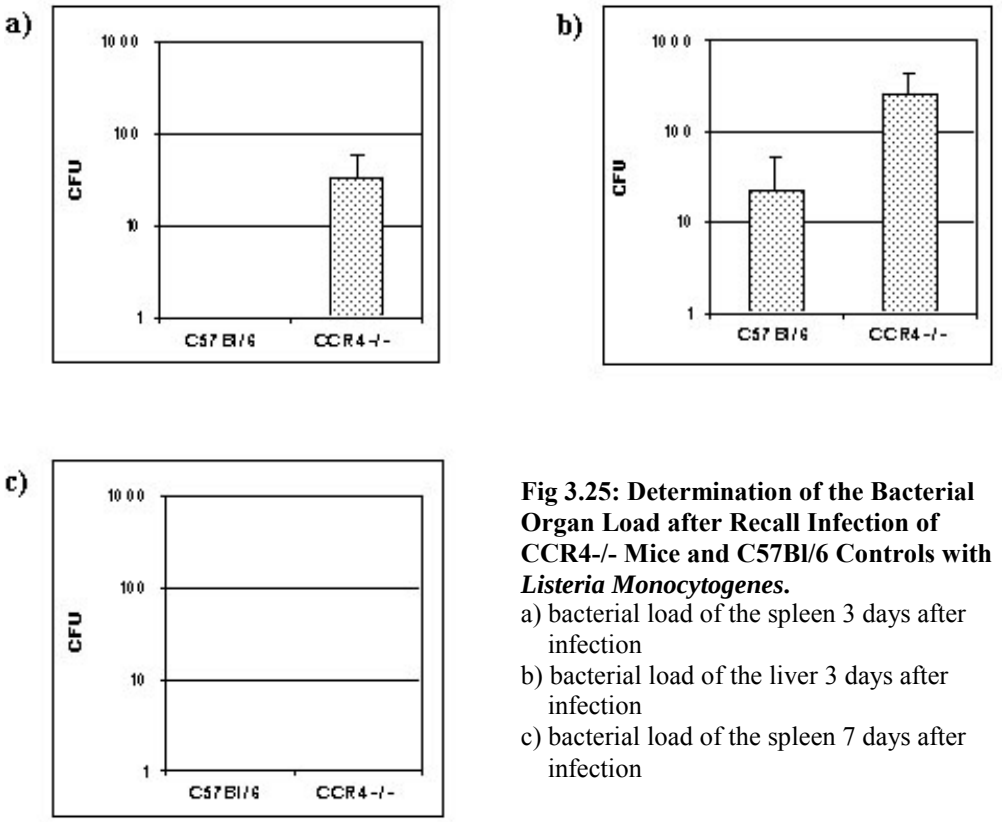


Fig 3.25: Determination of the Bacterial Organ Load after Recall Infection of CCR4-/- Mice and C57Bl/6 Controls with *Listeria Monocytogenes.*

a) bacterial load of the spleen 3 days after infection
b) bacterial load of the liver 3 days after infection
c) bacterial load of the spleen 7 days after infection

Evaluation of the results showed that the bacterial clearance in the liver is slightly less efficient in the CCR4$^{-/-}$ mice than in wt mice after systemic primary and recall infection with *L. monocytogenes*.

Taken together, the experiments conducted showed that CCL17 and CCR4 deficiency have only a minor influence on innate immune responses against systemic infection with *L. monocytogenes*. CCL17 deficiency leads to a slight loss of efficiency in bacterial clearance during primary infection but to a marginally increased effectiveness in bacterial clearance during recall responses. On the other hand, CCR4 deficiency results in a marginally decreased efficiency in bacterial clearance during primary and recall infection.

3.3.3 Effect of CCL17- and CCR4-Deficiency on Specific T Cell Responses after Infection with *Listeria Monocytogenes*

As demonstrated above CCL17- and CCR4-deficiency have only a minor influence on innate immune responses after systemic primary infection with listeria. But as this chemokine receptor-ligand pair plays an important role in chemotaxis of T cells I also investigated whether CCL17- and CCR4-deficiency have an influence on specific T cell responses against *Listeria Monocytogenes* after systemic primary and recall infection. For that purpose, groups of six age and weight matched female C57Bl/6 control mice and $CCL17^{-/-}$ mice or $CCR4^{-/-}$ mice, respectively, were infected i.v. with 2000 CFU of the ova- expressing *L. monocytogenes* strain L.m.ova. Seven days after primary infection three mice of each group were sacrificed and T cell responses were analysed by tetramer staining and intracellular cytokine staining.

Ova-expression by listeria results in presentation of the immunodominant peptide SIINFEKL via MHC class I to $CD8^+$ T cells. This, in turn, leads to induction of SIINFEKL-specific $CD8^+$ T cells that are directed against L.m.ova-infected cells which can be detected by tetramer staining. Re-stimulation of splenic T cells *in vitro* with the immunogenic peptides SIINFEKL and $LLO_{188-201}$ leads to production of IFNy and TNFα by SIINFEKL-specific $CD8^+$ T cells and LLO-specific $CD4^+$ T cells which can be detected by intracellular cytokine staining.

To analyse T cell responses after recall infection, the three remaining mice of each group were re-infected i.v. five weeks after primary infection with 200 000 L.m.ova. Five days after recall infection the mice were sacrificed and T cell responses were analysed as described. FACS blots of tetramer staining analysis of one representative wt and ko mouse after primary and recall infection are shown in Fig. 3.26 and 3.27. Results of the tetramer staining analysis are shown in Fig 3.28 and 3.29. FACS blots of intracellular cytokine staining analysis for IFNγ and TNFα of one representative wt and ko mouse after primary and recall infection are shown in Fig. 3.30-3.33. Results of the intracellular cytokine staining analysis are shown in Fig 3.36 and 3.37.

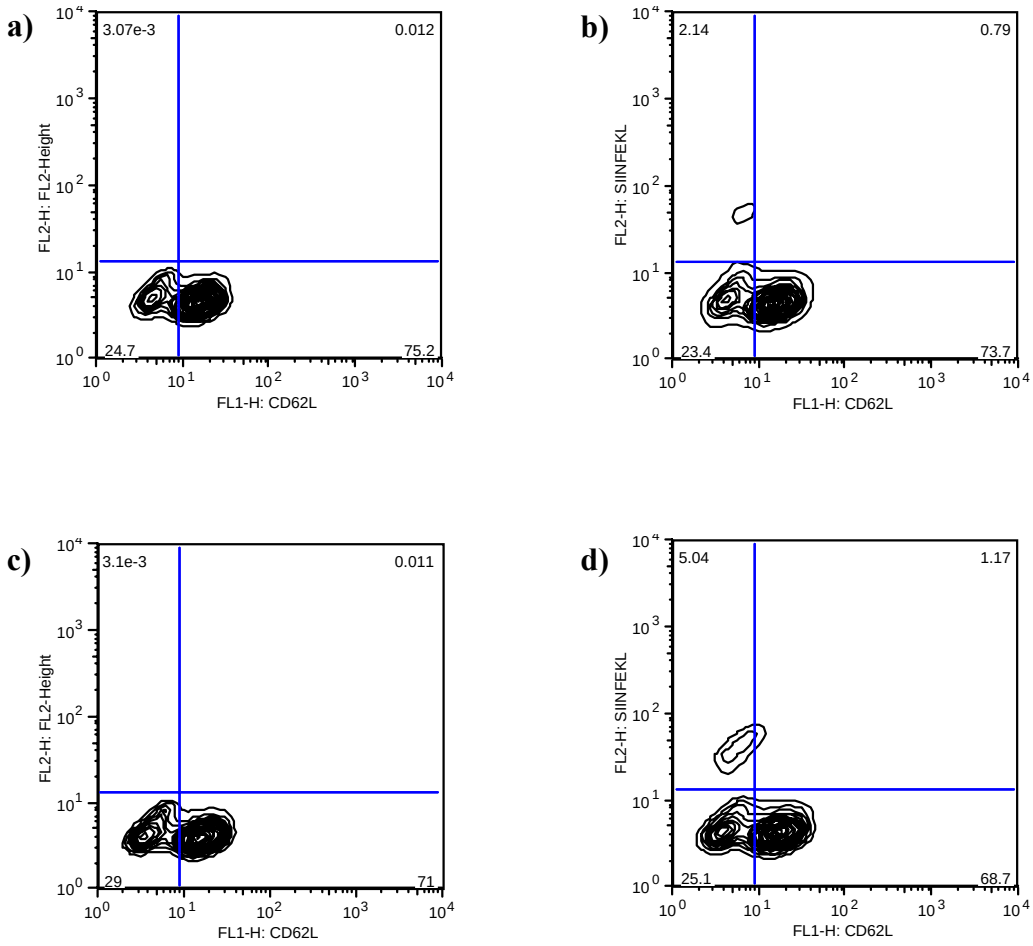


Fig. 3.26: Representative FACS Blots for Analysis of $CD8^+$ T cells for CD62L Expression and SIINFEKL Specific Tetramer Staining after Systemic Primary Infection with *L .monocytogenes*. $CCR4^{-/-}$ mice and C57Bl/6 control mice were primary infected with L.m.ova bacteria. Tetramer staining analysis was carried out 7 days after infection. Cells were stained with anti-CD8 and anti-CD62L surface antibodies and SIINFEKL specific tetramer or no tetramer (unstained controls). Fig 3.28a) C57Bl/6 wt mouse, unstained control; Fig 3.28b) C57Bl/6 wt mouse, stained with SIINFEKL specific tetramer; Fig 3.28c) $CCR4^{-/-}$ mouse, unstained control; Fig 3.28d) CCR4 -/- mouse, stained with SIINFEKL specific tetramer.

