The role of IL-21R signaling during adaptive immune responses

a dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZÜRICH
for the degree of
Doctor of Natural Sciences

presented by
ANJA STEFANIE FRÖHLICH
Dipl. Biol., FAU
born June 21, 1975
Citizen of Nürnberg (D)

accepted on the recommendation of
Prof. Dr. Manfred Kopf, examiner
Prof. Dr. Annette Oxenius, co-examiner

2008
# Index

## 1. ABSTRACT  

## 2. ZUSAMMENFASSUNG  

## 3. INTRODUCTION  

### 3.1. THE IMMUNE SYSTEM  

#### 3.1.1. THE INNATE IMMUNE SYSTEM  

#### 3.1.2. ADAPTIVE IMMUNE RESPONSES  

#### 3.1.2.1. Antigen presenting cells (APCs)  

#### 3.1.2.2. B lymphocytes  

#### 3.1.2.3. T lymphocytes  

#### 3.1.3. T CELL RESPONSES  

### 3.2. CYTOKINES AND CYTOKINE FAMILIES  

#### 3.2.1. THE COMMON γ-CHAIN RECEPTOR FAMILY  

#### 3.2.2. THE MEMBERS OF THE γ-CHAIN FAMILY OF CYTOKINES  

#### 3.2.2.1 IL-2 and IL-15  

#### 3.2.2.2 IL-4  

#### 3.2.2.3 IL-7  

#### 3.2.2.4 IL-9  

#### 3.2.3. IL-21 AND ITS RECEPTOR  

### 3.3. EXPERIMENTAL DISEASE MODELS  

#### 3.3.1. ALLERGIC AIRWAY INFLAMMATION (ASTHMA)  

#### 3.3.2. HELMINTH INFECTIONS  

#### 3.3.3. EXPERIMENTAL LEISHMANIASIS  

#### 3.3.4. VIRUS INFECTIONS  

### 3.4. AIM OF THIS THESIS  

## 4. RESULTS  

### 4.1. IL-21 RECEPTOR SIGNALING IS INTEGRAL FOR THE DEVELOPMENT OF TH2 EFFECTOR RESPONSES IN VIVO  

#### 4.1.1. ABSTRACT  

#### 4.1.2. INTRODUCTION  

#### 4.1.3. MATERIAL AND METHODS  

#### 4.1.4. RESULTS  

#### 4.1.5. DISCUSSION  

### 4.2. IL-21R SIGNAL IS ESSENTIAL FOR THE MAINTENANCE OF CD8 T CELL RESPONSES IN PERSISTENT LCMV DOCILE INFECTION  

#### 4.2.1. ABSTRACT  

---

**Page Counts:**

- ABSTRACT: 4
- ZUSAMMENFASSUNG: 6
- INTRODUCTION: 8
- THE IMMUNE SYSTEM: 8
- THE INNATE IMMUNE SYSTEM: 8
- ADAPTIVE IMMUNE RESPONSES: 10
- Antigen presenting cells (APCs): 10
- B lymphocytes: 11
- T lymphocytes: 12
- T CELL RESPONSES: 12
- CYTOKINES AND CYTOKINE FAMILIES: 19
- THE COMMON γ-CHAIN RECEPTOR FAMILY: 20
- THE MEMBERS OF THE γ-CHAIN FAMILY OF CYTOKINES: 22
- IL-2 and IL-15: 22
- IL-4: 23
- IL-7: 23
- IL-9: 24
- IL-21 AND ITS RECEPTOR: 24
- EXPERIMENTAL DISEASE MODELS: 29
- ALLERGIC AIRWAY INFLAMMATION (ASTHMA): 29
- HELMINTH INFECTIONS: 29
- EXPERIMENTAL LEISHMANIASIS: 30
- VIRUS INFECTIONS: 31
- AIM OF THIS THESIS: 35
- RESULTS: 36
- IL-21 RECEPTOR SIGNALING IS INTEGRAL FOR THE DEVELOPMENT OF TH2 EFFECTOR RESPONSES IN VIVO: 36
- ABSTRACT: 37
- INTRODUCTION: 37
- MATERIAL AND METHODS: 39
- RESULTS: 45
- DISCUSSION: 59
- IL-21R SIGNAL IS ESSENTIAL FOR THE MAINTENANCE OF CD8 T CELL RESPONSES IN PERSISTENT LCMV DOCILE INFECTION: 64
- ABSTRACT: 65
# Index

4.2.2. **INTRODUCTION** 66

4.2.3. **MATERIAL AND METHODS** 67

4.2.4. **RESULTS** 73

4.2.5. **DISCUSSION** 120

5. **APPENDIX** 127

5.1. **ABBREVIATIONS** 127

5.2. **ACKNOWLEDGEMENTS** 130

5.3. **REFERENCES** 133

**CURRICULUM VITAE** 157
1. Abstract

Cytokines are the key players in orchestrating innate and adaptive immune responses to pathogens. During cell-mediated immune responses, they direct naïve CD4 T cells to diverse subsets including Th1, Th2, Th17 and regulatory T cells. Cytokines belonging to the common γc receptor chain family such as Interleukin-2 (IL-2), IL-4, IL-7, IL-9 and IL-15 play crucial roles in activation, proliferation, differentiation and maintenance of T cells. IL-21 is the most recent described member of this family and is mainly produced by activated CD4 T cells. Its in vivo role during adaptive immune responses is still not well understood. The aim of this study was to address T cell responses to various pathogens, allergens and self-molecules (autoimmunity) using IL-21R-deficient mice. We found that IL-21R signaling was essential for the development of Th2-driven allergic airway inflammation as shown by impaired Th2 type effector responses such as pulmonary eosinophilia and airway hyperresponsiveness, as well as reduced frequency of Th2 cells in the lung of IL-21R-deficient mice. Similarly, Th2-mediated immunity to helminth infections was impaired indicated by reduced eosinophilia, basophilia and type-2 granuloma in the absence of IL-21R. Adoptive T cell transfer experiments revealed a central role of IL-21R signaling in supporting survival and/or migration of Th2 cells into peripheral tissues. In contrast, Th1 immune responses against *Leishmania major* infection and Th17-driven Experimental Autoimmune Myocarditis (EAM) were unimpaired in IL-21R-deficient mice. Furthermore, IL-21R was not required for efficient anti-viral CD4 and CD8 T cell responses to influenza virus, vaccinia virus (VV) and acute lymphocytic choriomeningitis virus strain WE (LCMV WE). In contrast, IL-21R-deficient mice were highly susceptible to persistent viral infection induced by LCMV strain Docile (LCMV DO) indicated by exacerbated CD8 T cell exhaustion and failure to control viral replication following the acute effector response. Analysis of mixed bone marrow chimeras (WT/KO → WT) revealed that IL-21R promoted CD8 T cells expansion and function in a cell autonomous manner. Our data suggest that impaired CD8 T cell function in IL-21R-deficient mice is not due to deregulation of PD-1 surface expression by virus-specific CD8 T cells or IL-10 production. Furthermore, regulatory T cells (Tregs) seem not be responsible for inhibition of CD8 T cell responses, although frequency of Treg is dramatically increased in IL-21R-deficient mice infected with LCMV DO.
Overall, our studies helped to understand the function of IL-21/IL-21R in T cell-mediated immune responses and related inflammatory diseases, which is of potential therapeutic value.
2. Zusammenfassung

Zytokine sind für die Koordinierung der angeborenen und adaptiven Immunantwort gegen Pathogene zuständig. Während zell-vermittelter Immunantworten dirigieren sie naive CD4 T Zellen in Richtung der verschiedenen T Helfer Antworten, zu denen Th1, Th2, Th17 oder regulatorische T Zellen gehören. Zytokine, die zur Familie gehören, die die γc Rezeptorkette gemeinsam haben wie Interleukin-2 (IL-2), IL-4, IL-7, IL-9 und IL-15, erfüllen wichtige Aufgaben während der Aktivierung, Proliferation und Differenzierung von T Zellen sowie in der Aufrechterhaltung der T Zell Antwort. Das zu letzter beschriebene Mitglied dieser Familie heißt IL-21 und wird von aktivierten CD4 T Zellen produziert. Seine Rolle während adaptiver Immunantworten in vivo ist immer noch nicht gut verstanden. Das Ziel dieser Studie war, T Zell Antworten auf verschiedene Pathogene, Allergene und körpereigene Moleküle (Autoimmunität) in IL-21 Rezeptor knockout (KO) Mäuse zu untersuchen. Wir fanden heraus, dass die Signalgebung des IL-21 Rezeptors für die Entstehung der Th2 Antwort nach Erzeugung von allergischer Atemwegsentzündung (Asthma) essenziell war. Die Th2 Effektorantwort in IL-21R KO Mäusen, welche pulmonale Eosinophilie, die Anzahl der Th2 Zellen in der Lunge sowie Überempfindlichkeit der Atemwege beinhaltete, war reduziert. Im gleichen Masse war die Th2 Immunantwort nach Infektion mit Helminthen in Abwesenheit des IL-21 Rezeptors vermindert, welches auf Grund von reduzierter Eosinophilie, Basophilie und Bildung von Typ-2 Granulomen festgestellt wurde. Adoptiver T Zell Transfer zeigte auf, dass die Signalgebung eine zentrale Rolle bei der Unterstützung des Fortbestandes oder der Migration von Th2 Zellen in peripheren Geweben spielt. Im Gegensatz dazu waren Th1 Antworten, die durch Infektion mit Leishmania major verursacht wurden, sowie die durch Th17 Zellen vermittelte autoimmune Myokarditis in den IL-21 Rezeptor KO Mäusen nicht beeinträchtigt.

Darüber hinaus waren IL-21 Rezeptor Signale für effiziente anti-virale CD4 und CD8 T Zell Antworten auf Influenza Virus, Vaccinia Virus sowie einer akuten Form des lymphocytären Choriomeningitis Virus (LCMV WE) nicht erforderlich. Im Unterschied dazu waren IL-21 Rezeptor KO Mäuse extrem anfällig für persistente Virusinfektionen, wie sie von dem LCMV Stamm Docile verursacht werden. Dies manifestierte sich durch übermäßige Beeinträchtigung der CD8 T Zell Antwort und dem Unvermögen die Virusreplikation über die akute Phase hinaus
zu kontrollieren. Analyse von gemischten Knochenmarkschimären (WT/KO → WT) zeigte dass der IL-21 Rezeptor in autonener Weise die Expansion und Funktion von CD8 T Zellen begünstigte. Unsere Daten deuten darauf hin, dass die beeinträchtigte Funktion der CD8 T Zellen von IL-21 Rezeptor KO Mäusen nicht auf die Deregulierung von PD-1 auf der Oberfläche von virus-spezifischen CD8 T Zellen oder auf IL-10 Produktion zurückzuführen ist. Darüber hinaus scheinen regulatorische T Zellen nicht für die Inhibierung der CD8 T Zell Antwort verantwortlich zu sein, obwohl deren Anzahl in IL-21 Rezeptor KO Mäusen, die mit LCMV Docile infiziert wurden, stark angestiegen war.
Zusammenfassend haben unsere Studien dazu beigetragen die Funktion von IL-21/IL-21 Rezeptor in T Zell vermittelten Immunantworten und den damit verbundenen Entzündungskrankheiten zu verstehen, was von potenziellem therapeutischen Wert ist.
3. Introduction

3.1. The immune system

Throughout life organisms are exposed to foreign invaders with the ability to cause disease and damage to the host. To deal with such pathogens, defense mechanisms are present in one form or another in all multicellular organisms. According to their long lifespan and habitant diversity a complex network of defense mechanisms has evolved in vertebrates, which is most efficient and elaborate in mammals. This immune system generally provides an effective defense against microbes and foreign substances thus maintaining the health of the host. Extensive research in the field of immunology has given insight into the complexity of this system but still, new factors and cell types belonging to this network are regularly discovered. One of the major goals of immunology is to develop strategies that lead to treatment of, and protection from, harmful diseases.

3.1.1. The innate immune system

The first defense against microbes is mechanical barriers such as epithelial cell layers including the skin, the respiratory and the intestinal tract. Intestine and respiratory epithelial cell layers are covered with goblet cell derived mucus, which can trap pathogens and consequently lead to their expulsion. In addition, enzymes and peptides in mucus and body fluids (e.g. tears, saliva) like lysozyme or lactoferrin provide antimicrobial protection. The low stomach pH together with enzymes like pepsin hinder microorganisms to reach the intestine, where their colonialization can be further impeded by bacteria inhabiting the intestinal tract.