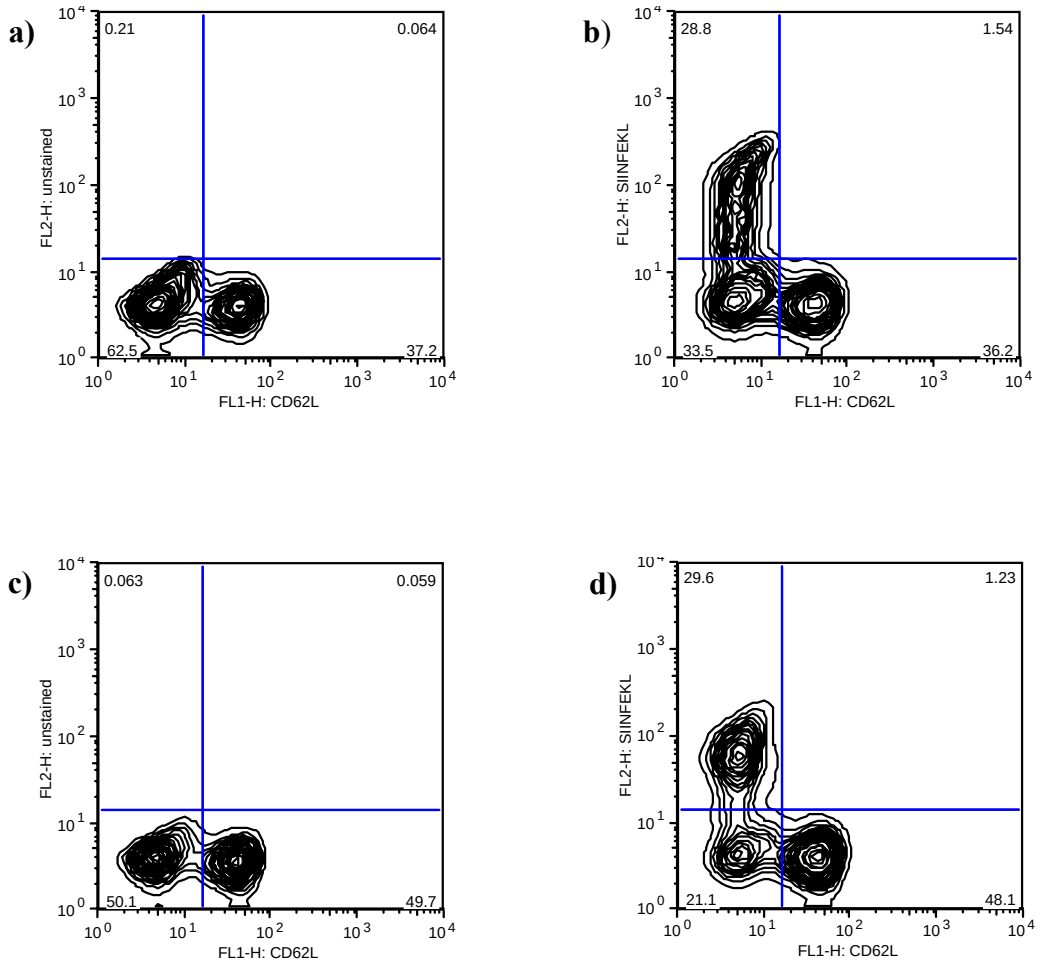


Fig. 3.27: Representative FACS Blots for Analysis of CD8$^+$ T cells for CD62L Expression and SIINFEKL Specific Tetramer Staining after Systemic Recall Infection with *L. monocytogenes*. CCR4$^{-/-}$ mice and C57Bl/6 control mice were primary infected with L.m.ova bacteria and re-infected five weeks later. Tetramer staining analysis was carried out 5 days after recall infection. Cells were stained with anti-CD8 and anti-CD62L surface antibodies and SIINFEKL specific tetramer or no tetramer (unstained controls). Fig 3.29a) C57Bl/6 wt mouse, unstained control; Fig 3.29b) C57Bl/6 wt mouse, stained with SIINFEKL specific tetramer; Fig 3.29c) CCR4$^{-/-}$ mouse, unstained control; Fig 3.29d) CCR4 -/- mouse, stained with SIINFEKL specific tetramer

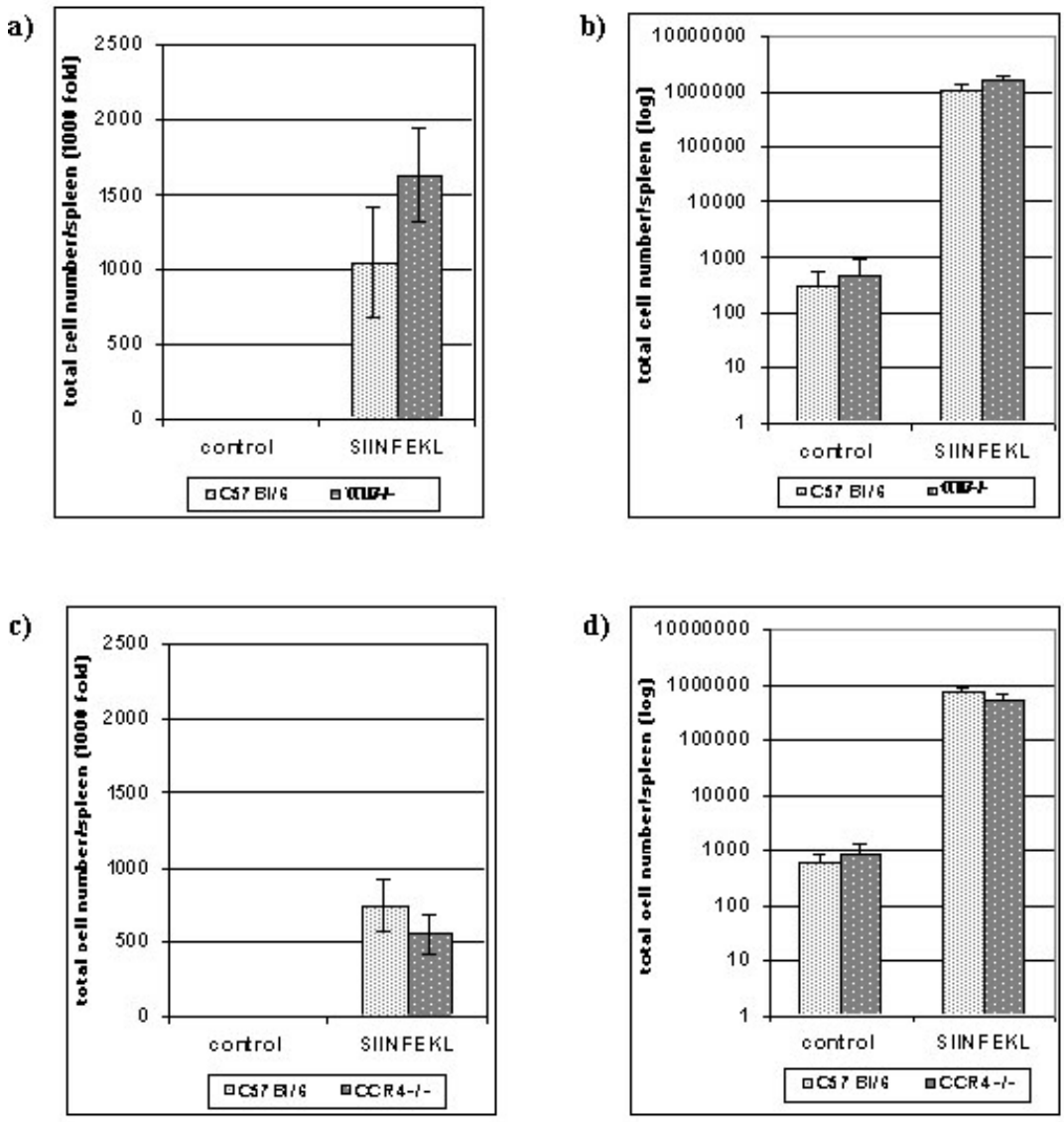


Fig. 3.28: Total Number of $CD8^+$ $CD62L^-$ SIINFEKL Specific T cells in the Spleen after Systemic Primary Infection of $CCL17^{-/-}$ Mice, $CCR4^{-/-}$ Mice and C57Bl/6 Control Mice with Ova-Expressing *Listeria Monocytogenes*. Fig.3.30a) and b): Analysis of $CCL17^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (a) and logarithmic (b) scale. Fig.3.30c) and d): Analysis of $CCR4^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (c) and logarithmic (d) scale.

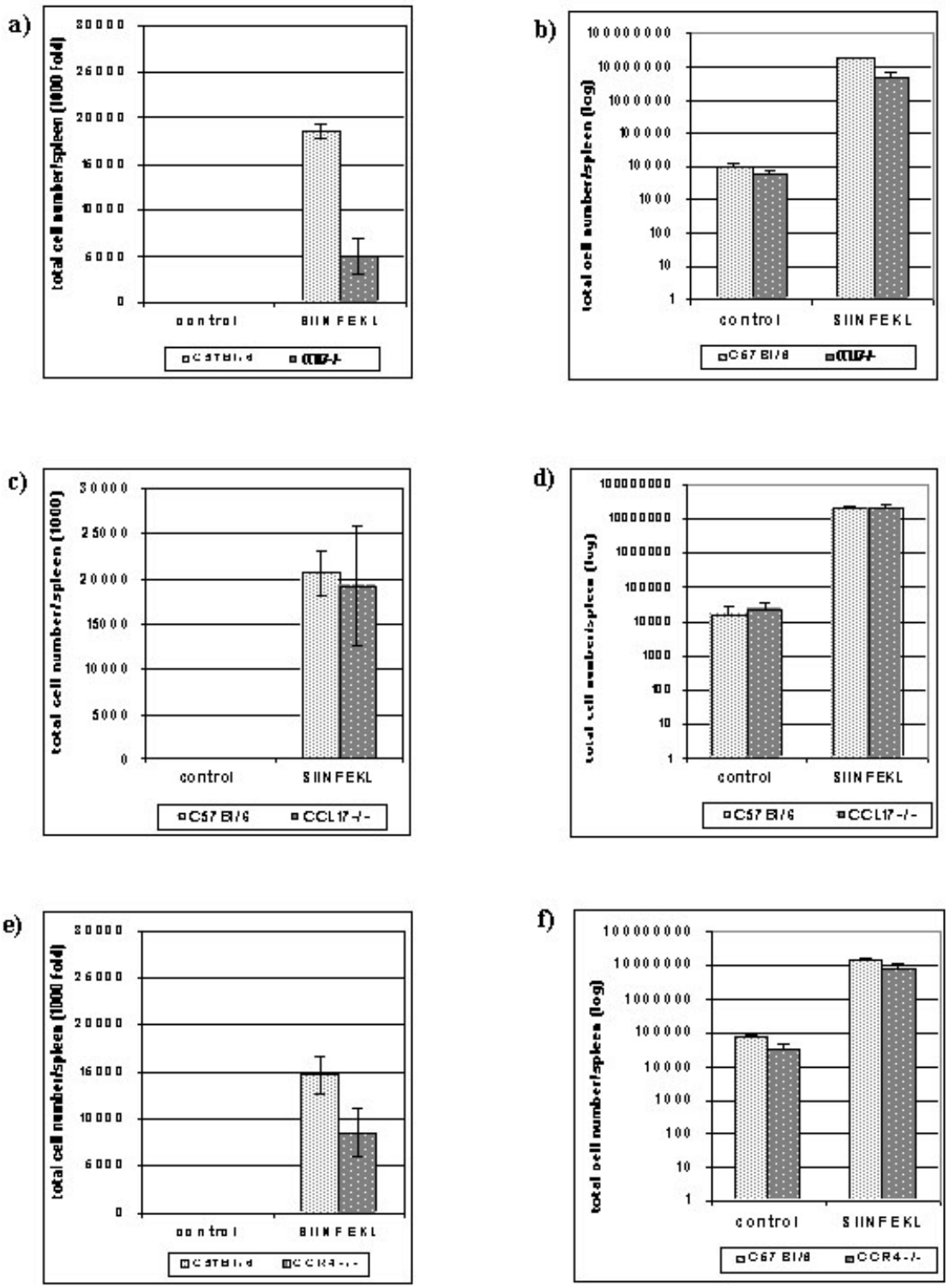


Fig. 3.29: Total Number of $CD8^+$ $CD62L^-$ SIINFEKL Specific T cells in the Spleen after Systemic Recall Infection of $CCL17^{-/-}$ Mice, $CCR4^{-/-}$ Mice and C57Bl/6 Control Mice with Ova-Expressing *Listeria Monocytogenes*. Fig.3.29a-d): Analysis of $CCL17^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (a, c) and logarithmic (b, d) scale. Fig.3.29e and f): Analysis of $CCR4^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (e) and logarithmic (f) scale.

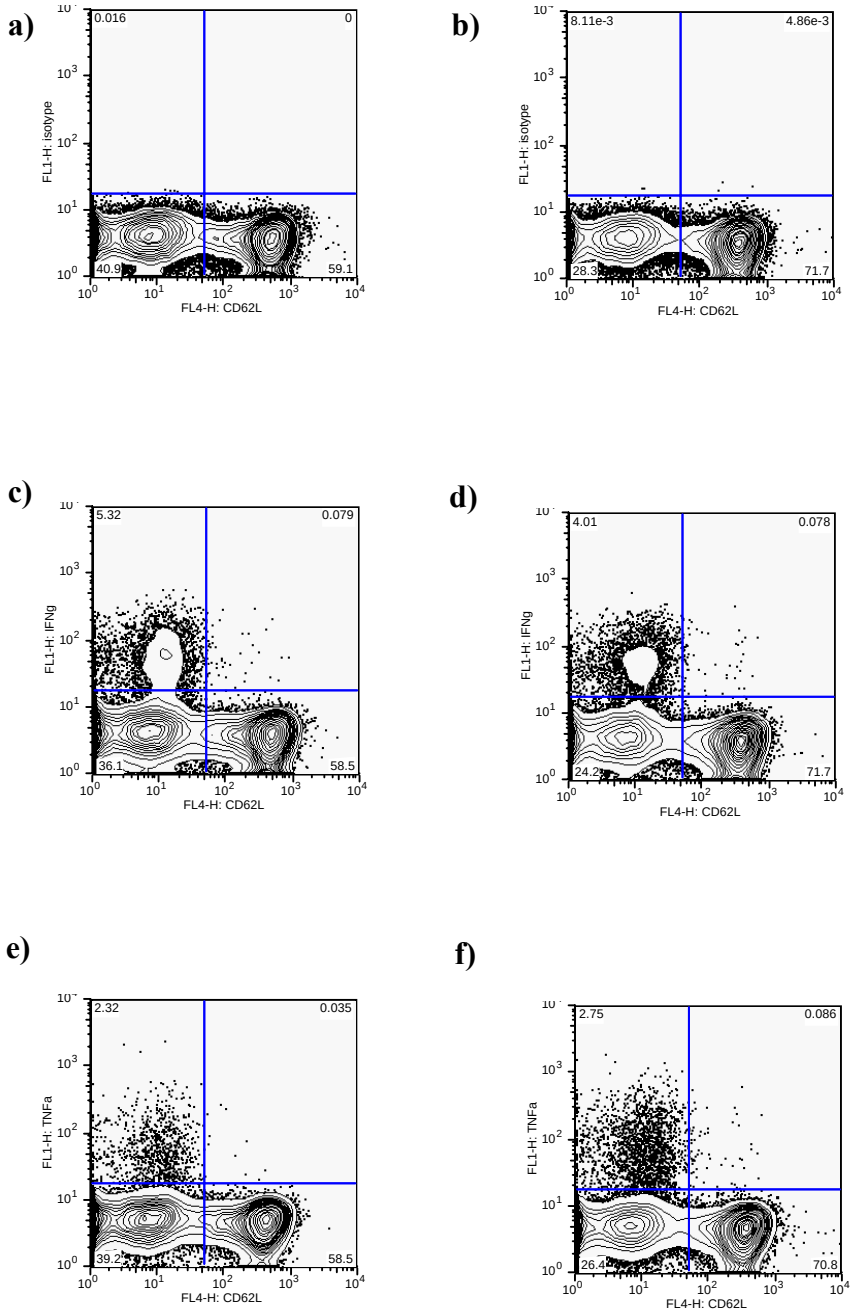


Fig. 3.30: Representative FACS Blots for Analysis of SIINFEKL-specific CD8$^+$ T cells for CD62L Expression and Intracellular IFNγ and TNFα Expression after Systemic Primary Infection with *L. monocytogenes* and Re-stimulation *in vitro* with SIINFEKL Peptide. CCR4$^{-/-}$ mice and C57Bl/6 control mice were primary infected with L.m.ova bacteria. ICS analysis was carried out 7 days after infection and 5 h of re-stimulation with SIINFEKL peptide *in vitro*. Fig 3.32a) C57Bl/6 wt mouse, isotype control; Fig 3.32b) CCR4$^{-/-}$ mouse, isotype control; Fig 3.32c) C57Bl/6 wt mouse, IFNγ; Fig 3.32d) CCR4 -/- mouse, IFNγ; Fig 3.32e) C57Bl/6 wt mouse, TNFα; Fig 3.32f) CCR4 -/- mouse, TNFα.

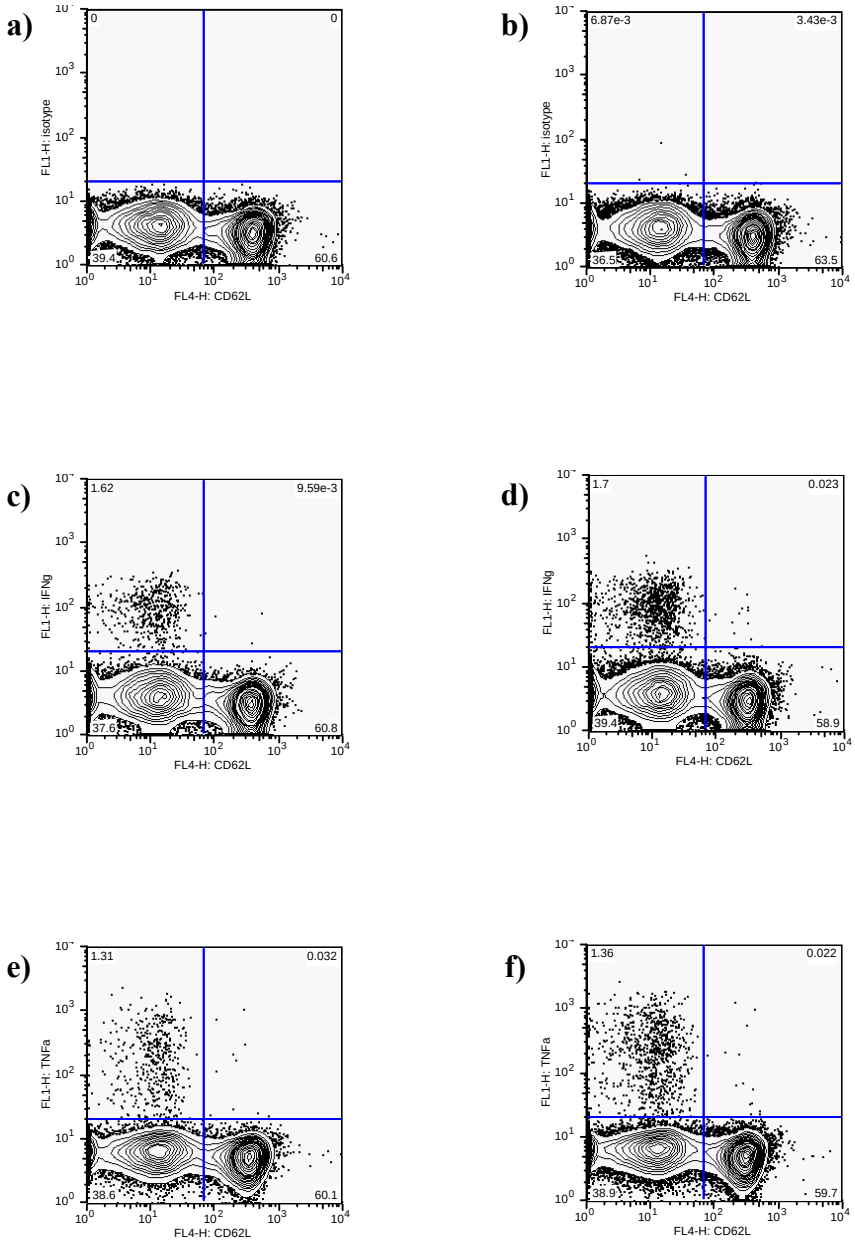


Fig. 3.31: Representative FACS Blots for Analysis of $LLO_{188-201}$-specific $CD4^+$ T cells for CD62L Expression and Intracellular IFNγ and TNFα Expression after Systemic Primary Infection with *L. monocytogenes* and Re-stimulation *in vitro* with $LLO_{188-201}$ Peptide. $CCR4^{-/-}$ mice and C57Bl/6 control mice were primary infected with L.m.ova bacteria. ICS analysis was carried out 7 days after infection and 5 h of re-stimulation with $LLO_{188-201}$ peptide *in vitro*. Fig 3.33a) C57Bl/6 wt mouse, isotype control; Fig 3.33b) $CCR4^{-/-}$ mouse, isotype control; Fig 3.33c) C57Bl/6 wt mouse, IFNγ; Fig 3.33d) CCR4 -/- mouse, IFNγ; Fig 3.33e) C57Bl/6 wt mouse, TNFα; Fig 3.33f) CCR4 -/- mouse, TNFα.