Once a pathogen has overcome the first line of defense it gets recognized by humoral and cellular components of the innate immune system. Blood-soluble proteins of the complement
Introduction

system initiate a cascade that can mediate direct lysis of pathogens by initiation of a membrane attack complex (MAC) or facilitate their uptake by macrophages via opsonization. Three pathways lead to MAC formation. The alternative and the lectin pathway can be initialized in the absence of pathogen-specific antibodies and are therefore seen as part of the innate immune system. The lectin pathway is activated by binding of mannose-binding lectin (MBL) to mannose residues on glycoproteins or carbohydrates from various microorganisms like bacteria, fungi and some viruses. The alternative pathway is initiated by cell surface constituents, which are foreign to the host, and initiate a cascade similar to the ones of the classical pathway. In contrast, the classical pathway is antibody-mediated and can therefore be considered as part of the adaptive immune system (1).

Many cell types contribute to innate immunity such as neutrophils, macrophages, monocytes, natural killer cells (NK cells) and dendritic cells (DCs). NK cells can lyse infected target cells by perforin and granzyme release, and are potent producers of anti-viral cytokines like TNFα and IFNγ (2). Macrophages are distributed throughout tissues where they take up antigen by phagocytosis and release inflammatory mediators such as cytokines and chemokines that direct adaptive immune responses and recruit other cells. Neutrophils migrate within a few hours from the blood to the site of infection where they engulf the pathogen. They are important for defense against bacteria and fungi through their secretion of antimicrobial peptides and radical oxygen intermediates (ROI) (3). DCs take up antigen, migrate to the draining lymph nodes and initiate adaptive immune responses (4).

Recognition of pathogens by cells of the innate immune system is mediated by a variety of receptors that bind to pathogen-associated molecular patterns (PAMPs) and are named pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are the best described groups of PRRs and recognize microbial patterns such as bacterial cell wall components (TLR 2,4,6), flagella (TLR 5) or bacterial DNA (TLR 9). Single-stranded RNA (ssRNA) triggers TLR 7/8 and double-stranded RNA (dsRNA) is bound by TLR 3 (5). Triggering of TLRs leads to the activation of the transcription factor NF-κB and production of inflammatory cytokines like type I IFNs and IL-12 (6).

Another important group of PRRs are the NOD proteins (nucleotide-binding oligomerization domain). These proteins are cytosolic and two members of this family, NOD1 and NOD2 recognize motifs from bacterial peptidoglycans (7).
Upon PRR triggering, macrophages and DCs are activated, up-regulate costimulatory molecules and secrete various cytokines and chemokines, which give detailed instructions to naïve CD8 and CD4 T cells during antigen presentation (4).

### 3.1.2. Adaptive immune responses

Adaptive immunity relies on the generation of a large receptor repertoire by rearranging germline encoded variable gene segments. Cells bearing specific receptors get activated and expand upon antigen encounter leading to the generation of effector cells and a pool of memory cells, which persist for a long time after pathogen clearance. B and T cells are key effector cells of the humoral and cell-mediated immune response, respectively.

#### 3.1.2.1. Antigen presenting cells (APCs)

While all nucleated cells express MHC class I molecules on their surface, only so called antigen presenting cells (APCs) additionally express MHC class II molecules.

While B cells and macrophages up-regulate MHC class II molecules upon antigen encounter, DCs express them already at low levels in an immature state (8, 9). Uptake of antigen and triggering of PRRs induces DC maturation leading to up-regulation of MHC class II and costimulatory molecules like B7.1 (CD80) and B7.2 (CD86) (5). Additionally, DC activation induces up-regulation of chemokine receptor CCR7, which mediates migration to the secondary lymphoid organs (10). Here, they present their antigen in context with MHC class I and MHC class II to the T cell receptor (TCR) of naïve CD8 and CD4 T lymphocytes, respectively, and, together with costimulation via CD28 on T cells, initiate their specific differentiation program. Cytokines (IL-12, type I interferons), secreted by the activated DCs, orchestrate the fate of the resulting immune response and license T cells to efficiently combat the pathogen. In this way, DCs determine the quality and the magnitude of the response. For these reasons DCs are
considered to be crucial players in the initiation and regulation of adaptive immune responses (11).

3.1.2.2. B lymphocytes

B cells are the primary effector cells of the humoral immune response. Naïve B cells arise from the bone marrow (BM) after rearrangement of the immunoglobulin (Ig) heavy and light chain segment and negative selection. The naïve B cell receptor (BCR) comprises membrane-bound IgM (mIgM) and mIgD. Activation of naïve B cells can occur in two different ways: T helper cell dependent (TD) and T helper cell independent (TI) activation, the latter does not require help from activated antigen-specific CD4 T cells. Many bacterial and viral pathogens display molecules with TI-characteristics and once such a molecule binds to the BCR, its ability to cross-link mIgs leads to polyclonal B cell activation (12).

TD activation requires CD4 T cell help and needs two subsequent signals. Once an antigen cross-links mIg (signal 1), the B cell up-regulates expression of MHC class II molecules and costimulatory B7.1, which interact with the TCR and CD28, respectively, on effector or memory CD4 T cells. The T cell starts to up-regulate CD40L, which triggers CD40 on the B cell (signal 2) (13). These interactions induce the B cell to up-regulate receptors for various cytokines and chemokines, which provide essential signals for differentiation, proliferation and migration. Furthermore, CD40/CD40L interaction is essential for switching from IgM to other antibody subclasses (IgG, IgE, IgA), which is regulated by various factors and cytokines (14, 15). After expansion and somatic hypermutation in germinal centers, activated B cells differentiate into plasma cells, which secrete high amounts of antibodies, or become long-lived memory B cells (16).
3.1.2.3. T lymphocytes

T lymphocytes are the primary effector cells of cell-mediated immune responses. Their T cell receptors (TCR) recognize peptides in context with MHC class I or class II molecules. T cells can be divided into CD4 T cells (T helper cells) or CD8 T cells (cytotoxic T cells). Both subsets develop in the thymus where they undergo rearrangement of genes for the TCR γ, δ, and β chains and become either γδ or αβ T cells, the latter comprises around 97-99% of the whole T cell population (17). Following the β chain rearrangement, immature T cells undergo extensive proliferation followed by α chain rearrangement, a step that increases diversity of the T cell repertoire. In this stage they express both co-receptors, CD4 and CD8. During their further maturation, T cells undergo selective processes, which ensure recognition of foreign antigens and unresponsiveness to self-antigens. Mature CD4 and CD8 T cells exit the thymus and home to secondary lymphoid organs (18).

3.1.3. T cell responses

3.1.3.1. T helper subsets

T helper cells (Th) cells have been traditionally divided into Th1 and Th2 cells based on their cytokine expression profiles and regulatory functions (19). Th1 cells secrete IFNγ and TNFα which are essential effector cytokines against intracellular pathogens. In contrast, Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which are associated with immune responses against helminths. Various factors can influence the direction of the polarization towards Th1 or Th2 subsets: the strength of TCR signal given by the antigen affinity for the TCR (20), the concentration of antigen (21, 22), the nature of the APC (23, 24), the genetic host background (25, 26), the duration of the signal (27), the cytokine environment (28, 29) and the costimulation (30).
IL-12 has been shown to promote Th1 cell differentiation by activating the signal transducer and activator of transcription 4 (STAT4). Induction of TCR cross-linking and IL-12 signaling lead to expression of the transcription factor T-bet, a master regulator of Th1 cell differentiation, which strongly enhances IFNγ production and suppresses production of Th2 cytokines (31). However, strong TCR stimulation and TLR-ligation can compensate for IL-12 (32). Recently, new factors have been shown to be involved in Th1 induction. IL-18 and IL-27, which are primarily produced by DCs and macrophages, serve as cofactors in IL-12-induced Th1 development and enhance IFNγ production by effector T cells (33, 34).

Th1 cells are essential for control of viruses (35) and intracellular pathogens like *Leishmania major* as the prototypic Th1 cytokine IFNγ enhances the induction of inducible nitric oxide synthase (iNOS) in macrophages leading to parasite elimination (36). Moreover, IFNγ supports B cell class switching from IgM to IgG2a (37).

In contrast, the presence of IL-4 drives Th2 cell differentiation by activation of STAT6 (38) and the lineage decision transcription factor GATA3, a master regulator of Th2 cell differentiation (39). Once the cell is programmed to Th2, c-maf binds to the *il4* promoter and further augments IL-4 production (40). Presence of IL-4 during priming inhibits responsiveness to the Th1-polarizing cytokine IL-12 by down-regulating the IL-12Rβ chain on CD4 T cells (39). Besides IL-4, Th2 cells secrete a broad range of other cytokines including IL-5, IL-9, IL-10 and IL-13. Th2 responses are beneficial as they promote expulsion of nematodes (41) but can also have detrimental effects as they support the development of allergic diseases such as asthma (42).
The most recently discovered Th lineage is called Th17 and is characterized by the production of IL-17. Differentiation to Th17 cells is promoted by the presence of TGFβ, IL-6, IL-23 and IL-21, whilst IFNγ, IL-4 or retinoic acid inhibit their development (44-48). RORγT is the key transcription factor orchestrating the differentiation of Th17 cells from naïve CD4 T cells and is required for the maintenance of IL-17 production (49). The IL-17 signaling pathway differs from Th1 or Th2 cells as activation of c-Jun-N-terminal kinase (JNK) and NF-κB occurs via TNF receptor-associated factor 6 (TRAF6) (50). Th17 cells play a major role in the induction of autoimmune diseases (47, 51-53) and in some bacterial infections (54, 55). Moreover, it was demonstrated that Th17 cells are the most efficient producers of IL-21 (56).

A further subset of CD4 T cells has been described, which, in contrast to the other subsets, suppresses immune responses and thus is essential in the prevention of autoimmune diseases (57). Due to their regulatory function these cells have been termed regulatory T cells (Tregs). Sakaguchi et al. first described the existence of these CD4 T cells with regulatory function expressing the IL-2Rα chain (CD25) (58). Later, it was shown that the expression of the transcription factor Foxp3 is a hallmark for Tregs (59). Mice deficient for Foxp3 suffer from lethal autoimmune syndromes caused by the absence of Tregs (60, 61). Natural Tregs (nTregs) represent approximately 1-10% of the CD4 T cell population and derive exclusively from the αβ T cell lineage (62). Treg differentiation from naïve T cells is promoted by the presence of TGFβ, IL-2 and retinoic acid (48) while it is inhibited by IL-21 (56). Inducible Tregs (iTregs)
acquire their suppressive function only through T cell activation and can be divided into several subclasses according to their surface markers and cytokine production. Some of them have been shown to mediate disease suppression by the production of IL-10 (termed Treg1, Tr1) (63) or TGFβ (termed T helper 3, Th3) (64). Also, signals provided by PD-1L (programmed death 1-ligand) on endothelia can induce Foxp3+CD25+CD4+ Tregs (65). It is still controversial whether Tregs mediate their suppressive function via cell-cell contact or by the secretion of suppressive cytokines like IL-10, TGFβ or IL-35 (66, 67).

Figure 3.2:
Reciprocal development of Foxp3 and Th17 cells, adapted from (68).

3.1.3.2. CD8 T cells

CD8 T cells recognize antigens presented by MHC class I molecules. Activation of CD8 T cells leads to the generation of cytotoxic T lymphocytes (CTLs) with the capacity to recognize and eliminate altered self-cells (e.g. virus infected or tumor cells.). Since almost every nucleated cell expresses MHC class I molecules, an activated CTL can lyse any cell in the body displaying its specific antigen. The phases of CD8 T cell responses can be divided into several phases: priming and expansion, contraction and memory (69).
Introduction

Figure 3.3: Kinetic of CD8 T cell responses after pathogen encounter, adapted from (69).

Activation and costimulation

Priming and activation of naïve CD8 T cells occurs when the TCR interacts with an MHC I-peptide complex on mature APCs (70). CD40-CD40L ligation is a key step in T cell activation as blockade of this pathway leads to reduced CD8 T cell priming (71, 72). The requirement of CD40-CD40L engagement for function and expansion of CD8 T cell can be overcome during some viral or bacterial infections through ligation of PRRs (73-75). Similar to CD4 T cell activation, CTL activation is promoted by costimulatory molecules belonging to the CD28 super-family like CD28 and ICOS, or inhibited by CTLA-4 and PD-1 (30). Within the last years, PD-1 has received attention as its blockade has been shown to protect CD8 T cells from exhaustion (76).

Cytotoxicity

CTLs mediate killing of target cells through two distinct pathways, one of which involves the induction of lytic granules filled with cytolytic proteins (perforins, granzymes) shortly after their
activation. Once the CTL recognizes an antigen/MHC class I complex on a cell surface, the integrin receptor LFA-1 binds to ICAMs on the target cell forming a conjugate. The Golgi stacks and the storage granules move to the junction with the target cell. Ca\textsuperscript{2+} influx triggers the exocytosis of perforin into the synaptical space. Conformational changes in perforin allow the formation of pores on the target cell surface and granzymes can enter the cells (77). In addition, granzyme B can enter the cell via binding to a mannose 6-phosphate receptor. This complex gets endocytosed by the target cell allowing granzyme entry (78). Once inside the target cell, the granzymes initiate a cascade of caspases that lead to apoptosis (79).

The second way of CTL killing is the induction of apoptosis via the Fas/FasL pathway. FasL is expressed on activated CTLs and its ligation to Fas on the target cell also activates the caspase cascade, which in consequence induces apoptosis of the target cell (80).

Although these two pathways are independent they complement each other and, dependent on the disease model, one or the other seems to be more important.

**Contraction**

The contraction phase of the CTL response usually correlates with pathogen clearance. Around 90-95% of effector T cells are eliminated (81). Various molecules are involved in this process of contraction. While IL-2 promotes expansion of T cells during priming it has been shown that during the contraction phase it induces alterations in genes that encode apoptosis-related proteins (82, 83). This process is called activation-induced cell death (AICD).

**Memory CD8 T cell subsets**

Some of the T cells generated during an immune response remain as long-lived memory T cells. They are specific for a previously encountered antigen and possess the ability to reside inside the body over years. Kinetics of secondary responses to a familiar antigen are characterized by a reduced lag-time for expansion, re-acquisition of effector function and a much higher amplitude of the expansion phase. Thus, the activation-threshold of memory T cells is much lower than that of naïve T cells (84). In general, memory T cells have been characterized according to their...
anatomical location, expression of cell surface markers and effector functions (85). Memory T cells expressing L-selectin (CD62L) and CCR7 home to the lymph nodes and are termed central memory cells (T_{CM}) while memory cells that lack these homing receptors can be found in the peripheral tissues and are termed effector memory cells (T_{EM}). The IL-7Rα (CD127) has been shown to be expressed on naïve and memory CD8 T cells and has also been used to characterize different populations of memory T cells (86). In this report, CD8 T cells are subclassed into CD62L-CD127- effector T cells (T_{E}), CD62L-CD127+ effector memory T cells (T_{EM}) and CD62L+CD127+ central memory T cells (T_{CM}).

**CD8 T cell memory generation**

The generation of memory CD8 T cells is not well understood. In a model of LCMV infection, it was proposed that during the effector phase some CD8 T cells highly express the IL-7Ra chain and that these cells can develop into long-lived memory CD8 T cells with an elevated level of anti-apoptotic molecules like Bcl-2 and Bcl-XL (87). Several models have been proposed to determine how memory T cells may arise from effector cells (88, 89). CD4 T cell help has been shown to be essential for memory generation and maintenance in many infectious models (90-93) although there is also evidence that memory generation can occur in the absence of CD4 T help (94).

**Memory maintenance**

Maintenance of long-lived memory CD8 T cells is dependent on signals provided by IL-15 and IL-7 (95-97). The cooperation of both cytokines is necessary to efficiently maintain memory CD8 T cell responses, but both cytokines alone can drive homeostatic proliferation. Thus, dependent on the infection model, different pathways may support the maintenance of CD8 memory T cells.
3.2. Cytokines and cytokine families

Cytokines are low molecular weight glycoproteins secreted by leukocytes and various other cells in the body. Many cytokines are referred to as interleukins (ILs), which indicates their secretion by leukocytes and action upon others (or other cell types).

Cytokines secreted by cells upon stimulation can act on the producing cell itself (autocrine) or on a neighbouring cell (paracrine). Endocrine action (on distant cells) is rare due to their short half-life. Generally, cytokines are responsible for shaping the immune response against a certain pathogen allowing the initiation and maintenance of the immune response. Like hormones, only a low concentration of cytokines is needed to exert biological effects as they bind with high affinity to their specific receptors.

According to their structures, cytokines can be divided in four groups: the hematopoietin family (e.g. IL-2, IL-4), the interferon family (e.g. IFNα, IFNγ), the chemokine family (e.g. IL-8, RANTES) and the tumor necrosis factor family (e.g. TNFα, LTα).

Although cytokine receptors are variable in structure, they can be classed into five families of receptor proteins: the immunoglobulin superfamily receptors (e.g. IL-1R), the class I cytokine receptor family (hematopoietin receptor family, e.g. IL-2R, IL-21R), the class II cytokine receptors (interferon receptor family, e.g. IFNγR), the TNF receptor family (e.g. TNFα, CD40L) and the chemokine receptor family (e.g. IL-8R).

The majority of cytokines signal through the class I cytokine receptor family (hematopoietin receptor family). The receptors are transmembrane glycoproteins and contain characteristic external structures, which contain a set of four conserved cysteine residues and a sequence of WSXWS (W=tryptophan, S=serine, X=any amino acid) (98).

Due to the sharing of a signal-transduction unit, class I cytokine receptors can be subclassed into the GM-CSF receptor subfamily, which share a common β unit, the IL-6 receptor subfamily, sharing a common gp130 subunit and the IL-2 receptor family, which shares a common γ subunit and is therefore also called the common γ-chain (γc) family.
3.2.1. The common $\gamma$–chain receptor family

![Overview of the $\gamma$c receptor family of cytokines and their specific receptors. All of them share the common $\gamma$ chain; IL-2 and IL-15 additionally share a $\beta$ chain. Adapted from (99).]

The common $\gamma$c family of cytokines is composed of IL-2, IL-4, IL-7, IL-9, IL-15 and their latest family member IL-21. Their receptors share the $\gamma$-chain (CD132). While every cytokine receptor is composed of its specific $\alpha$ chain, IL-2 and IL-15R share an additional $\beta$ chain (CD122). Deficiency for $\gamma$c expression severely impairs the biological function of all these cytokines (100). The important role of the $\gamma$c has been shown when it was demonstrated that mutations in the IL-2R$\gamma$ chain are responsible for X-linked severe immunodeficiency in humans (XSCID) (101). Later, studies in mice deficient for $\gamma$c demonstrated that this phenotype is characterized by absence of T cells, NK cells and functional B cells (100).

The signal transduction of the $\gamma$c receptor occurs via the JAK/STAT pathway. Cytokines bind to homodimeric or heterodimeric receptors to which Janus kinases (JAKs) are constitutively associated. After receptor dimerization, JAKs trans-phosphorylate each other leading to phosphorylation of cognate tyrosines on the receptor allowing STATs to bind. In this way the latter become phosphorylated, they dimerize and translocate to the nucleus where they regulate gene expression (98). As $\gamma$c signaling occurs via the cytokine receptor associated protein tyrosine kinase 3 (JAK3), mice deficient for this kinase reveal the same SCID phenotype as $\gamma$c deficient mice (102, 103). Due to their strong impact on T cell responses, $\gamma$c cytokines have been the focus of extensive immunological research. The latest member, IL-21 and its receptor,
was discovered in 2000 (104, 105). Since then, a lot of in vivo and in vitro studies have been performed to define the role of IL-21 and its receptor in adaptive immune responses.

**Figure 3.5:**

Signaling via the JAK/STAT pathway by a typical type I cytokine (e.g. IL-2), adapted from (106).
3.2.2. The members of the γ-chain family of cytokines

3.2.2.1 IL-2 and IL-15

The receptors for IL-2 and IL-15 are heterotrimeric and both contain a subunit known as the IL-2β chain (CD122). CD122 together with the γc forms an intermediate-affinity receptor for both cytokines (107, 108). For the formation of the high affinity forms the receptors need a third, unique receptor unit: IL-2Rα (CD25) and IL-15Rα (109). Although they share two receptor units, their distinct functions can be maintained through the distribution of the α chains. IL-2Rα is expressed on B and T cells and presence of IL-2 leads to its up-regulation and, in consequence, to the formation of the high-affinity receptor (110). In contrast, IL-15 exerts its actions most efficiently when bound to IL-15Rα on DCs, monocytes and stromal cells. Type I or II interferons, as well as TLR stimulation, lead to up-regulation of both IL-15 and its α chain. From there, it is presented to cells expressing the IL-2Rβ chain and the γc chain to form the high affinity IL-15 receptor, a phenomenon that is called trans-presentation (111).

Both cytokines stimulate T and B cell proliferation, CTL generation and proliferation as well as activation of NK cells (112). In adaptive immune responses, they have distinct and often competing roles. IL-2 enhances proliferation in vitro and in vivo (113, 114) and is essential for generation and maintenance of Foxp3+CD25+CD4+ Tregs (62). Moreover, it down-regulates T cell responses by the induction of AICD (82). Deficiency of IL-2 or IL-2Rα in mice results therefore in uncontrolled polyclonal expansion of T and B cells due to the lack of AICD and Tregs (115).

IL-15 or IL-15Rα deficient mice, in contrast, do not show uncontrolled proliferation, but have a dramatic reduction in thymic and peripheral NK cells, NKT cells, intestinal intraepithelial lymphocytes and memory CD8 T cells (95, 116). As previously mentioned, IL-15 is important for the maintenance of memory CD8 T cells upon LCMV infection (96). More recently it has been reported that IL-15 signaling also plays a role in the maintenance of memory CD4 T cells (117).
3.2.2.2 IL-4

IL-4 is the prototypic Th2 cytokine. The presence of IL-4 during Th priming leads to the development of IL-4-producing Th2 cells. Notably, IL-4 can be also produced by mast cells and basophils (118) (119). Within the γc family of cytokines, it is the main cytokine that activates STAT6 underlining its role in driving Th2 differentiation and IgE class switching (38). The IL-4Rα chain also functions as a component of the IL-13R and, like IL-4, IL-13 signals via STAT6 and contributes via GATA-3 activation to the Th2 phenotype (120). The impact of IL-4 in immune responses became clear following the analysis of the respective knock-out mice. IL-4 deficient mice had normal T and B cell development, however, they exhibited reduced serum IgG1 levels and IgE was not detectable upon infection with the nematode Nippostrongylus brasiliensis (Nb) (121, 122). Moreover Th2 associated cytokines like IL-3, IL-5, IL-9 and IL-10 were absent in this experimental setup. Reduction in eosinophilia after Nb infection was due to a lack of IL-5 producing Th2 cells. In contrast, Th1 responses were not affected by IL-4 deficiency (122).

3.2.2.3 IL-7

The primary role of IL-7 is to maintain homeostatic proliferation and therefore survival of naïve and memory T cells. The important role of IL-7 for the survival of memory CD8 T cells is partially attributed to the induction of anti-apoptotic Bcl-2 (123, 124). During acute LCMV infection, it has been shown that effector CD8 T cells with high surface IL-7R expression give rise to long-lived memory CD8 T cells (87).
3.2.2.4 IL-9

IL-9 is an effector cytokine produced by Th2 cells during asthma and helminth infections. Initially it was shown to be a growth factor for T cell and murine mast cell lines (125, 126). IL-9 deficient mice failed to develop goblet cell hyperplasia and were impaired in pulmonary mast cell expansion induced by *Schistosoma mansoni* infection. IL-9 plays a minor role in the initiation or maintenance of Th2 responses, but is a feature of them by modulating mucus production and mast cell proliferation.

3.2.3. IL-21 and its receptor

The IL-21 receptor (IL-21R) was initially described as a novel interleukin receptor strongly related to the IL-2Rβ and IL-4Rα chain. It contained the conserved WSXWS region and was therefore classed to the type I cytokine receptor family. It was physically adjacent to the IL-4Rα chain on chromosome 16 and its expression was most pronounced in the thymus and the spleen (104, 105). Upon activation, T, B and NK cells up-regulated the IL-21R and, like IL-2Rβ, IL-21R associated with JAK1 to mediate activation of STAT1, STAT3 and, to a lesser extend, STAT5α/β (127). Mature IL-21 has a relative molecular mass of 14.4 kDa and consists of a 131-residue four-helix-bundle cytokine domain with significant homology to IL-2, IL-4 and IL-15. In contrast to its receptor, IL-21 could not be detected in naïve tissues, but stimulation of T cells with αCD3 and αCD28 strongly induced IL-21 expression in CD4 T cells, but not in CD8 T cells or in B cells. Investigation of its biological activity showed that IL-21 could strongly costimulate the CD40-CD40L induced proliferation of B cells. Moreover IL-21 acted in synergy with IL-2, IL-7 and IL-15 to induce T cell proliferation with or without αCD3 stimulation. IL-21 alone had no impact on T cell proliferation in the absence of αCD3 stimulation. In vitro, IL-21 promoted expansion and differentiation of NK cells from BM progenitors in synergy with Flt3 and IL-15 (105). Later it was proven that the γc was a required signaling component of the IL-21R (128, 129).
Introduction

Sources of IL-21 and its role in innate immune responses

Since its discovery, many in vitro studies were performed to better characterize the impact of IL-21 on both innate and adaptive immunity. It was suggested that IL-21 induces functional maturation of NK cells resulting in enhanced IFNγ production and perforin expression. In NKT cells, IL-21 enhanced survival in vitro, led to up-regulation of granzyme B expression and enhanced cytokine production in the context of αCD3/CD28 stimulation. In contrast, BMDCs generated in the presence of IL-21 were functionally inhibited as they remained in an immature state and were not able to activate T cells (130, 131). Some of the sources of IL-21 have already been identified. Recent studies demonstrated that NKT cells and stromal cells can be a source of IL-21 (132,133). In addition, it has been shown that the main source of IL-21 is activated Th2 cells (134) and recently Th17 cells were identified as the most potent producers of IL-21 suggesting a role for IL-21 in inflammatory diseases (56).