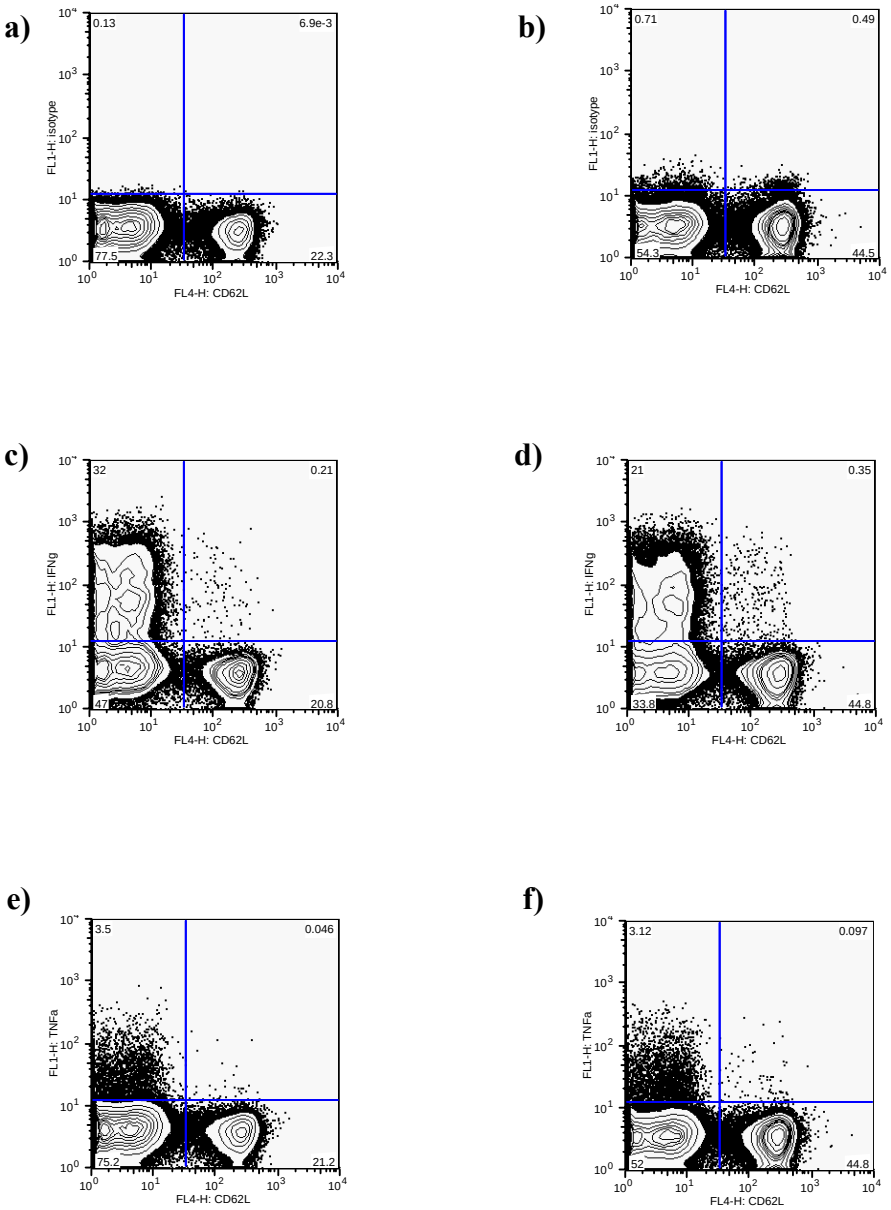


Fig. 3.32: Representative FACS Blots for Analysis of SIINFEKL-specific CD8$^+$ T cells for CD62L Expression and Intracellular IFNγ and TNFα Expression after Systemic Recall Infection with *L. monocytogenes* and Re-stimulation *in vitro* with SIINFEKL peptide. CCR4$^{-/-}$ mice and C57Bl/6 control mice were primary infected with L.m.ova bacteria and re-infected five weeks later. ICS analysis was carried out 5 days after infection and 5 h of re-stimulation with SIINFEKL peptide *in vitro*. Fig 3.34a) C57Bl/6 wt mouse, isotype control; Fig 3.34b) CCR4$^{-/-}$ mouse, isotype control; Fig 3.34c) C57Bl/6 wt mouse, IFNγ; Fig 3.34d) CCR4 -/- mouse, IFNγ; Fig 3.34e) C57Bl/6 wt mouse, TNFα; Fig 3.34f) CCR4 -/- mouse, TNFα.

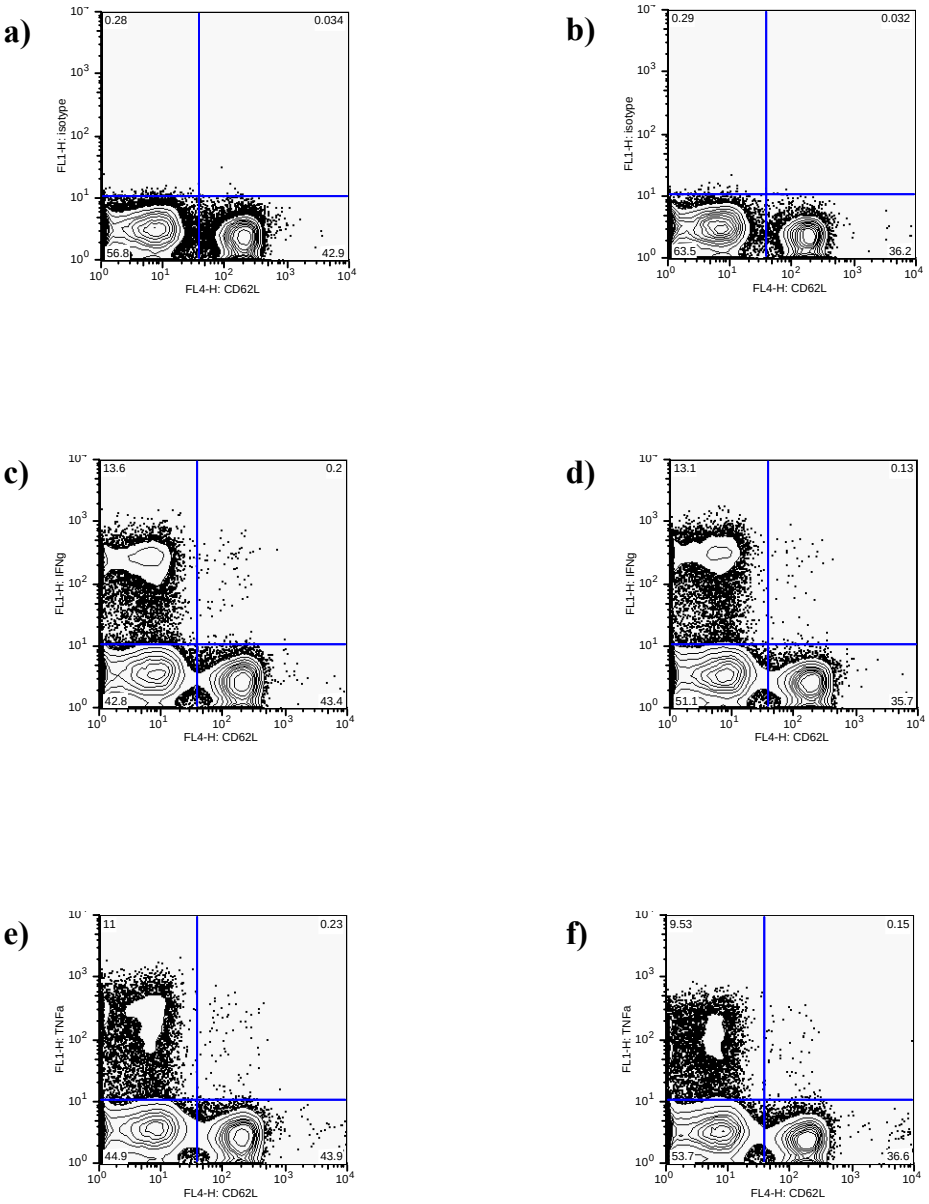


Fig. 3.33: Representative FACS Blots for Analysis of $LLO_{188-201}$-specific $CD4^+$ T cells for CD62L Expression and Intracellular IFNγ and TNFα Expression after Systemic Recall Infection with *L. monocytogenes* and Re-stimulation *in vitro* with $LLO_{188-201}$ Peptide. $CCR4^{-/-}$ mice and C57Bl/6 control mice were primary infected with L.m.ova bacteria and re-infected five weeks later. ICS analysis was carried out 5 days after infection and 5 h of re-stimulation with $LLO_{188-201}$ peptide *in vitro*. Fig 3.35a) C57Bl/6 wt mouse, isotype control; Fig 3.35b) $CCR4^{-/-}$ mouse, isotype control; Fig 3.35c) C57Bl/6 wt mouse, IFNγ; Fig 3.35d) CCR4 -/- mouse, IFNγ; Fig 3.35e) C57Bl/6 wt mouse, TNFα; Fig 3.35f) CCR4 -/- mouse, TNFα.