The role of IL-21 in antibody production

The role of IL-21 in B cell responses has been widely investigated. IL-21R-deficient mice exhibited normal B cell development, however, naïve adult mice had reduced serum IgG1 and elevated serum IgE levels (135). In line with these data, treatment of wild-type mice with IL-21 led to decreased IgE levels in the serum and further analysis revealed that IL-21 induced reduction of germline Ce transcripts and therefore negatively regulated IgE isotype class switching (136). Still, IgE production was dependent on IL-4 as in IL-4/IL-21R double deficient mice IgE production was abrogated (135).

IL-21 and CD4 T cell responses

The literature concerning the impact of IL-21 on CD4 T cells is controversial. As mentioned above, the first source of IL-21 was described as being activated Th2 cells (134). In humans IL-21 mRNA transcripts were predominantly found in Th1 cells and most strikingly in follicular T
helper cells (T(FH)), a CD4 T cell subset homing to B cell areas in secondary lymphoid organs and providing B cell help (137). Presence of IL-21 during priming of naïve murine CD4 T cells under Th1 conditions led to the impairment of IFNγ production via suppression of Eomesodermin (Eomes) (138). In human cells, however, TCR activation in the presence of IL-21 induced genes associated with Th1 cells like IFNγ, T-bet and IL-12Rβ (139). CD4 T cells from IL-21R-deficient mice could be polarized towards Th1 or Th2 under the respective condition excluding a role for IL-21 in driving T helper cell polarization (135). However, Th1 driven DTH responses were increased in IL-21R-deficient mice due to enhanced IFNγ levels (134). Induction of Th2 responses in IL-21R-deficient mice during helminth infection was impaired and no Th1 bias was observed (140).

IL-21 appears to play a key role in the differentiation of Th17 cells. In vitro generation of this subset from naïve CD4 T cells required the presence of IL-6 and TGFβ. It has been described that IL-6 induced IL-21 expression, which acted in an autocrine manner to increase its own level and, in consequence, IL-17 production together with IL-23 and TGFβ (46). In the process of Th17-priming, IL-21 could compensate for the presence of IL-6 and rendered IL-6-deficient mice able to mount a potent Th17 response. In addition, IL-21 treatment reduced the number of peripheral Tregs (141). The importance of IL-21 in Th17 generation has been demonstrated in vivo as IL-21 deficiency impaired Th17 development and resulted in protection against experimental autoimmune encephalomyelitis (EAE). In this study, the presence of IL-21 also suppressed the generation of Foxp3+ Tregs (56).

**IL-21 in CD8 T cell responses**

In vivo and in vitro effects of IL-21 on CD8 T cells have been studied. IL-21 did not substantially induce the proliferation of naïve and memory CD8 T cells, but in context with IL-15 or IL-7, cells stimulated with IL-21 proliferated intensively (105, 142). In a mixed lymphocyte reaction (MLR), IL-21 and IL-15 enhanced IFNγ production and could increase cytotoxicity (143). In a system with OT-I cells, priming in the presence of IL-21 led to a unique effector phenotype characterized by distinct surface molecule expression and increased cytolytic function, but reduced IFNγ production (144). Another report using cells derived from rhesus
monkeys showed that IL-21 could induce apoptosis of Ag-specific CD8 T cells by down-regulation of Bcl-2 and suggested therefore a role for IL-21 during the CD8 contraction phase (145). In contrast, it was also demonstrated in mice that IL-21 is essential for survival of naïve T cells and that increased proliferation in presence of IL-21 is a result of enhanced Bcl-2 expression (133).

Due to the positive in vitro effects of IL-21 on NK and CD8 T cell cytotoxicity and proliferation, it was suggested that IL-21 administration could be beneficial for tumor treatment. Indeed, most in vivo studies concerning CD8 T cell responses and IL-21 involve tumor models. In mouse studies, B16 melanomas secreting IL-21 were completely rejected in vivo due to activation of CD8 T cells and NK cells. It has been shown in this model that CD4 T cell help was dispensable and that the positive effect was due to increased perforin expression (146). Similar beneficial effects of IL-21 were obtained in a model of murine mammary adenocarcinoma, in which the tumor cells secreted IL-21 (147). In a thymoma model, IL-21 treatment led to enhanced proliferation of tumor-specific CD8 T cells and, compared to IL-2 treatment, increased cell survival (148). In established B16 melanomas, transfer of specific CD8 T cells together with peptide vaccination and a combination of IL-21 and IL-15 treatment led to tumor regression (142). Priming of tumor-specific CD8 T cells in the presence of IL-21 did not induce an effector phenotype, but, upon adoptive transfer, these cells were able to mediate tumor regression (149). Positive effects of IL-21 in tumor treatment were also shown in humans recently (150). Taken together, these studies implicate a potent role for IL-21 in expansion and effector function of CD8 T cells.

However, the role of IL-21 in anti-viral responses has yet to be analysed extensively. Plasmid-delivered IL-21 and IL-15 could increase the magnitude and longevity of CD8 T cell responses to a DNA vaccine encoding the HIV-1 envelope glycoprotein in line with the effects seen in tumor models (151). Comparison of the effects of IL-21 and IL-15 on CD8 T cells from HIV-infected patients showed that IL-21 up-regulated perforin expression in virus-specific memory CD8 T cells, but did not increase activation, proliferation or degranulation (152).

Further studies of anti-viral responses in IL-21R deficient mice are rare. One publication showed impaired cytotoxic T cell responses upon infection with recombinant vaccinia virus. This included reduction in percentages of specific CD8 T cells and reduced cytotoxicity in the absence of IL-21R signaling (142).
Figure 3.6:
The pleiotrophic effects of IL-21 on various immune cells, adapted from (153).
3.3. Experimental disease models

3.3.1. Allergic airway inflammation (asthma)

Allergic asthma is driven by Th2 mediated immune responses against environmental allergens leading to inflammation of the respiratory tract (154).

Once inhaled, an allergen is taken up by lung DCs, which migrate to the draining lymph node (LN) (155). During allergic responses, T cells are primed by DCs to differentiate into Th2 cells, which secrete IL-4 and induce class switch to antigen-specific IgE isotype by B cells in the lymph node. Th2 cells migrate out of the draining lymph nodes to the lung and secrete, apart from IL-4, IL-13, which induces bronchoconstriction and mucus production, IL-9 for mast cell differentiation and IL-5 for the differentiation and recruitment of eosinophils (156). Re-exposure to the same antigen leads to cross-linking of specific IgE on sensitized mast cells, which triggers the release of mediators of bronchoconstriction (e.g. histamine, prostaglandin). Mast cells also release cytokines linked to allergic inflammation like IL-4, IL-5 and IL-13 and can contribute to airway remodeling (157) (158).

Beneficial effects of IL-21 administration during immunization and challenge of mice with OVA has been reported previously. OVA-specific IgE and IgG1 levels were significantly reduced in IL-21 treated mice and eosinophilia was reduced in the lung whilst the number of T cells and macrophages seemed unaffected. These findings indicate an important regulatory role for IL-21 in allergic airway inflammation (136).

3.3.2. Helminth infections

Th2 responses are beneficial for the clearance of helminthes (159). Nippostrongylus brasiliensis (Nb) and Heligmosomoides polygyrus (Hp) are two well-characterized models of helminth infection. Whilst Hp larvae are directly administered via oral gavage, Nb larvae are injected subcutaneously, migrate to the lung and cause airway remodeling leading to similar symptoms
as in asthmatic mice (160). After passing the lung, Nb larvae are coughed up, swallowed and pass to the gut (161).

Once in the gut, parasite antigens are presented to CD4 T cells in mesenteric LN or other gut-associated lymphoid tissues where they drive the induction of Th2 effector cells. These orchestrate the immune response through production of cytokines like IL-4, IL-13, IL-9 and IL-5 and, in consequence, induce IgE and IgG1 class switching in B cells. IL-5 triggers eosinophilia and, together with IL-4, IL-9, IL-13 and IgE crosslinking, can result in enhanced mast cell and basophil development. IL-4 and IL-13 stimulate smooth muscle contractility, increase intestinal permeability and elevate goblet cell mucus production (41). Together, these effects can contribute to parasite expulsion. Moreover, IL-4 and IL-13 can drive development of alternatively activated macrophages (AAMs), which contribute to the regulation of the immune response and also have a role in clearing cell debris and wound healing (162). As the larva of Hp develops in the gut submucosa, an immune cell infiltrate is rapidly formed around it and separates it from the tissue. It is composed of AAMs, neutrophils, CD4 T cells, DCs and eosinophils and is termed type-2 granuloma (163).

The involvement of IL-21 in Th2 driven anti-helminth responses has been demonstrated. IL-21R deficient mice showed diminished Th2 cytokine driven inflammation upon infection with *Nb* and *Schistosoma mansoni* (*S. mansoni*). Notably, upon *S. mansoni* infection, granuloma size and liver fibrosis were reduced in the absence of IL-21R signaling. In vitro, IL-21 could increase the expression of IL-4R and IL-13Rα1 on macrophages resulting in elevated FIZZ1 and arginase-1 activity upon stimulation with IL-4 and IL-13, which are features of AAMs. Therefore it was concluded that IL-21 amplifies activation of AAMs and Th2 effector function. Still, there was no influence of IL-21 on parasite expulsion (140).

### 3.3.3. Experimental leishmaniasis

*Leishmania major* (*L. major*) is a protozoan parasite naturally transmitted by a sand fly and causes cutaneous leishmaniasis. In the murine model, *L. major* promastigotes are injected subcutaneously into the ear or the footpad. The parasite is taken up by macrophages where it replicates and induces the development lesion through inflammation (164). The majorities of
Inbred mouse strains, like C57BL/6, develop small lesions at the inoculation site and cure spontaneously 6-12 weeks after infection. This outcome is due to the development of a cell-mediated Th1 response (characterized by enhanced IFNγ and TNFα production) which activates macrophages to produce iNOS and thus promotes parasite killing (36, 165). In contrast, other strains like BALB/c mice mount a detrimental Th2 response characterized by increased IL-4 production and consequently high IgG1 and IgE antibody titers (164). Thus, these mice are susceptible to infection and develop progressive lesions, which never heal, and the parasite disseminates to internal organs leading to systemic disease and death. The importance of IL-4 as a susceptibility factor has been demonstrated as IL-4 deficient mice on a BALB/c background are able to control *L. major* infection (166). Other susceptibility factors are IL-10, TGFβ and IL-13 (167, 168, 169).

This model provides an ideal tool to analyse whether Th responses in cytokine-deficient mice are biased towards Th1 or Th2 responses. So far, the role of IL-21R signaling in experimental leishmaniasis has not been addressed.

### 3.3.4. Virus infections

Viruses are strictly intracellular pathogens that can cause acute or chronic infections in the host. The innate immune response to viruses involves the activation of cells through TLRs and other PRRs leading to the release of type I interferons and activation of DCs. (170). During adaptive immunity, CTLs mediate specific killing of infected host cells and, together with CD4 T cells, are an important source of IFNγ, which is a key anti-viral cytokine through its ability to enhance viral elimination. Cytopathic viruses cause cell destruction and as large amounts of virus are released, antibodies play a pivotal role in its neutralization. During infection with non-cytopathic viruses viral elimination is mediated by virus-specific CD8 T cells which eliminate infected host cells by lysis or/and cytokine secretion (171). In addition, CD4 T cells play a critical role in providing help for the generation of antibodies and the maintenance of CD8 T cells during the memory phase or in persistent infections.
**Lymphocytic choriomeningitis virus (LCMV)**

The non-cytopathic lymphocytic choriomeningitis virus (LCMV) belongs to the family of arenaviruses. The natural host of LCMV is the mouse where it is transmitted from mother to the offspring via the placental or congenital route. Dependent on the virus strain, LCMV leads to various outcomes: LCMV WE, Armstrong and low doses of LCMV Docile (LCMV DO) lead to acute infections with rapid viral clearance whilst high dose infections with the variants Docile or clone-13 cause persistent infections (172, 173).

During LCMV infection, the generation of CTL responses is essential for viral clearance as mice lacking CD8 T cells are not able to control the virus (174). CTLs act on host cells via Fas- and perforin-mediated killing mechanisms (175), via type I interferons and, to a lesser extend, via IFNγ (176).

CTL generation is not dependent on CD4 help as CD4 deficient or depleted mice can still mount virus-specific CD8 T cell responses and can control low doses of acute LCMV. However, CD4 T cells play a pivotal role in mice infected with high doses of LCMV, as in the absence of CD4 T cells mice are not able to control the virus or sustain an effector CD8 T cell response (91). In addition, generation of CD8 memory is impaired in the absence of CD4 T cell help (91, 177). During chronic LCMV infections, CD4 T cells also play an essential role in the maintenance of CD8 T cells, as the number of specific CD8 T cells starts to decline during the course of infection when CD4 help is absent (91, 174).

In the LCMV model, antibodies play a minor role in early control of the virus as neutralizing antibodies to LCMV only arise around 50-80 days after infection with acute LCMV (171). During chronic LCMV infections however, neutralizing antibodies are elicited even later (91, 178). Similar to the other models, antibody generation is dependent on CD4 T cell help as mice deficient for or depleted of CD4 T cells are not able to generate neutralizing antibodies. As a result, the virus re-emerges at around 3 months after infection (177). µMT mice, which lack B cells, have a delayed initial CD8 T cell response, and similarly to CD4 T cell deficient mice, they show recrudescence of viremia at a later stage of infection (177).

A phenomenon that has been widely studied during LCMV infections is exhaustion of CD8 T cells (173). In general, exhaustion means loss of cytotoxic function, impaired proliferation, reduced cytokine production (IL-2, IFNγ, TNFα) and, in consequence, leads to cell deletion.
The extent of exhaustion directly correlates with the viral load and continued exposure to antigen, demonstrated during chronic LCMV infection (179). Absence of CD4 T cell help during infection results in even more pronounced CD8 T cell exhaustion underlining again the cooperation between CD8 and CD4 T cells (180). CD8 T cells are the first to show signs of exhaustion, CD4 T cells are also affected during later stages of infection. It has been suggested that the reason for exhaustion lies in protection of the host from otherwise excessive immunopathology caused by CTLs (181). Exhaustion of T cells is not limited to chronic LCMV infection as it has also been demonstrated for CD8 T cells from HIV-infected patients (182). Thus, chronic LCMV models give insights into possible mechanisms underlying viral chronicity.

Recently, several factors have been implicated in LCMV persistence. T cells from mice infected with persistent LCMV showed increased expression of PD-1. Blockade of the PD-1/PD-1L pathway resulted in enhanced CD8 T cell activity even in the absence of CD4 T cell help (76). IL-10 has been previously described to act in a regulatory manner as it suppresses cytokine production and T cell proliferation. Persistent LCMV infection induces enhanced IL-10 production in APCs leading to impaired T cell responses. Disruption of the il10 gene resulted in the maintenance of effector T cell responses, elimination of the virus and the development of anti-viral memory responses. Therapeutic treatment with anti-IL-10R antibodies restored T cell function and enhanced viral elimination confirming the phenotype of IL-10 deficient mice (183, 184).

CD27 is a member of the tumor necrosis factor receptor family and acts in a costimulatory manner to elicit B and T cell responses. As the ligand for CD27, CD70, is continuously expressed in chronic viral infections like HIV, the role of CD27 in chronic LCMV infection has been analysed (185). CD27-deficient mice efficiently mounted neutralizing antibodies 20-30 days after infection, which consequently led to control of the virus. Moreover, CD8 T cells showed pronounced anti-viral cytokine production and cytotoxicity. Similar results were obtained when mice were treated early during infection with anti-CD70 antibodies (178). Thus, blockade of CD27 could also provide a treatment strategy for overcoming exhaustion evident during chronic infections.
Influenza

The cytopathic influenza virus belongs to the Orthomyxoviridae family of RNA viruses. Influenza A viruses can be classified based on their surface proteins hemagglutinin (HA or H) and neuramidase (NA or N). Influenza virus infects the epithelial cells of the airways and is typically localized and confined to the respiratory tract. Sublethal doses of influenza infection in mice result in acute infection with clearance of the virus from the lung by day 7-10. The clearance of influenza virus is mediated through the tight interplay between CD4 T cells, CD8 T cells and B cells. Cytotoxic T cells play a major role in viral clearance via MHC class I dependent cytotoxic mechanisms including perforin and Fas-dependent killing (186). Absence of CD8 T cells after infection with the pathogenic influenza A/Puerto Rico/8/34 leads to increased viral replication and morbidity, while a milder infection only delayed viral clearance (187). Antibody production is essential for the control of influenza as mice deficient in B cells succumb to infection (188). CD4 T cell help is needed for B cell isotype switching, but not for the initiation of CD8 T cell responses (189, 190). Similar to infection with LCMV, influenza-specific CD4 T cells are necessary for the maintenance of memory CD8 T cells (191). So far the role of IL-21R signaling in influenza A infection has not been analysed.

Vaccinia

Vaccinia virus (VV) is a double-stranded DNA (dsDNA) virus that belongs to the family of poxviruses (Poxviridae). It replicates in the cytoplasm of host cells and requires therefore a large genome encoding enzymes and proteins involved in viral DNA replication (192). The ovaries are the primary target organs of VV. Both humoral and cellular immune responses have been thought to play a role in protection, but mostly antibodies confer protection to VV (193). Although VV induces a potent primary CD8 T cell response followed by long-term memory (194), one report demonstrates that CD8 T cell deficient mice survive even high doses of VV (195). In line with this, it was shown that in vivo depletion of CD4 T cells or B cells in IgH-/− mice abrogated protection due to impaired antibody production, while depletion of CD8 T cells alone had minor consequences when antibody titers were normal. Still, there was a
contribution of CD8 T cells to protective immunity in the absence of CD4 T cells as MHC class II deficient mice depleted of CD8 T cells showed enhanced disease development compared to untreated MHC class II mice (196). It was further shown that CD4 T cell help for CD8 T cells is needed at different stages of infection (197).

In summary, control of VV infection is more dependent on CD4 T cell help and antibodies than on CTLs.

In context of IL-21, a previous report showed that IL-21R deficient mice mounted an impaired CD8 T cell response against a recombinant VV carrying the HIV gp160 as shown by reduced specific CD8 T cell numbers and cytotoxicity (142).

### 3.4. Aim of this thesis

We sought to study adaptive immune responses in the absence of IL-21R signaling by using IL-21R-deficient mice. In particular, we took advantage of the disease models described above to study the role of IL-21R signaling in the generation of Th1-mediated (*Leishmania major*), Th1-associated CTL (viral infection models) and Th2 (allergic airway inflammation, helminth infection) immune responses. In this way we aimed to contribute to a better characterization of IL-21R signaling within the complex cytokine network.
4. Results

4.1. IL-21 receptor signaling is integral for the development of Th2 effector responses in vivo

Anja Fröhlich\textsuperscript{1*}, Benjamin J. Marsland\textsuperscript{1*}, Ivo Sonderegger\textsuperscript{1}, Michael Kurrer\textsuperscript{2}, Marty Hodge\textsuperscript{1}, Nicola L. Harris\textsuperscript{4}, Manfred Kopf\textsuperscript{1#}

\textsuperscript{1}Institute of Integrative Biology, Molecular Biomedicine, ETH Zürich, Switzerland

\textsuperscript{2}Department of Pathology, University Zürich, Switzerland

\textsuperscript{3}Inflammation Division, Millennium Pharmaceuticals, Incorporated, Cambridge, MA

\textsuperscript{4}Institute of Integrative Biology, Environmental Biomedicine, ETH Zürich, Switzerland

*Authors contributed equally

#Address correspondence to: Manfred Kopf, Molecular Biomedicine, Swiss Federal Institute of Technology Zürich, Wagistrasse 27, 8952 Zürich-Schlieren, Switzerland.
Phone: +41-44-633 6470
Email: Manfred.Kopf@ethz.ch
4.1.1. Abstract

Interleukin 21 (IL-21) is a member of the common γ-chain family of cytokines, which influence a broad spectrum of immunological responses. A number of studies have examined the function of IL-21, however, its specific role in Th1/Th2 cell differentiation and related effector responses remains to be clarified. Thus, we generated IL-21R-deficient mice and have investigated the role of IL-21R signaling using a series of in vivo experimentally induced disease models. We first addressed the role of IL-21R signaling in Th2 immune responses by examining allergic airway inflammation, *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* anti-helminth responses. In each of these systems, IL-21R signaling played a clear role in the development of Th2 responses. Comparatively, IL-21R signaling was not required for the containment of *Leishmania major* infection, or the development of Experimental Autoimmune Myocarditis, indicative of competent Th1 and Th17 responses, respectively. Adoptive transfer of T cells and analysis of IL-21R+/+/IL-21R-/- chimeric mice revealed that IL-21R signaling was central to Th2 cell survival or migration to peripheral tissues. Overall, our data shows IL-21 plays a crucial role in supporting polarized Th2 responses in vivo, whilst appearing superfluous for Th1 and Th17 responses.

4.1.2. Introduction

Cytokines are key players in shaping immune responses against both self and foreign antigens, influencing the induction of either tolerance or effective immunity. Whilst cytokines have a broad spectrum of activities, distinct levels of redundancy due to the sharing of common receptors, or receptor subunits help ensure appropriate immune responses develop. Interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15 belong to the type I cytokine family, their receptors share the common γ-chain and signal via the JAK/STAT pathway (101, 198-201). These cytokines play crucial roles in T cell responses including cell proliferation, differentiation, and maintaining memory populations. IL-21 is the most recently described member of this cytokine family (104, 105), and a series of studies have shown that it has pleiotropic effects upon the development of immune responses. The main source of IL-21 is activated CD4 T cells, however numerous other
Results

Cell types express the receptor (105). It has been shown to promote expansion of natural killer (NK) cells and augment their antitumor activity (146) but also to inhibit dendritic cell activation and maturation in vitro (130). Furthermore, IgE isotype switching is deregulated in the absence of IL-21-receptor signaling indicative of a regulatory role for IL-21 (135). The data to date concerning the role of IL-21 in Th1 and Th2 responses is controversial (99). Addition of IL-21 to in vitro T cell cultures has been shown to have no influence on the production of IL-4 and IL-5, whilst in vivo, exogenous IL-21 can reduce the infiltration of eosinophils into the airways (generally considered to be IL-5-dependent) (136). A further study has shown that IL-21 mRNA was exclusively detected in vitro and in vivo polarized Th2 cells, but not Th1 cells (134). This study also showed that IL-21 inhibited IFNγ-production by Th1 cells in vitro although neither TNFα production nor T-bet expression was compromised (134). In contrast, Strengell et al. showed that IL-21 could induce expression of genes involved in Th1 responses including IFNγ, the IL-12Rβ chain and T-bet (139). In an attempt to clarify the role of IL-21 in both Th1 and Th2 immune responses, we have generated mice deficient in the IL-21 receptor, and extensively assessed their ability to mount Th1 and Th2 responses in vivo. We found IL-21R expression was central to the development of Th2-driven allergic airway inflammation as shown by impaired Th2 cytokine production, infiltration of eosinophils and airways hyperresponsiveness. In support of prior publications antibody isotype switching was also deregulated, characterized by reduced IgG1 and IgA and increased IgE production. In line with this data, Th2 responses against the parasites Nippostrongylus brasiliensis and Heligmosomoides polygyrus were similarly impaired. However, this immune deregulation was not a general phenomenon as the development of Th1 immune responses against Leshmania major, and Th17-driven Experimental Autoimmune Myocarditis (EAM) were normal. Overall, our data from in vivo disease models supports a central role for IL-21 signaling in Th2, but not Th1 or Th17 immune responses.
4.1.3. Material and Methods

Mice and Pathogens

C57BL/6 and BALB/c wild-type mice were obtained from Charles River (Germany). IL-21R-deficient mice were generated as described below and backcrossed more than 5 generations onto the C57BL/6 background and 4 generations onto the BALB/c background. Mice were maintained specific pathogen free in BioSupport (Zurich) animal facility in isolated ventilated cages. Animals used in experiments were between 8 and 10 weeks of age. N. brasiliensis was maintained by passage through Lewis rats, H. polygyrus was passaged through IL-4/- mice on a C57BL/6 background. Mice were infected subcutaneously with 550 live N. brasiliensis L3 worms or orally with 200 L3 H. polygyrus worms and sacrificed at day 14 post infection. For infection with L. major, stationary phase promastigotes were collected from in vitro culture in biphasic Novy-Nicolle-McNeal (NNN) blood agar medium, and 1-2×10^6 parasites were injected subcutaneous into the right hind footpad. For induction of delayed-type hypersensitivity (DTH), SLA (equal to 5×10^6 L. major promastigotes diluted in PBS) was injected in a volume of 50 µl into the previously not infected footpad. The footpad swelling was monitored with a metric caliper (Kroepelin, Schluchtern, Germany). Mice were sacrificed and tissues taken at the indicated time points. All animal experimental procedures were approved by the Zürich animal committee.