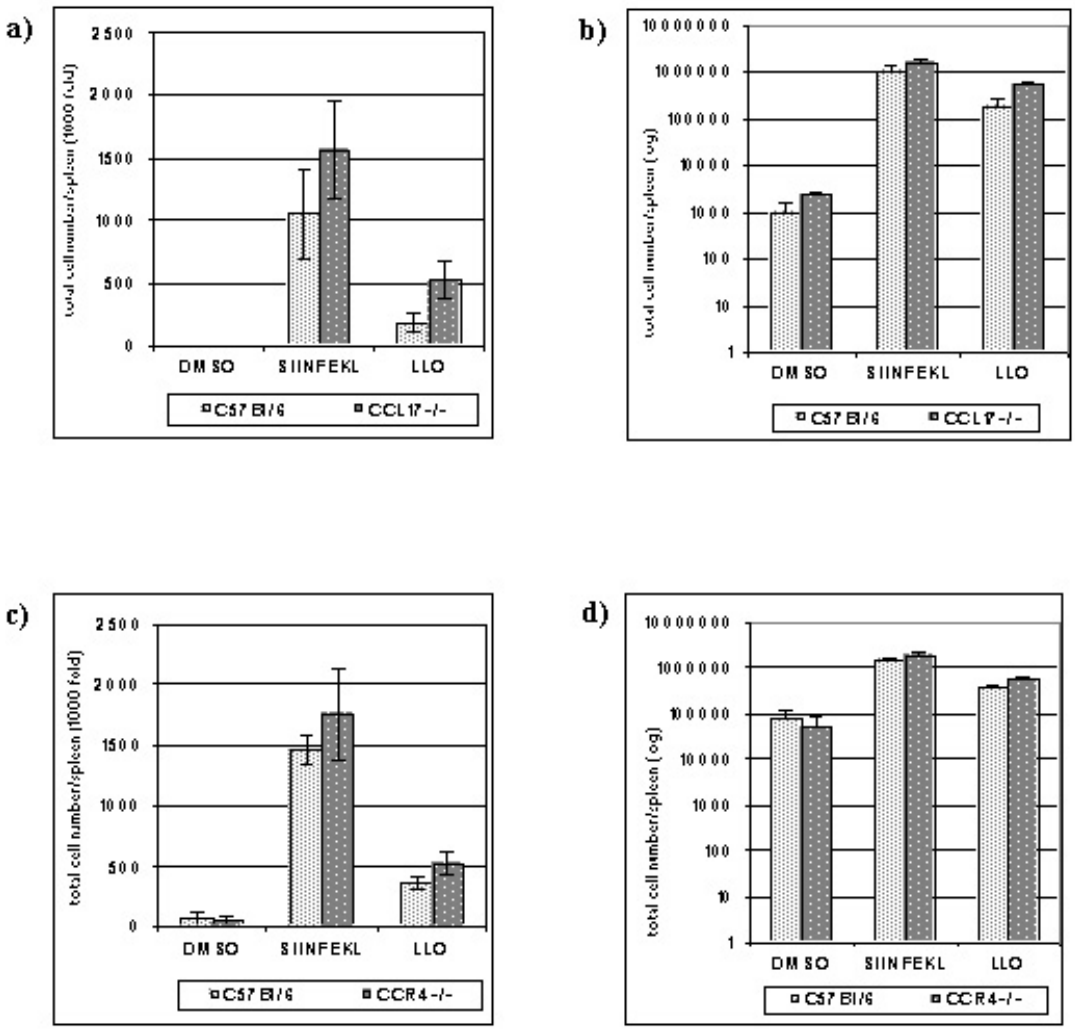


Fig. 3.34: Total Number of $CD8^+$ $CD62L^-$ $IFN\gamma^+$ and $CD4^+$ $CD62L^-$ $IFN\gamma^+$ T cells in the Spleen after Systemic Primary Infection of $CCL17^{-/-}$ Mice, $CCR4^{-/-}$ Mice and C57Bl/6 Control Mice with Ova-Expressing *Listeria Monocytogenes* and Re-stimulation *in vitro* with SIINFEKL or $LLO_{188-201}$ Peptide, Respectively. Fig.3.36a) and b): Analysis of $CCL17^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (a) and logarithmic (b) scale. Fig.3.36c) and d): Analysis of $CCR4^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (c) and logarithmic (d) scale.

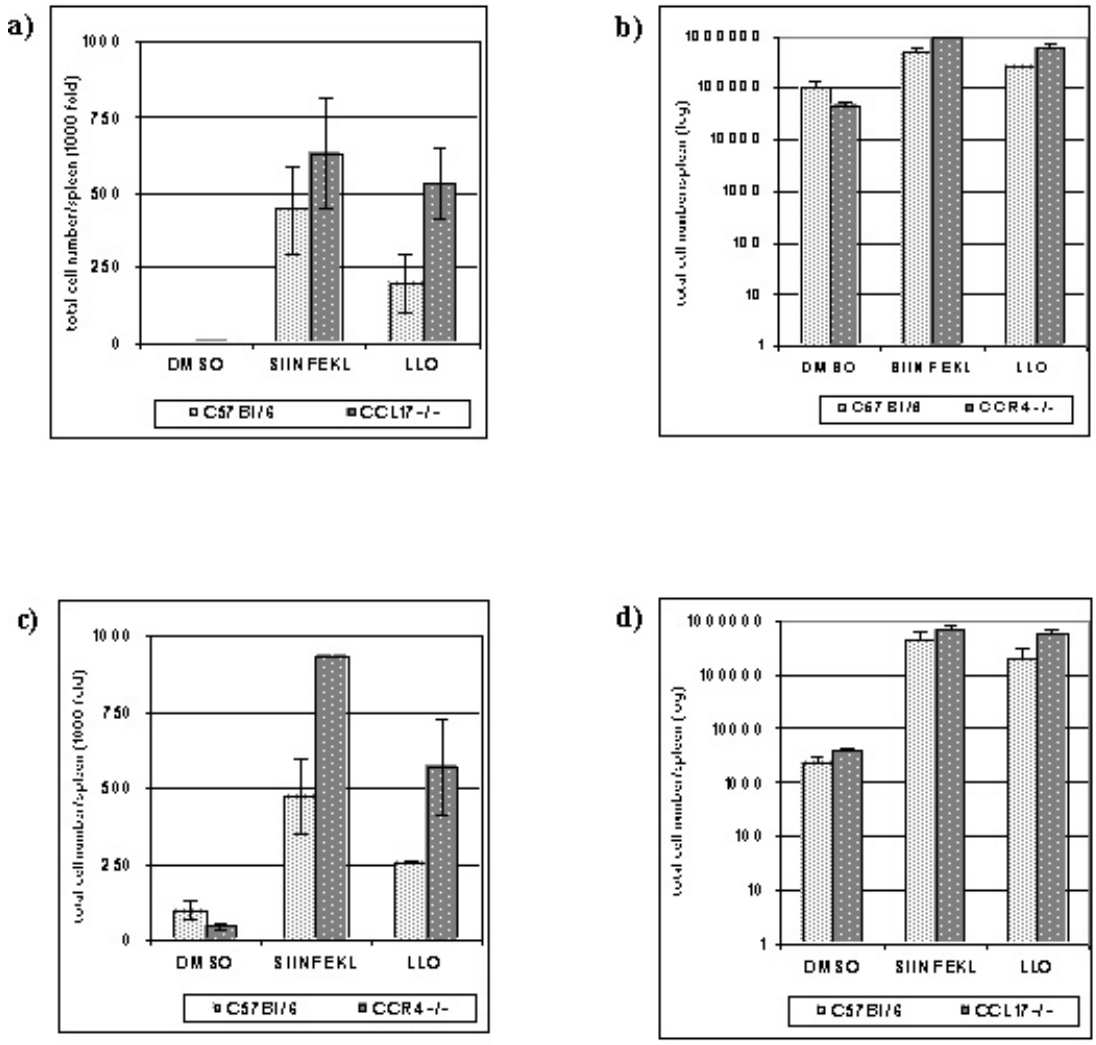


Fig. 3.35: Total Number of $CD8^+$ $CD62L^-$ $TNF\alpha^+$ and $CD4^+$ $CD62L^-$ $TNF\alpha^+$ T cells in the Spleen after Systemic Primary Infection of $CCL17^{-/-}$ Mice, $CCR4^{-/-}$ Mice and C57Bl/6 Control Mice with Ova-Expressing *Listeria Monocytogenes* and Re-stimulation *in vitro* with SIINFEKL or $LLO_{188-201}$ Peptide, Respectively. Fig.3.37a) and b): Analysis of $CCL17^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (a) and logarithmic (b) scale. Fig.3.37c) and d): Analysis of $CCR4^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (c) and logarithmic (d) scale.

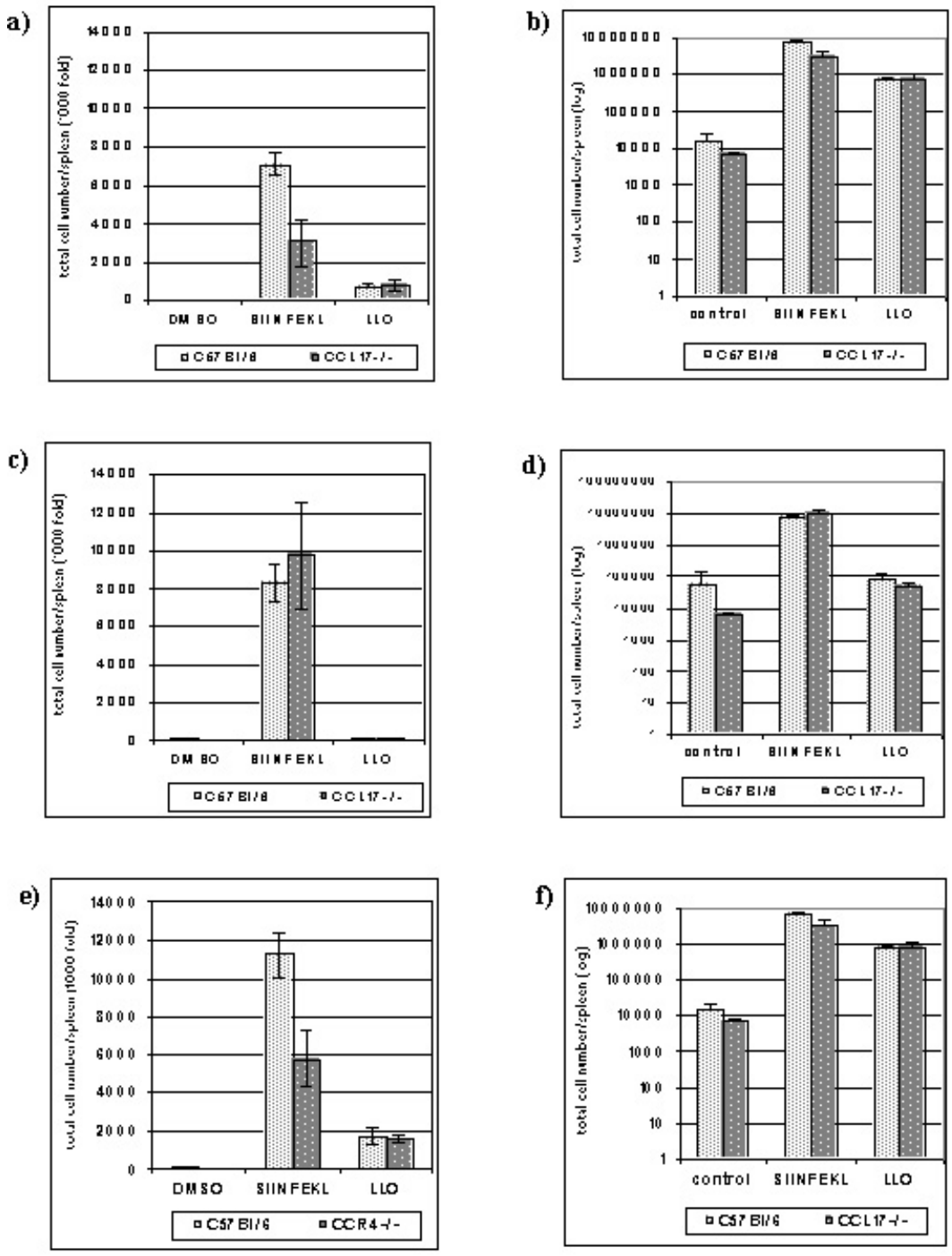


Fig. 3.36: Total Number of $CD8^+$ $CD62L^-$ $IFN\gamma^+$ and $CD4^+$ $CD62L^-$ $IFN\gamma^+$ T cells in the Spleen after Systemic Recall Infection of $CCL17^{-/-}$ Mice, $CCR4^{-/-}$ Mice and C57Bl/6 Control Mice with Ova-Expressing *Listeria Monocytogenes* and Re-stimulation *in vitro* with SIINFEKL or $LLO_{188-201}$ Peptide, Respectively. Fig.3.38a-d): Analysis of $CCL17^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (a, c) and logarithmic (b, d) scale. Fig.3.38e) and f): Analysis of $CCR4^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (e) and logarithmic (f) scale.