Genotyping of IL-21R-deficient mice

DNA was isolated from tails of mice by tissue lysis and DNA precipitation. For the PCR reaction the following primers were used: IL-21R F 5’-GCT AGC ACT TCT ATA GGC AAA GGG-3’, IL-21R 5’-CAG CTG AGC TTT GTG GGA GG-3’ and NEO 3193 5’-CGA GAC TAG TGA GAC GTG CTA CTT CC-3’. Using Platinum Taq (Invitrogen) the reaction was performed in a T 3000 Thermocycler (Biometra) over 35 cycles with an annealing temperature of 58°C.
**Results**

**OVA-induced airway inflammation**

Mice were immunized by intraperitoneal injection (i.p.) with 100 µg OVA (grade V; Sigma, St. Louis, MO) in 200 µL alum adjuvant (SERVA, Electrophoresis GmbH, Heidelberg, Germany). 10 days later, mice were challenged on 4 following days by intranasal inoculation (i.n.) with 100 µg OVA in 50 µL PBS.

**Measurement of airway responsiveness**

One day after last i.n. challenge with OVA, mice were placed in individual unrestrained whole body plethysmograph chambers (Buxco Electronics, Inc., Petersfield, United Kingdom). Airway responsiveness was assessed in mice by inducing airflow obstruction with aerosolized Methacholine-Chloride (MetCh). This procedure estimates total pulmonary airflow in the upper and lower respiratory tracts. The chamber pressure was used as a measure of the difference between thoracic expansion (or contraction) and air volume removed from (or added to) the chamber during inspiration (or expiration). Pulmonary airflow obstruction was assessed by measuring PenH using BioSystem XA software (Buxco Electronics, Inc.). Measurements of MetCh responsiveness were obtained by exposing mice for 3 min to incremental doses of aerosolized MetCh (Aldrich Chemie, Steinheim, Germany) and monitoring the breathing pattern for 5 min after initiation of aerosol dose.

**Collection and analysis of BAL cells**

Two days after the last i.n. challenge with OVA, mice were sacrificed by CO₂ inhalation. The trachea was cannulated and BAL was performed by flushing the airways 3 times with 0.4 ml PBS. Total BAL cells were counted using a Coulter Counter (IG Instruments, Zurich, Switzerland) and spun onto glass slides using a Cytospin 2 (Shandon Southern Products Ltd, England). Following fixation with methanol for 2.5 min, cells were stained with undiluted May-Grünwald solution (Fluka, Buchs, Switzerland) for 3 min. A further staining was performed in a
50% May-Grünwald solution for 3 min. In a last step cells were stained in 7% Giemsa solution (Fluka) for 12 min. Slides were rinsed with water and air-dried overnight. Dried cells were embedded in Eukit solution under glass cover slips. Percentages of macrophages, lymphocytes, neutrophils and eosinophils were determined microscopically by counting 200 cells/sample using standard morphological and cytochemical criteria. Total numbers of eosinophils were derived by multiplying the total number of leukocytes by the percentage of eosinophils identified morphologically.

**Generation of bone marrow chimeras**

Recipient mice were lethally irradiated (9.5 Gy) by using a 60Cobalt source and were injected i.v. with 1 x 10^7 Thy 1 depleted bone marrow (BM) cells that were collected from tibias and femurs of donor mice. After 6 weeks, reconstitution of the hematopoietic system was assessed by FACS analysis.

**Adoptive transfer of SMARTA2 (Sm2) T cells**

Naïve CD4 T cells were isolated from spleens of Sm2 or IL-21R/- sm2 mice and 1x10^7 T cells were adoptively transferred to C57BL/6. One day after transfer mice were immunized i.p. with 50 µg GP13 peptide in alum adjuvant. Ten days after immunization mice were challenged intranasally with 25 µg of gp61 on two consecutive days. Five days after the first challenge mice were sacrificed and cells isolated for analysis.

**TCR Transgenic T Cell-DC coculture**

Naive CD4 T cells were isolated from spleens by MACS bead separation following the manufacturers instructions (Miltenyi Biotech) and were found to be >90% CD4+ CD62L^high by subsequent FACS analysis. DCs were isolated from spleens of naive wild-type or IL-21R-deficient mice as described previously (21). Isolated T cells (6.5 x 10^4 cells/well) and DCs (1.4 x 10^4 cells/well) were cultured in 96-well plates in the presence of gp61 peptide at a concentration of 10nM. After 24 h T cells were analysed for expression of activation markers: cells were
Results

incubated with a biotinylated anti-CD40L antibody in IMDM at 37°C. After 4 h cells were washed twice with PBS/0.1% BSA and incubated with PE labeled anti-CD62L, APC labeled anti-CD25 and PerCP labeled streptavidin (BD Pharmingen) for 25 min at 4°C. Cells were washed extensively and analysed by flow cytometry. To assess proliferation, purified SMARTA 2 T cells were labelled with 5µM CFSE (Molecular Probes) by incubation at room temperature for 7 min. After extensive washing with IMDM, CFSE-labelled T cells were cultured in the presence of GP13 and DCs as described above. After 3 days T cells were harvested, stained with PerCP labeled anti-CD4 antibody (BD Pharmingen) and the CD4 T cells were analysed for CFSE staining by flow cytometry.

ELISA measurement of antibodies and cytokines

At the indicated time points BAL fluid, serum or culture supernatant was analysed for total IgE, specific IgG1, specific IgA, IL-4 or IFNγ. 96-well plates (Maxisorp; Nunc) were coated with anti-IgE, anti-IFNγ or anti IL-4 at 5 µg/mL in 50 µL PBS overnight at 4°C. For antigen-specific ELISAs plates were coated with OVA at 50µg/ml or with soluble leishmania antigen (SLA) at 10µg/ml overnight at 4°C. Between all of the following steps, plates were washed 5 times with PBS. Coated plates were blocked with PBS/1% BSA for 2 h at room temperature. Samples from individual mice/ cultures were serially diluted in PBS/0.1% BSA as indicated, followed by incubation at room temperature for 2 h. Thereafter, alkaline phosphatase labeled goat anti-mouse antibodies to IgE, IgG1, IgA, IL-4 or IFNγ (Southern Biotechnology Associates, Inc.), were added at room temperature for 2 h, followed by addition of the substrate p-nitrophenyl phosphate (Sigma-Aldrich). Optical density (OD) was determined at 405 nm.

Proliferation assay

A single cell suspension was made from the indicated lymph nodes by gentle teasing through 70 µm nylon cell strainers (Falcon ®) and cells washed through the strainers with IMDM into 50
ml (Falcon ®) tubes. Antigen at the indicated concentrations was diluted in 96 well plates and 5x10^5 cells were added per well. Cells were cultured for 72 hours at 37°C with [³H] thymidine added (1 µCi/well) for the last 12 hours. Total [³H] thymidine incorporation was measured as an indicator of cell proliferation.

**Intracellular cytokine staining and FACS analysis**

Approximately 5x10^5 cells from either BAL samples or in vitro culture were stimulated with PMA (10⁻⁷ M) and Ionomycin (1 µg/ml). For the final two hours, Brefeldin A (10 µg/ml) was added to the cultures. Thereafter, cells were washed with PBS/0.1% BSA and incubated with anti-CD32/CD16 mAb for 30 min at 4°C to block Fc binding. After another washing step, cells were stained with PerCp-labeled anti-CD4 mAb (BD Pharmingen) for 15 min at 4°C. For basophil detection blood samples were stained with FITC-labeled IgE and with PE-labeled CD49b after blocking. Subsequently, cells were washed with PBS/0.1% BSA, then again in PBS and fixed with 2% paraformaldehyde for 20 min at room temperature. For intracellular staining fixed cells were then incubated in permeabilization buffer (0.5% saponin/PBS/1% BSA) containing APC-labeled anti-IFNγ and PE-labeled anti-IL-4 mAb (BD Pharmingen) for 30 minutes at room temperature. Cells were washed twice in permeabilization buffer and then resuspended in PBS/1% BSA and analysed by flow cytometry (FACSCalibur; Becton Dickinson) and FlowJo software (Tree Star, Inc).

**Induction of Experimental Autoimmune Myocarditis and histological assessment**

A murine heart muscle specific peptide derived from α-myosin H chain (Ac-RSLKLMATLFLSTYASADR-OH) was used as antigen. The peptide (purity 85%; ANAWA Biochemical Services and Products, Switzerland) was dissolved in CFA (Difco, Detroit, MI) and emulsified 1/1 with PBS. Mice were immunized s.c. with 100 µg/0.2 ml on days 0 and 7. Sham-immunized controls were injected with CFA emulsified with PBS alone. Fourteen days after the second immunization mice were sacrificed, and their hearts removed, fixed in 4%
neutral buffered formalin, and processed for Haematoxylin and Eosin staining. The glass slides were coded and evaluated by a pathologist. For diagnosis of myocarditis, an inflammatory infiltrate forming foci between muscle fibers or surrounding individual myocytes, with or without associated myocyte necrosis or apoptosis, was considered essential. A vague increase in interstitial cellularity was not considered sufficient for diagnosis. Myocarditis was scored on a semi quantitative scale using grades from 0 to 4 (0, no inflammatory infiltrates; 1, small foci of inflammatory cells between myocytes or inflammatory cells surrounding individual myocytes; 2, larger foci of 100 inflammatory cells or involving 30 myocytes; 3, 10% of a myocardial cross-section involved; and 4, 30% of a myocardial cross-section involved).

**Statistics**

Statistical significance was analysed by the Student’s t test. Unless otherwise indicated data represents mean +/- standard deviation with data giving P values < 0.05 being considered statistically significant.
4.1.4. Results

Generation of IL-21R-deficient mice

IL-21R-deficient mice were generated by homologous recombination. The targeting vector for IL-21R contained regions of Exon 2 and 5 and was designed to replace the intervening IL-21R coding sequence with a 3.3kb Neomycin resistance cassette (Figure 4.1.1 A). The target vector was electroporated into 129Sv ES cells and recombinant ES clones were identified by Southern blot analysis of genomic DNA and microinjected into C57BL/6 blastocysts. Germ line transmission of the targeted allele was confirmed by Southern blot of tail genomic DNA. The resulting chimera mice were backcrossed onto either C57BL/6 or BALB/c strains. Mice were genotyped by PCR as described in the methods (Figure 4.1.1 B). The homozygous IL-21R-deficient mice were viable and fertile and showed no gross developmental abnormalities. The absence of IL-21R mRNA in cells derived from knockout mice was confirmed by RT-PCR (Figure 4.1.1 C). Proportions of monocyte, lymphocyte and granulocyte populations appeared normal in the IL-21R-deficient mice, however circulating IgE levels were increased approximately 2 fold in the IL-21R-deficient mice (data not shown).

Figure 4.1.1:

Generation of IL-21R knockout mice. (A) The genomic structure of the IL-21-receptor gene, and the targeting vector used for homologous recombination, resulting in the disruption of exons 2-5 of the wild-type gene. (B) Mice
were genotyped by PCR with the wild-type gene yielding a 503 bp product and the knock-out gene yielding a 752 bp product. (C) IL-21R mRNA from splenocytes of wild-type and IL-21R-deficient mice was assessed by RT-PCR.

**IL-21R signaling is required for Th2 responses against Helminth parasites**

Given the controversy over the role of IL-21 in Th2 immune responses, we chose to investigate two well-defined *in vivo* models of Th2 immunity, specifically, infection with the Helminth parasites *Nippostrongylus brasiliensis* (Nb) and *Heligmosomoides polygyrus* (Hp). C57BL/6 and IL-21R-deficient mice on a C57BL/6 background were infected subcutaneously with Nb and 14 days later the infiltration of cells into the airways was assessed. The infiltration of both eosinophils and lymphocytes was reduced in the absence of IL-21R signaling (Figure 4.1.2 A), supporting a role for IL-21 in the development of Th2 cells and their effector function. In addition, IgA and IgG1 levels in BAL fluid and serum were decreased in IL-21R-deficient mice, whilst IgE was similar (Figure 4.1.2 B and data not shown). We next examined the immune response against the intestinal Helminth, Hp. C57BL/6 and IL-21R-deficient mice on a C57BL/6 background were infected via oral gavage with Hp. On day 14 post infection, the proportion of basophils both in the blood and the spleen was significantly reduced in the absence of IL-21R signaling (Figure 4.1.3 A). Similarly, the proportion of eosinophils circulating in the blood was also reduced in the IL-21R-deficient mice as compared to wild-type controls (Figure 4.1.3 B). Intestinal granuloma formation was impaired both in size (Figure 4.1.3 C) and number (Figure 4.1.3 D) in the IL-21R-deficient mice following Hp infection. Splenocytes were isolated and restimulated for 3 days in the presence of Hp excretory secretory (HES) antigens, followed by a short restimulation with PMA and Ionomycin. Surprisingly, intracellular cytokine staining and FACS analysis revealed that there was no significant difference in the proportion of IL-4 or IFNγ producing CD4 T cells (Figure 4.1.3 E) although total cell counts were reduced overall (data not shown). These data indicated that the Th2 cells, which had survived the 3d culture, were still capable of producing IL-4 and IFNγ, even in the absence of IL-21R signaling. In contrast, measurement of IL-4 and IFNγ in the supernatant of the cultures by ELISA showed significantly reduced levels of IL-4 (Figure 4.1.3 F) and increased levels of IFNγ (Figure 4.1.3 G) in the absence of IL-21R signaling. Taken together, these data indicate that whilst T cells can differentiate into Th2 cells in the absence of IL-21R
Results

signaling, they have impaired expansion or survival, and consequently in vivo and in vitro Th2 responses are impaired.

Figure 4.1.2:

IL-21R signaling is required for Th2 responses against *Nippostrongylus brasiliensis*. C57BL/6 and IL-21R-deficient mice were infected subcutaneously with Nb. On day 14 post infection BAL was performed and (A) the total number of eosinophils, lymphocytes and macrophages was determined by total and differential cell counts. (B) Total IgA, IgG1 and IgE in the BAL fluid were assessed by ELISA. Data is from a representative experiment using 5 mice per group. Similar results were obtained in an independent experiment.
IL-21R signaling is required for Th2 responses against *Heligomosomoides polygyrus*. C57BL/6 and IL-21R-deficient mice were infected by oral gavage with *H. polygyrus*. On day 14 post infection (A) the proportion of basophils in the blood and spleen were determined by FACS analysis. (B) The proportion of eosinophils circulating in the blood was determined by differential cell counts of blood smears. (C) H&E staining of intestinal sections with granuloma indicated by arrows. (D) Granulomas were counted under a dissecting microscope. Splenocytes were cultured for 3 days in the presence of HES, followed by a short restimulation with PMA and ionomycin. (E) The proportion of CD4 T cells producing IFNγ and IL-4 was determined by intracellular cytokine staining and FACS analysis. (F) IL-4 and (G) IFNγ from supernatant of the sample cultures was measured by ELISA. Similar results were obtained in a repeat experiment.
IL-21R signaling is required for the development of Th2-driven allergic airway inflammation

Systemic immunization followed by airway challenge with ovalbumin (OVA) is a standard method for inducing Th2-driven allergic airway inflammation, airways hyperresponsiveness and recruitment of eosinophils. It has previously been reported that administration of exogenous IL-21 during immunization and challenge of mice with OVA impairs the ensuing recruitment of eosinophils into the airways (136). Suto et al showed that addition of IL-21 to T-helper cell cultures did not influence Th1 or Th2 differentiation (136) whilst other reports indicate that in vitro, IL-21 suppresses Th1 development and has no effect on Th2 cells (134). In an attempt to clarify such conflicting results, we immunized IL-21R-deficient and BALB/c control mice with OVA followed by intranasal challenge with OVA as described in the Methods. In contrast to the report showing exogenous IL-21 can suppress eosinophil recruitment (136), the absence of IL-21R signaling in our system resulted in reduced eosinophil recruitment as compared to BALB/c controls (Figure 4.1.4 A). Lymphocyte migration was similarly impaired, indicating that IL-21 in fact supports lymphocyte expansion/migration and eosinophil maturation/migration to the airways. Expression of IL-21R on eosinophils was not detectable by RT-PCR indicating a direct signal from IL-21 upon eosinophils was unlikely (data not shown). Ex vivo restimulation of OVA-specific lymphocytes led to reduced proliferation suggesting expansion or survival of cells in vivo might also be impaired in the absence of IL-21R signaling (Figure 4.1.4 B). In line with this data, IL-21R-deficient mice were less responsive to inhalation of increasing concentrations of MetCh indicating that this IL-13-dependent airways hyperresponsiveness was also influenced by the absence of IL-21R signaling (Figure 4.1.4 C). Notably, reduced levels of IL-13 in the BAL fluid of IL-21R-deficient mice were also detected (data not shown). OVA-specific IgG1 (Figure 4.1.4 D) and IgA (Figure 4.1.4 E) in the BAL fluid and serum (data not shown) were reduced in the absence of the IL-21R; however IgE production was enhanced (Figure 4.1.4 F) as previously reported (135). Overall, these data indicate that IL-21 plays an important role in the development of Th2-mediated allergic airway inflammation.
**Results**

Figure 4.1.4:

**IL-21R signaling is required for the development of Th2-driven allergic airway inflammation.** BALB/c and IL-21R-deficient mice were immunized i.p. with OVA protein adsorbed in alum adjuvant, followed 10 days later by i.n. challenge with OVA on 4 consecutive days. Two days after the final i.n. challenge: (A) The number of eosinophils, lymphocytes and macrophages that had infiltrated into the airways was determined by BAL and differential cell counts; (B) Lung draining lymph nodes were removed and total lymph node cells cultured in the presence of OVA protein for 72 hours. $[^{3}H]$ thymidine incorporation over the last 12 hours of culture was measured as an indicator of cell proliferation. (C) One day post i.n. challenge, mice were exposed to increasing concentrations of Acetyl-$\beta$-Methacholine-Chloride (MetCh) and airways hyperresponsiveness determined using a full body unrestrained plethysomograph. (D) OVA-specific IgG1, (E) IgA and (F) total IgE was measured in BAL fluid. Data is from representative experiments using 4-7 mice per group. Similar results were obtained in 3 independent experiments.
IL-21R-deficient Th2 cells exhibit reduced recruitment or accumulation in the airways

To assess whether the defect in Th2 responses was due to impaired T cell priming by dendritic cells, we utilized an in vitro system where either C57BL/6 or IL-21R-deficient DCs were cultured together with TCR transgenic Sm2 CD4 T cells in the presence of gp61 peptide. After 24 h we analysed the T cells for expression of activation markers (Figure 4.1.5 A) and proliferation (Figure 4.1.5 B). No differences between cells primed by C57BL/6 or IL-21R-deficient DCs were found, indicating IL-21R signaling in DCs was not required for T cell activation. We then analysed the ability of CD4 T cells lacking the IL-21R to migrate to draining lymph nodes and inflamed peripheral tissue in vivo. TCR transgenic Sm2 or IL-21R-deficient Sm2 CD4 T cells, which can be tracked in vivo by expression of Vα2, were adoptively transferred into C57BL/6 mice. Recipient mice were immunized with gp61 peptide in alum adjuvant and after 10 days challenged with gp61 peptide intranasally. We analysed draining LNs and the BAL for presence of transgenic CD4 T cells. Whilst there was no difference in the percentage of Vα2+CD4+ cells in the draining LN of both groups (Figure 4.1.5 C) we found less Vα2+CD4+ cells in the BALs of mice receiving IL-21R-deficient Sm2 T cells (Figure 4.1.5 D). Total numbers of CD4 T cells in BALs were comparable in both groups, likely representing the non-specific migration of endogenous memory CD4 T cells to the site of inflammation. In addition, eosinophil numbers in the airways of mice receiving IL-21R-deficient Sm2 cells were reduced, supporting our previous findings with Nb infection and OVA induced allergic airway inflammation (Figure 4.1.5 E). Thus, whilst homing or survival of IL-21R-deficient Sm2 cells to the draining LN appears to be normal, the recruitment or survival of IL-21R-deficient CD4 T cells to the site of inflammation is impaired. As a consequence of the reduced number of Th2 cells in the airways, there is a reduced influx of eosinophils.
Normal T cell priming but reduced T cell recruitment to the airways in the absence of IL-21R signaling. DCs isolated from either C57BL/6 or IL-21R-deficient mice were cocultured with CFSE labeled Sm2 T cells in the presence of gp61 peptide. (A) After 24h cells were collected and stained for the activation markers CD62L, CD25 and CD40L. (B) Proliferation was assessed by FACS analysis of CFSE stained cells. Sm2 or IL-21R-deficient Sm2 cells were adoptively transferred into C57BL/6 mice. Mice were immunized with gp61 peptide in alum adjuvant. After 10 days mice were challenged i.n. on 4 consecutive days. One day after the final challenge the (C) DLNs and (D) BALs were analysed for the percentage of the transferred Sm2 cells (Vα2+CD4+) by FACS. (E) The number of eosinophils in the BAL was determined by differential cell counts. Numbers in histograms represent geometric mean; numbers above gates represent percentage of cells in gate. Similar results were observed in 3 independent experiments.

Whilst it was likely that CD4 T cells were primarily accountable for the reduced Th2 responses, we also sought to clarify the contribution of non-hematopoietic cells. Accordingly, we lethally irradiated C57BL/6 mice or IL-21R-deficient mice and reconstituted the hematopoietic compartment with bone marrow (BM) derived from either C57BL/6 or IL-21R-deficient mice. After successful reconstitution we induced the model of allergic airway inflammation as described above. Analysis of draining LNs showed no significant differences in the CD4 T cell compartment (Figure 4.1.6 A). In the BAL however a clear reduction of CD4 T cells in both groups that received IL-21R-deficient derived BM as compared to the groups that received
C57BL/6 BM was evident (Figure 4.1.6 B). In line with this, the number of eosinophils was strongly reduced in IL-21R-deficient BM recipients (Figure 4.1.6 C). IL-4 production by BAL derived CD4 T cells was reduced in IL-21R-deficient BM recipients, whilst IFNγ production was comparable or elevated (Figure 4.1.6 D). These findings support the conclusion that the absence of IL-21R on CD4 T cells is the primary factor that leads to the overall reduced Th2 response. Notably, the impairment of CD4 T cell recruitment to the airways was slightly greater when IL-21R was absent in both the hematopoietic and non-hematopoietic compartments, indicating a secondary role for cells of the non-hematopoietic systems in the recruitment or accumulation of CD4 T cells to the airways.

Figure 4.1.6:
The absence of IL-21R on hematopoietic cells is primarily responsible for impaired Th2 immune responses in the lung. Reconstituted bone marrow chimeras were immunized i.p. with OVA adsorbed in alum adjuvant, followed 10 days later by i.n. challenge with OVA on 4 consecutive days. Two days after the final challenge: The
Results

The number of CD4 T cells in (A) DLN and (B) BAL was determined by FACS analysis. (C) Eosinophils that had infiltrated into the airways were determined by differential counts. (D) The proportion of CD4 T cells producing IL-4 and IFN\(\gamma\) was determined after PMA/IONOMycin restimulation by intracellular cytokine staining and FACS analysis.

**Immune response to Leishmania major is unaltered in IL-21R-deficient mice on a susceptible BALB/c background**

As Th2 responses to helminth infection and allergic airway inflammation were impaired in the absence of IL-21R signaling, we assessed the outcome of *L. major* infection in IL-21R-deficient mice on a BALB/c background. Genetically susceptible BALB/c mice mount a detrimental Th2 response that results in uncontrolled parasite replication and dissemination to visceral organs. We infected BALB/c mice, IL-21R-heterozygous and IL-21R-deficient littermates with 5x10^5 parasites into the right footpad. Assessment of footpad swelling revealed no difference within the groups (Figure 4.1.7 A). Similarly, parasite titers in footpads were comparable between IL-21R-heterozygous and IL-21R-deficient mice (Figure 4.1.7 B). IL-4 and IFN\(\gamma\) production by restimulated draining lymph node (dLN) cells was comparable in the groups analysed (Figure 4.1.7 C, D). Similarly, IL-10 production after restimulation with PMA/IONOMycin was comparable (Figure 4.1.7 E), frequencies of Foxp3+CD25+CD4+ regulatory T cells in the draining LNs were similar (Figure 4.1.7 F).
Results

Figure 4.1.7:

**Immune response to *L. major* is not altered in IL-21R-deficient (B/c) mice.** BALB/c mice, IL-21R+/+ and IL-21R-/- littermates were infected with 5x10^5 *L. major* promastigotes into the right footpad. Footpad swelling was monitored longitudinally (A). The dots represent the averages ± SD of 4-5 mice per group. (B) At day 35 after infection parasite load from the footpads was assessed by limiting dilution in the groups indicated. Draining LN cells were restimulated with SLA (3 lysed parasites/cell) for 48hrs. Supernatants were analysed for IL-4 and IFNγ by ELISA (C, D). Draining lymph node cells were restimulated in the presence of PMA/Ionomycin for 4 hrs, after 2 hours Brefeldin A was added. (E) IL-10 was detected by intracellular staining and flow cytometry. (F) Percentages of Foxp3+CD25+CD4 T cells, assessed by flow cytometry. Dots represent single mice, the lines the averages. Similar results were obtained in at least 2 independent experiments.