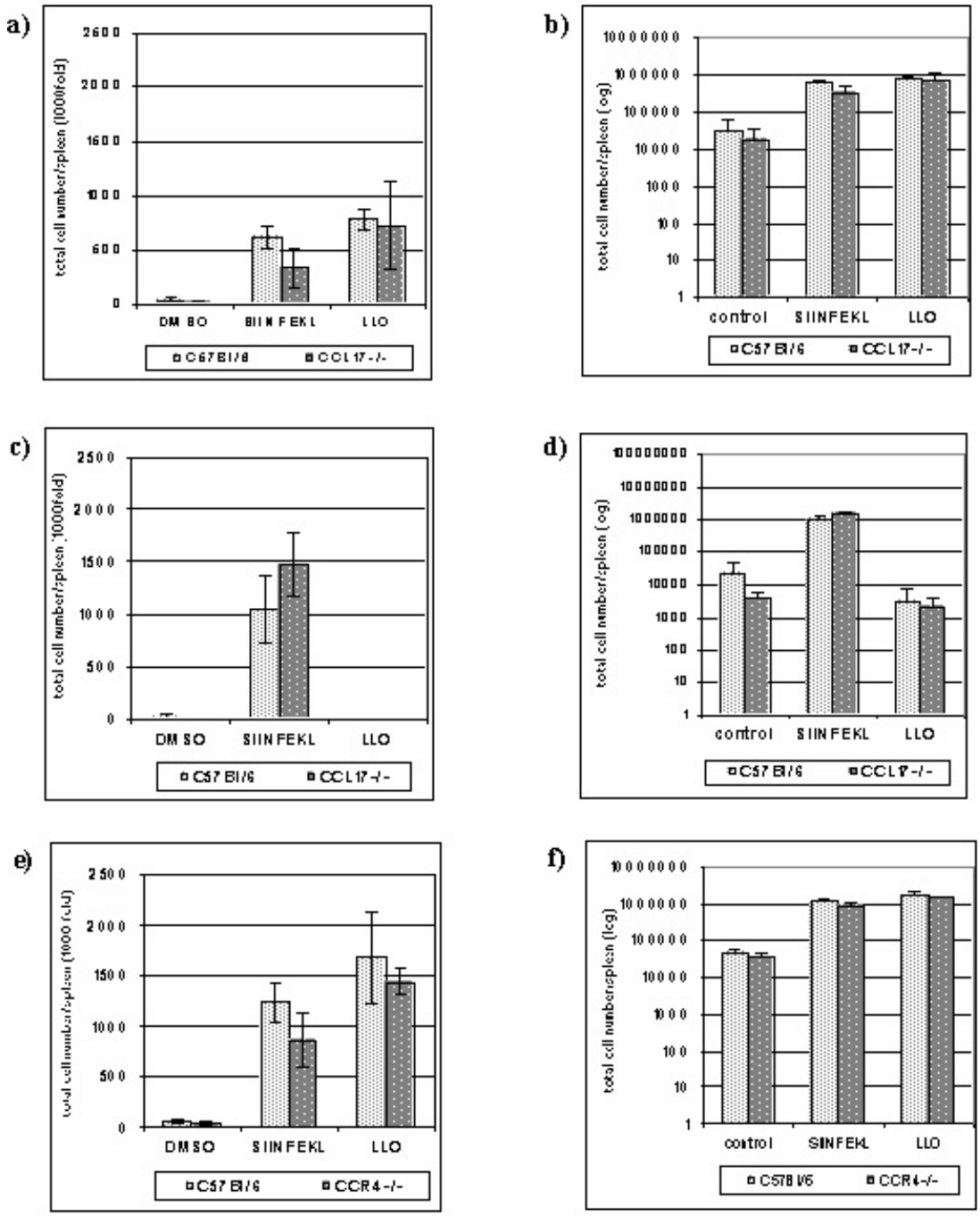


Fig. 3.37: Total Number of $CD8^+$ $CD62L^-$ $TNF\alpha^+$ and $CD4^+$ $CD62L^-$ $TNF\alpha^+$ T cells in the Spleen after Systemic Recall Infection of $CCL17^{-/-}$ mice, $CCR4^{-/-}$ mice and C57Bl/6 control mice with Ova-Expressing *Listeria Monocytogenes* and Re-stimulation *in vitro* with SIINFEKL or $LLO_{188-201}$ Peptide, Respectively. Fig.3.39a)-d): Analysis of $CCL17^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (a, c) and logarithmic (b, d) scale. Fig.3.39e) and f): Analysis of $CCR4^{-/-}$ mice and C57Bl/6 control mice. Results are shown in linear (e) and logarithmic (f) scale.

Tetramer staining results demonstrate that primary infection with L.m.ova induces a marginally higher number of SIINFEKL-specific $CD8^+$ T cells in $CCL17^{-/-}$ mice than in wt mice and equal numbers of SIINFEKL-specific $CD8^+$ T cells in $CCR4^{-/-}$ mice and wt mice. Intracellular cytokine staining shows that, after primary infection, $CCL17^{-/-}$ mice and $CCR4^{-/-}$ mice exhibit slightly higher numbers of IFNy and TNFα producing $CD8^+$ T cells after re-stimulation with SIINFEKL than wt mice. $CCL17^{-/-}$ mice also induce slightly higher numbers of IFNy and TNFα producing $CD4^+$ T cells after re-stimulation with LLO compared to wt mice while $CCR4^{-/-}$ mice exhibit comparable amounts of IFNy producing $CD4^+$ T cells but marginally higher amounts of TNFα producing $CD4^+$ T cells. As none of the differences between knock out mice and wild type mice is significant, CCL17- and CCR4-deficiency do not have an influence on the induction of L.m.ova specific $CD8^+$ T cells after primary infection or the number of IFNy and TNFα producing listeria specific $CD8^+$ and $CD4^+$ T cells.

Examination of the acquired data after recall infection reveals a contradictory picture. The results of the first experiment in the $CCL17^{-/-}$ mice, which is shown in Fig 3.29 a) and b), demonstrate that $CCL17^{-/-}$ mice induce far less SIINFEKL-specific $CD8^+$ T cells compared to wt mice. In the second experiment, which is displayed in Fig 3.29 c) and d), $CCL17^{-/-}$ mice induce comparable numbers of SIINFEKL-specific $CD8^+$ T cells compared to wt controls. $CCR4^{-/-}$ mice, on the other hand, induced marginally less SIINFEKL-specific $CD8^+$ T cells in this experimental setting as shown in Fig 3.29 e) and f). Analysis of the first experiment in the $CCL17^{-/-}$ mice, which is displayed in Fig 3.36 a) and b) further demonstrates that $CCL17^{-/-}$ mice show fewer numbers of IFNy and TNFα producing $CD8^+$ T cells after re-stimulation with SIINFEKL and comparable numbers of IFNy and TNFα producing $CD4^+$ T cells after re-stimulation with LLO than wt mice. In the second experiment, which is shown in Fig 3.36 c) and d), $CCL17^{-/-}$ mice induced marginally higher numbers of IFNy and TNFα producing $CD8^+$ T cells after re-stimulation with SIINFEKL than wild type controls while both groups of mice induce almost no IFNy and TNFα producing $CD4^+$ T cells after re-stimulation with LLO. $CCR4^{-/-}$ mice show fewer numbers of IFNy and TNFα producing $CD8^+$ T cells after re-stimulation with SIINFEKL than wt mice and comparable numbers of IFNy and TNFα producing $CD4^+$ T cells after re-stimulation with LLO as displayed in Fig 3.36 e) and f).

Taken together, CCL17- and CCR4-deficiency do not play a significant role in the induction of specific T cell responses against *Listeria Monocytogenes* after systemic primary infection. It has also been shown that CCL17- and CCR4-deficiency do not have a reproducible influence on specific T cell responses against *Listeria Monocytogenes*.

4 DISCUSSION AND OUTLOOK

The focus of this PhD work was the analysis of the functional relevance of the chemokines CCL17 and CCL22 for dendritic cell biology. It has been proven that CCL17 and CCL22 both bind to the receptor CCR4 (Imai et al., 1997; Imai et al., 1998) but there is also evidence that CCL17 binds to the receptor CCR8, which has been verified to bind to CCL1 (Bernardini et al., 1998; Ross et al., 1997). Additionally, CCL22 has been proposed to bind to a second receptor which has yet to be identified (Struyf et al., 1998; Mantovani et al., 2000). Analysis of the $CCR4^{-/-}$ mice indicates that CCL3, which is the known ligand of the receptor CCR3, might also bind to CCR4. An overview of the confirmed and proposed receptor-ligand pairs is displayed in Fig. 4.1.

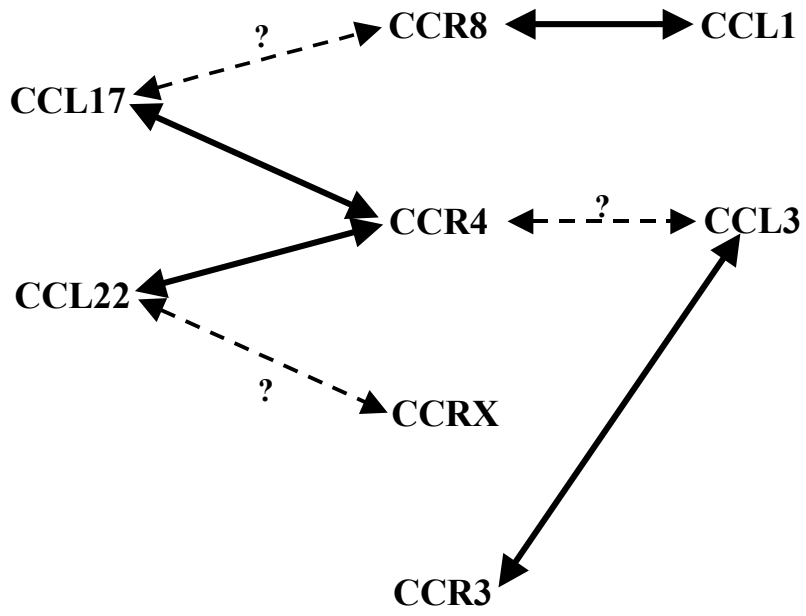


Fig 4.1: An overview of the confirmed and proposed receptors for the CC chemokines CCL17, CCL22, CCL1 and CCL3

CCL17 and CCL22 are expressed on a basal level by immature DCs and expression is strongly up-regulated during maturation so that DCs are the main producers of these chemokines (Lieberam and Forster, 1999; Alferink et al., 2003; Ross et al., 1999; Schaniel et al., 1998). It has been demonstrated that CCL17 and CCL22 act on and therefore induce chemotaxis of activated T cells, preferably of the T_H2 and T_C2 subtype (Sallusto et al., 1998 b, c; Bonecchi et al., 1998) but also monocytes, macrophages and NK cells (Schuh et al., 2002; Jakubzick et al., 2004; Inngjerdingen et al., 2000) via interaction with the receptor CCR4. As a consequence, CCL17 and CCL22 contribute to the specific guidance of their target cells of the innate and adaptive immune system to their designated tissues and organs where they can interact with other cells and execute their effector functions.

In order to further clarify the biological function of CCL17 and CCL22 *in vivo*, three different approaches were chosen for the investigation. In my first approach, I aimed at the generation of CCL17/CCL22 double knock out mice to be able to study the effect of the combined deficiency of these chemokines *in vivo*. In my second approach I analysed the contribution of CCL17 and CCL22 to Langerhans cell migration from the skin to the draining lymph nodes *in vivo*. Finally, in my third approach, I investigated the role of CCL17 and CCL22 during systemic infection with *Listeria monocytogenes* by examining the survival, the bacterial clearance and specific T cell responses *in vivo*. The results of my work are discussed in the following sections.

4.1 Part I: Generation of a CCL17/CCL22-Double-Knock Out Mouse

Knock out mice, which are mutant animals with a specific gene disruption, are a valuable system to study the function of specific genes *in vivo* and are today widely and successfully used. The $CCL17^{-/-}$ mice, which had been generated in our lab (see section 1.9 and Alferink et al., 2003) were readily available to study the influence of CCL17-deficiency *in vivo*. CCL22, which has been shown to also bind to CCR4, is still present in the $CCL17^{-/-}$ mice, so that is possible that CCL22 compensates for the lack of CCL17.

The CCR4$^{-/-}$ mice which had been generated by Y. Chvatchko (see Chvatchko et al., 2000) were also available in our institute, to indirectly study the biological role of CCL17 and CCL22 in vivo as the knock out mice are unable to respond to these chemokines via CCR4. But as it has been proposed that CCL17 may also bind to CCR8 and that CCL22 might also ligate with another, yet unknown, chemokine receptor it is possible that effects which are mediated by the ligation of CCL17 and CCL22 to these receptors are still functional in CCR4$^{-/-}$ mice. Hence, it was not possible to investigate the whole range of immunological effects which might be caused by the combined deficiency of CCL17 and CCL22 in the knock out mice which were available at the time. As a consequence, it was decided to generate a CCL17/CCL22-double-knock-out mouse.

As the genomic loci of CCL17 and CCL22 are both located on murine chromosome 8 in close vicinity, the likelihood of a crossing over was extremely small so that it was impossible to gain a double knock out by crossing the already available CCL17$^{-/-}$ mouse with a newly generated CCL22-/- mouse. But since the 50A8 ES cell line, which has a targeted disruption of the CCL17 gene (see section 1.9) and which had been used to generate the knock out mice, was already available it was decided to re-target these ES cells with a suitable replacement vector for CCL22.

Successful targeting of the CCL22 locus would result in CCL17/CCL22-deficient ES cells, if the integration of the CCL22 vector construct had taken place on the same chromosome. The obtained ES cell line could be used to generate a homozygous mutant mouse strain which allows analysis of the combined deficiency of CCL17 and CCL22 *in vivo*.

During the course of this project, I designed and generated two CCL22 gene replacement vectors, which differed in their SAH. Both vectors were used to re-target the CCL17-deficient 50A8 ES cell line. In total, I screened 1728 selected ES cell clones for homologuous recombination but no positive clone could be identified.

The reason for the failure to generate a CCL17/CCL22 double knock out mouse is not known. One plausible explanation is that the inability to identify a positive clone is due to screening problems. This reason can be excluded, though, as the majority of the clones was screened by a well established and reliable southern blot and not by PCR, which is a more error-prone method. The second possibility is, that there might be a problem with the targeting construct itself. Two CCL22 replacement vectors had been used to generate the double knock out mice which only differed in their SAH sequence but not in their LAH sequence. As a consequence, it might be possible that the chosen LAH sequence is not suitable for the task. Unfortunately, the CCL22 locus only has a length of approximately 6 kb which makes it difficult to design an alternative replacement vector with a different LAH sequence which still leads to the deletion of vital parts of the protein after homologue recombination. A third possibility is the fact that secondary structures of the target locus might prevent homologuous recombination. This assumption is contradicted by the fact that a CCL22 knock out mouse has been successfully generated in the group of T. Rolink (T. Rolink, personal communications). Although, to my knowledge, this mouse line has been generated several years ago, it has not been published so far. The efficiency of recombination and the phenotype of these mice is unknown.

4.2 Influence of CCL17/CCL22-CCR4 and CCL1-CCR8 Chemokine Receptor-Ligand Pairs on Langerhans Cells

Langerhans cells (LC) are the dendritic cell population of the epidermis. They form an organised network in the epidermal layer and serve as sentinels of the skin. They surveille the local microenvironment and assimilate foreign antigens. After antigen uptake, LCs migrate to the draining lymph nodes and present antigens to specific T lymphocytes leading to T cell activation. Thus, LCs are essential for the induction of cutaneous immune responses.

Immature LCs express the chemokine receptors CCR1, CCR5 and CXCR2 and therefore respond to the chemokines CCL3, CCL5 and CXCL8 (Vecchi et al., 1999; Sozzani et al., 1999) which are produced in the epidermis and thus retain immature LCs in the skin. After antigen uptake the LCs migrate from the skin to the draining lymph nodes and gradually mature which results in the loss of surface expression of CCR1, CCR5 and CXCR2. The maturing LCs up-regulate the surface expression of CCR7 leading to responsiveness to CCL19 and CCL21 which is essential for the directed migration from peripheral tissues to the draining lymph nodes (Sallusto et al., 1998; Sallusto et al., 1999). So far, it has not been determined whether additional receptor-ligand-pairs contribute to the migration of LCs.

Migration from the skin to the draining lymph nodes is essential for LCs to be able to excecute their function as APC and to initiate cutaneous immune responses.
It has been previously demonstrated that the cytokines IL-1ß, IL-18 and TNFα are necessary to induce mobilisation and migration of LCs (Cumberbatch et al., 1997; Cumberbatch et al., 2001) which, on the other hand, is inhibited by IL-10, TGF-ß1 and lactoferrin to maintain tissue homeostasis (Wang et al., 1999; Riedl et al., 2000; Cumberbatch et al., 2000).

The chemokine receptors CCR4 and CCR8 are both expressed on LC's (Qu et al., 2004; G. Randolph, unpublished data). It has been shown that these receptors are frequently co-expressed and there is evidence that CCR4 and CCR8 might function in a cooperative way. Additionally, it has been demonstrated that CCL1 can have a synergistic effect on the migratory response to CCR4 ligands (Iellem et al., 2001; Inngjerdingen et al., 2000). Langerhans cells also express the chemokines CCL1, CCL17 and CCL22 which are the ligands for the receptors CCR4 and CCR8(Katou et al., 2001; Tang and Cyster, 1999; Ancuta et al., 2000; Alferink et al., 2003). This expression might lead to an autrocrine stimulation that might help to induce Langerhans cell migration. For that reason, G. Randolph and her group (The Mt. Sinai School of Medicine, New York; unpublished data) decided to investigate the question whether CCL17, CCL22 and CCL1 indeed play a role in Langerhans cell migration *in vivo* by performing skin sensitisation experiments (FITC painting).

The group of G. Randolph were able to show, that treatment of wt mice with neutralising anti-CCL1 antibodies had no effect on LC migration *in vivo* while treatment of mice with a combination of anti-CCL17 and anti-CCL22 antibody had a minor inhibitory effect. Combined treatment of wt mice with anti-CCL17 and anti-CCL1 resulted in a strong reduction of Langerhans cell migration which was further enhanced by combined treatment with neutralising anti-CCL1, anti-CCL17 and anti-CCL22 antibodies. Additionally, they found that CCR8-/- mice showed a strong reduction of LC migration which was comparable to the reduction in migration after the combined treatment with anti-CCL17 and anti-CCL1.

Since LC migration had not yet been studied in the $CCR4^{-/-}$ mouse I decided to further analyse this question using skin sensitisation experiments (FITC painting). To investigate the role of CCL1, I additionally studied LC migration in $CCR4^{-/-}$ which had been treated with neutralising anti-CCL1 antibody. First of all, I was able to show that the migration of skin resident Langerhans cells from the skin to the draining lymph nodes was only marginally decreased in $CCR4^{-/-}$ mice. Thus, the CCL17/CCL22-CCR4 receptor ligand pairs have a negligible effect on Langerhans cell migration. Second, I demonstrated that migration of skin resident Langerhans cells to the cutaneous lymph nodes was marginally increased in $CCR4^{-/-}$ mice which had been treated with neutralising anti-CCL1 antibody compared to isotype treated C57Bl/6 control mice. Taken together, the evaluation of the experimental data indicates that the combined influence of the CCL17/CCL22-CCR4 receptor ligand pairs and the CCL1-CCR8 receptor ligand pair on Langerhans cell migration is negligible.

The data which were obtained by G. Randolph and her group indicated that CCL17 and CCL22 facilitate migration of Langerhans cells from the skin to the draining lymph nodes through the activation of CCR4. In contrast to these finding, my data show no difference in LC migration in $CCR4^{-/-}$ mice which contradicts the theory that CCR4 can induce migration. I also observed that neutralisation of CCL1 in the $CCR4^{-/-}$ mice does not result in a reduction of Langerhans cell migration. These findings are in conflict with the data that were obtained by G. Randolph as she showed the strongest reduction of LC migration after *in vivo* neutralisation of CCL1, CCL17 and CCL22. My data therefore contradict the hypothesis that CCR4 and CCR8 induce LC migration in a cooperative way.

Taken together, I was able to show that Langerhans cell migration is independent of CCL17- and CCL22-signalling via CCR4. I further demonstrated that neutralisation of CCL1 in $CCR4^{-/-}$ mice in vivo does not influence LC migration. These findings are in contrast with the proposal that CCL1-signalling via CCR8 is important to induce LC migration although it has to be kept in mind that there is evidence that CCL17 might also bind to CCR8 and might therefore compensate the lack of CCL1.

4.3 Role of CCL17/CCL22-CCR4 Chemokine Receptor-Ligand Pair During Systemic Infection with *Listeria Monocytogenes*

Listeria Monocytogenes (LM) is a gram-positive intracellular pathogenic bacterium which infects a wide range of hosts including humans, mice and other mammals (Murray et al., 1926; Pirie et al., 1927; Pamer, 2004). In immunology, LM infections are widely used in mouse models as listeria are well characterised and can be perfectly used to investigate the function of the mammalian immune system *in vivo*.

Innate immune responses are essential to the survival of the host during infection with *Listeria monocytogenes* (Unanue, 1997). It has been demonstrated that CCR4 knock out mice showed resistance to LPS-induced endotoxin shock in high and low dose models which might be due to a significant decrease in the $F4/80^{+}$ population in the knock out mice (Chvatchko et al., 2000). This finding leads to the assumption that CCR4 knock out mice might have a defect in innate responses. It has been further demonstrated that neutrophils, monocytes and macrophages are important for the bacterial clearance of listeria (Rogers and Unanue, 1993; North, 1970) while IFNy and TNF, which are produced by NK cells and macrophages, are vital for the primary defense against LM (Tripp et al., 1993). CCR4, the corresponding receptor for CCL17 and CCL22, is expressed on macrophages, monocytes and NK cells. Hence, it is possible that lack of CCR4-signaling weakens innate immune response against listeria which might result in a lack of survival in CCL17- and CCR4-deficient mice after systemic infection.

It had been initially observed by J. Alferink in our lab (unpublished data, see section 1.9) that CCL17$^{-/-}$ mice in C57Bl/6 background showed no difference in survival after infection with high and medium doses (10×LD_{50} and 10×LD_{50}) of *L. monocytogenes* compared to littermate controls. On the other hand, CCL17$^{-/-}$ mice showed better survival after infection with low doses (0.1 ×LD_{50}) of *L. monocytogenes* than littermate controls. These data encouraged me to further investigate the influence of CCL17-deficiency on survival after systemic listeria infection in C57Bl/6 and Balb/c genetic background.

In contrast to the previous data, my own findings showed that there was no difference in survival between CCL17$^{-/-}$ mice and control mice in C57Bl/6 and Balb/c background after systemic infection with high, medium and low doses of *L. monocytogenes*.

As CCL22, the second ligand for the receptor CCR4, is still expressed in CCL17 knock out mice, it is possible that CCL22 compensates for the lack of CCL17 during Listeria infection. Therefore, it was necessary to investigate whether the survival of mice was influenced by the lack of CCR4 expression. As CCR4$^{-/-}$ mice are not available in Balb/c background it was only possible to evaluate the effect of CCR4-deficiency in C57Bl/6 background. My results showed that there was no difference between CCR4$^{-/-}$ mice and C57Bl/6 control mice in the medium and low dose groups, but survival of CCR4$^{-/-}$ mice in the high dose group was marginally prolonged.

In summary, the experimental data demonstrate that CCR4 signalling is not important for the survival of mice after systemic infection with high, medium and low doses of *L. monocytogenes*.

Although CCR4-signaling has no effect on survival of mice after listeria infection, it could not be excluded that CCL17- and CCR4-deficiency have other negative influences on innate immune responses which might effect the bacterial clearance in spleen and liver after systemic infection with *L. monocytogenes*. In order to investigate this question, the bacterial organ load of spleen and liver was determined after primary and recall infection of CCL17$^{-/-}$ mice and CCR4$^{-/-}$ mice with *Listeria monocytogenes* compared to wild type controls.

Primary infection with a high dose (20 000 CFU) of the ova-expressing listeria strain L.m.ova showed, that the bacterial clearance in CCL17$^{-/-}$ mice is slightly more efficient than in wild type mice. Additionally, CCL17$^{-/-}$ mice were infected with a low dose (2000 CFU) of listeria and analysis of the data obtained confirmed the results of the high dose experiment although the result was difficult to quantify due to the low numbers of CFU detected. Investigation of the impact of CCL17-deficiency on the bacterial clearance during recall infection showed that the bacterial clearance is slightly less efficient in CCL17$^{-/-}$ mice than in control mice which is in contrast to primary infection. Analysis of the effect of CCR4-deficiency on the bacterial clearance after primary infection with a low dose of L.m.ova and after recall infection revealed that the bacterial clearance is marginally less efficient in the CCR4$^{-/-}$ mice than in wild type mice although the results were again difficult to quantify because of the low numbers of CFU detected.

Taken together, the experiments conducted demonstrated that CCL17- and CCR4-deficiency only have a negligible influence on innate immune responses against systemic infection with *L. monocytogenes*.

Neutrophils, monocytes and macrophages are essential for the bacterial killing while NK cell-derived IFNy and TNF is important for the primary defence against listeria. Chemokines are responsible for the guidance of immune cells to the site of infection and as CCR4 is expressed on macrophages, monocytes and NK cells we hypothesised that CCL17- and CCR4-deficiency might result in reduced recruitment of these cell types to the site of infection. This, in turn, could lead to reduced survival and bacterial clearance in the knock out mice. However, the experimental data I obtained during this work clearly showed that survival and bacterial clearance is independent of CCR4 signalling. Hence, it is likely that other chemokine receptor-ligand pairs are more important for the recruitment of monocytes, macrophages and NK cells than CCL17/CCL22-CCR4. One good candidate is CCR2 which is expressed by monocytes, macrophages and, in inflammatory conditions, by NK cells (Maghazachi, 2003; Charo and Peters, 2003; Linton and Fazio; 2003) so that it might be worthwhile to study listeria infection in the CCR2 knock out mice.

Neutrophils are attracted by members of the CXC chemokine family, which contain the characteristic ERL motif (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8) All these chemokines have been shown to exclusively bind to the chemokine receptor CXCR2 (Baggiolini et al, 1997; Gale and McColl, 1999; McColl, 2003). Consequently, CXCR2 and its ligands might be important for the recruitment of neutrophils and therefore for bacterial clearance and survival of the host during systemic listeria infection. Hence, CXCR2 also is a good candidate to further study recruitment of important cells of innate immunity during infection with *Listeria monocytogenes*.

The adaptive immune system is also important for the control of infection and elimination of the bacteria from the body. (δ T cells help to control the inflammatory response after infection while humoral immunity provides a minor contribution to protective immunity (Edelson and Unanue, 2001; Egan and Carding, 2000). But the most important players of adaptive immunity against *L. monocytogenes* are the αβ T cells which have been shown to be critically involved in bacterial clearance and long-term immunity (Pamer, 2004).

Analysis of the αβ T cell-mediated immune responses revealed that $CD8^+$ T cells are more important for adaptive immune responses against listeria than the $CD4^+$ T cells (Ladel et al., 1994). Priming of $CD8^+$ T cells is mediated by DCs which is probably mediated by cross-presentation of antigens that are derived from infected macrophages as DCs themselves are not a significant *in vivo* reservoir of listeria (Lenz et al., 2000; Jung et al., 2002). Infection with L. monocytogenes leads to the induction of memory T cells which are able to mediate protective immunity. It has been demonstrated that $CD4^+$ T cells are important for the maintenance of memory $CD8^+$ T cells since mice which lack $CD4^+$ T cells show normal primary $CD8^+$ T cell responses but impaired long term preservation of memory $CD8^+$ T cells so that protective immunity against re-infection is gradually lost (Shedlock et al., 2003; Kusar et al., 2002; Sun et al., 2003). The exact mechanisms that are used by $CD4^+$ T cells to maintain memory $CD8^+$ T cells is not yet known.

As discussed above, CCL17- and CCR4-deficiency have only a minor influence on innate immune responses after systemic primary infection with listeria. Because this chemokine receptor-ligand pair plays an important role in chemotaxis of T cells, I also investigated if CCL17- and CCR4-deficiency have an influence on specific T cell responses against *Listeria Monocytogenes* after systemic primary and recall infection.

Evaluation of the $CD8^+$ T cell response during systemic primary and recall infection with L.m.ova reveals that neither CCL17-deficiency nor CCR4-deficiency influence the induction of SIINFEKL-specific $CD8^+$ T cells or the number IFNy and TNFα producing $CD8^+$ T cells after re-stimulation with SIINFEKL. Analysis of the $CD4^+$ T cell response during primary and recall infection demonstrates that CCL17-deficiency and CCR4-deficiency do not affect the induction of IFNy and TNFα producing $CD4^+$ T cells after re-stimulation with LLO. Taken together, CCR4 signalling is not necessary for the induction of specific T cell responses against *Listeria Monocytogenes* after systemic primary and recall infection.

I was not able to show an influence of CCL17- and CCR4-deficiency on Langerhans cell migration or innate and adaptive immune responses against *Listeria monocytogenes* during systemic infection. However, it has been demonstrated that disrupted CCR4 signalling does have an influence on immune responses. CCR4 knock out mice show resistance to LPS-induced endotoxin shock in high and low dose models (Chvatchko et al., 2000). They also exhibited aggressive anti-fungal responses which are characterised by enhanced neutrophil function and increased macrophage recruitment into the airways. However, the recruited neutrophils and macrophages are unable to kill the fungus, which leads to severe invasive lung disease and mortality in CCR4 knock out mice (Schuh et al., 2002). Analysis of the CCL17 knock out mice demonstrated that they show diminished T-cell-dependent contact hypersensitivity responses and deficiency in rejection of allogenic organ transplants (Alferink et al., 2003). CCL17 knock out mice also show resistance against nematode infection (A. Hörauf, personal communication). These findings contradict the theory that CCL17 and CCL22 are entirely redundant. Hence, the CCR4 knock out mice and the CCL17 knock out mice will remain an interesting topic of research in the future.

5 SUMMARY

During this PhD work I focused on the analysis of the functional relevance of the chemokines CCL17 and CCL22 for dendritic cell biology. First, I investigated the influence of the CCL17/CCL22-CCR4 chemokine receptor-ligand pairs on Langerhans cell migration *in vivo*. Additionally, I evaluated the role of the CCL1-CCR8 receptor ligand pair in this question as CCL17 is proposed to also bind to CCR8. I was able to show that Langerhans cell migration is totally independent of CCR4 signalling. Evaluation of the data which were obtained during this PhD work and by G. Randolph (unpublished data) strongly support that CCR8 signalling through CCL1 and CCL17 contributes to the induction of LC migration in vivo and that CCR4-independent signalling via CCL22 further induce migration. Hence, the experimental evidence further strengthens the proposal that CCL17 can bind to the chemokine receptor CCR8 as well as the assumption that CCL22 can ligate with an additional, yet unidentified, chemokine receptor. Second, I analysed the role of the chemokine receptor-ligand pair CCL17/CCL22-CCR4 in systemic infection with the intracellular pathogen *Listeria monocytogenes in vivo*. I could show that CCR4 signalling is not important for the survival of mice after systemic infection with high, medium and low doses of Listeria and that CCL17- and CCR4-deficiency only have a negligible influence on innate immune responses against systemic infection with *L. monocytogenes*. Analysis of T cell responses during systemic primary and recall infection revealed that CCR4 signalling is not necessary for the induction of specific $CD8^+$ and $CD4^+$ T cell responses against *Listeria Monocytogenes* after systemic primary and recall infection. In order to investigate whether CCL17 and CCL22 can additionally act on other chemokine receptors it was aspired to generate a CCL17/CCL22 double knock out mouse. For this purpose, a gene replacement vector was designed to inactivate CCL22 in CCL17-deficient embryonic stem cells.

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