**Leishmania major** specific IgG1 is reduced in the sera of IL-21R-deficient (B/c) mice

We were further interested whether antibody production upon *L. major* infection was altered in the absence of IL-21R signaling. Thus, we assessed *L. major* specific antibodies in the sera at day 35 after infection. *L. major* specific serum IgG1 was reduced in IL-21R-deficient mice, while IgG2a was unaffected (Figure 4.1.8 A and B). Surprisingly, assessment of IgE after infection showed no difference between the groups (Figure 4.1.8 C).
Results

Figure 4.1.8:

*L. major* specific IgG1 is reduced in IL-21R-deficient (B/c) mice. BALB/c mice, IL-21R+/+ and IL-21R−/− littermates mice were infected with 5x10⁵ *L. major* promastigotes. On day 35 after infection mice were sacrificed and serum was collected. (A, B) *L. major* specific antibodies in the sera were detected by ELISA on previously SLA coated plates (10µg/ml). (C) Total IgE was assessed in the serum of *L. major*-infected mice by ELISA. Dots represent the averages of 4-5 mice per group ± SD.

In summary, our results indicate that the absence of IL-21R signaling does not influence the outcome of *L. major* infection in B/c mice.

**IL-21R signaling is not required to mount a resistant Th1 response against *Leishmania major***

Th2 immune responses appear to be particularly sensitive to the absence of the IL-21R on T cells. In order to clarify the role of IL-21R signaling in Th1 immunity, we assessed the ability of IL-21R-deficient mice on a C57BL/6 background to respond to *L. major* infection. Wild-type C57BL/6 mice respond to *L. major* infection with a Th1 response dominated by the induction of IFNγ that enables macrophages to kill the parasite and clear the infection (164, 202, 203). IL-21R-deficient and wild-type C57BL/6 mice were infected with 2x10⁶ parasites in the right hind footpad. The inflammatory response was monitored weekly by measuring the swelling of the infected, as compared to uninfected footpad. No difference in footpad swelling was evident between IL-21R-deficient and wild-type control mice (Figure 4.1.9 A). Restimulation of lymphocytes isolated from the draining lymph node with *L. major* soluble antigens showed a
significantly increased proportion of IFNγ producing CD4 T cells from the IL-21R-deficient mice (Figure 4.1.9 B), which became more pronounced at later time points (data not shown). These data indicate that IL-21R expression is not required for the development of Th1 immunity but in fact, may suppress it.

Figure 4.1.9:
IL-21R-deficient mice exhibit a Th1 immune response following infection with *Leishmania major*. C57BL/6 and IL-21R-deficient mice on a C57BL/6 background were infected with 2 x 10⁶ promastigotes of *L. major* in the hind footpad. (A) At the time points indicated, the course of disease was monitored using metric callipers to determine footpad swelling. (B) Three weeks post infection, the draining lymph node was removed and isolated cells were restimulated with PMA and Ionomycin for 4 hrs. IFNγ production by CD4 T cells was assessed by FACS. Values show percentage of IFNγ producing cells, gated on CD4+ cells. Data is from a representative experiment using 3-5 mice per group.

**Delayed-type hypersensitivity (DTH) is impaired in IL-21R-deficient mice**

As described above *L. major* infection in IL-21R-deficient mice on a C57BL/6 background lead to normal footpad swelling and to elevated IFNγ production. Next, we assessed memory responses to secondary immunization. Challenge with *L. major*-specific antigens leads to the induction of a Th1-driven delayed-type hypersensitivity, which results in footpad swelling caused by accumulation of Th1 cells and macrophages at the site of challenge. C56BL/6 wild-type and IL-21R-deficient mice, which had resolved the infection, were challenged with soluble *Leishmania* antigen (SLA) into the contralateral footpad. IL-21R-deficient mice clearly exhibited lower footpad swelling (Figure 4.1.10 A). Analysis of cytokine production by draining
lymph node cells after specific re-stimulation showed no differences in IFNγ (Figure 4.1.10 B) or IL-2 production (Figure 4.1.10 C).

Figure 4.1.10:

*Delayed-type hypersensitivity (DTH) is impaired in IL-21R-deficient mice.* C57BL/6 and IL-21R-deficient mice were infected with $2 \times 10^6$ *L. major* promastigotes into the right footpad. 8 weeks after infection, recovered mice were challenged with SLA (corresponding to $5 \times 10^6$ live promastigotes) in the footpad and swelling was measured up to 3 days post challenge (A). The dots indicate the averages, the error bars the SD of 3-4 mice per group. Similar results were obtained at least in 1 similar experiment. Draining popliteal LN were removed and re-stimulated with SLA for 48hs; the supernatant was analysed for IFNγ (B) and IL-2-production (C) by ELISA. Dots represent individual mice, the lines the averages.

**The development of Th17-driven Experimental Autoimmune Myocarditis is independent of IL-21R signaling**

The role of Th1 and Th2 cytokines in the development of Experimental Autoimmune Myocarditis (EAM) has been controversial. Recently however, we found that EAM is in fact mediated by the Th17 cell subset (53). We sought to establish whether IL-21R signaling was involved in the development of Th17-mediated EAM. Mice were immunized with myosin peptide in CFA on days 0 and 7, and on day 21 were sacrificed and hearts taken for histological analysis. A comparable severity and prevalence of disease developed in both IL-21R-deficient and IL-21R-heterozygous littermate controls (Figure 4.1.11 A). Histological examination revealed the infiltrate in IL-21R-deficient mice and IL-21R-heterozygous littermate controls
consisted primarily of macrophages and lymphocytes, with some granulocytes also being present (Figure 4.1.11 B). No significant difference was detected in either the number or nature of the cell infiltrate. Overall, these data show that IL-21R signaling is superfluous for the development of Th17-mediated EAM.

All EAM experiments have been performed and analysed by Ivo Sonderegger.

**Figure 4.1.11:**
IL-21R-deficient mice develop Th17-mediated Experimental Autoimmune Myocarditis. IL-21R-heterozyous and IL-21R-deficient mice were immunized with myosin-peptide in CFA on days 0 and 7, and sacrificed 14 days later. Hearts were removed, fixed in 4% buffered formalin, and histological sections evaluated. (A) The severity of inflammatory infiltrates were assessed and scored as described in the methods. (B) Representative images of H&E stained IL-21R+/- and IL-21R-/- heart tissue. Original magnification 15x, 60x and 400x. Horizontal lines in (A) represent the median value for each group.

**4.1.5. Discussion**

Extensive research has shown that cytokines belonging to the type 1 cytokine family have a broad spectrum of activities, some of which might be considered conflicting. IL-4 for example is classically associated with the polarization and effector function of Th2 cells (204) and IL-4-deficient mice are defective in Th2 responses (166). However, it has also been reported that IL-4 promotes development of CD8 cytotoxic T cells in tumor (205) and viral (206) models (typically associated with Th1, not Th2 responses). Similarly, IL-2 drives both Th1 and Th2 cell proliferation, whilst being important for stabilizing the accessibility of the *IL-4* gene and consequently Th2, not Th1 differentiation (207). IL-21 appears to follow suit in this respect,
Results

60

exhibiting pleiotropic effects in innate and adaptive immunity. A body of literature now supports a role for IL-21 in NK T cell development and effector function (208-212), and B cell antibody isotype switching (135, 136, 213-216). Furthermore, addition of IL-21 has been shown to drive IFNγ production in synergy with IL-15 (139, 142). However defining a role for IL-21 in Th1 and Th2 development remains controversial, possibly due to the nature of the in vitro systems used and the administration of exogenous cytokines.

In this manuscript, we have generated IL-21-receptor-deficient mice to allow the role of IL-21 during in vivo Th1, Th2 and Th17 disease models to be addressed. We first addressed Th2 immune responses, which are known to drive allergic airway inflammation and immunity against Helminth parasites. In a model of OVA-induced allergic airway inflammation, IL-21R-deficient mice exhibited a reduced hyperresponsiveness upon inhalation of Methacholine. In this model, AHR is typically associated with the activity of the Th2 cytokine IL-13, indicating that the IL-13 response was impaired in the absence of IL-21R signaling. In support of this result, analysis of the BAL fluid revealed reduced levels of IL-13 (data not shown). It has been shown that recruitment of eosinophils to the lung is dependent on the in vivo activity of IL-5 (217, 218), but the question remained whether the reduced eosinophil recruitment was due to an inherent defect relating to the absence of the IL-21R, or whether it was a consequence of reduced lymphocyte migration into the airways and thus reduced release of IL-5. IL-21R expression was not detectible on eosinophils (data not shown) arguing against an inherent defect in these cells. Rather, the adoptive transfer experiments indicated that migration of IL-21R-deficient Sm2 T cells into the lung was impaired whilst their survival and accumulation in the draining LN was comparable to wild-type T cells. This data was supported by the BM chimeras, which similarly showed reduced CD4 T cell numbers in the BAL of IL-21R-deficient BM recipients. Notably, the cytokine profiles of IL-21R-deficient CD4 T cells that had reached the lung showed reduced IL-4, and enhanced IFNγ production. The consequence of lower numbers of Th2 cells, and their reduced IL-4 production is the likely mechanism underlying the reduced eosinophil recruitment and airways hyperresponsiveness. It remains to be definitively shown whether the reduced CD4 T cell numbers in the lung is due to a reduced survival and accumulation or to an impairment in cell migration, however given the nature of the common gamma chain family of cytokine receptors, impaired survival is the most plausible explanation.
In the absence of IL-21R signaling, T helper cell dependent antigen specific IgA and IgG1 in the BAL were decreased in line with the reduced number of eosinophils and lymphocytes. Whilst the impaired antibody response is likely to be secondary to the decreased CD4 T cell response, it remains possible that the absence of the IL-21R on B cells may play a direct role.

Suto and colleagues (136) have shown that addition of IL-21 leads to reduced IgE and eosinophil migration in a model of OVA-induced allergic airway inflammation. Whilst our data showing increased IgE production in the IL-21R-deficient mice supports their data, it is difficult to reconcile why eosinophil numbers and IgG1 production are reduced in both the absence of IL-21R and upon addition of IL-21. Further studies are required to clarify this issue, but possibilities include: IL-21 may play distinct roles at different stages of immune responses (potentially acting in synergy with different cytokines); its reported effect may be mediated through another cell type (e.g. NK cells); or it might additionally signal through an as yet unidentified receptor.

An important question is whether IL-21, like its closest relative IL-2, is primarily involved in driving proliferation or polarization of T cells. As indicated above, the most well accepted role for IL-2 is to enhance T cell proliferation, however it plays a clear role in Th2 differentiation. Our ex vivo proliferation experiments with OVA-specific T cells indicate that IL-21R signaling is critical for T cell proliferation. Indeed, addition of IL-21 to in vitro cultures has proven to enhance proliferation ((105, 143) and data not shown), as does its closest related cytokine, IL-2. However IL-21 did not play a major role in mediating Th2 cell differentiation in vitro (134). The primary role of IL-21 in T cell differentiation appears to support IL-4 mediated Th2 polarization, rather than to drive polarization itself. In line with this hypothesis, it has been shown that T cells isolated from IL-21R-deficient mice have no defect in polarization (135). However, exposure of naïve T cells to IL-21 followed by culture in Th1 polarizing conditions resulted in a down-regulation of IFNγ expression, whilst IL-21 did not affect already polarized Th1 cells (134). Our unpublished results are consistent with that report, as we also see a reduction in IFNγ production upon addition of IL-21, which becomes more pronounced in the presence of IL-4 (data not shown). It is plausible that this phenomenon is linked to the well-established Th1/Th2 counter-regulatory paradigm, with IL-21 playing a similar and supportive role to IL-4 in the suppression of Th1 development. However, considering IL-21 signals
Results

predominantly through STAT3 (129) and IL-4 signals through STAT6 (219), is it likely that the pathways underlying this may be distinct.

Although we observed a general reduction in Th2 responses, infection with *L. major* in susceptible IL-21R-deficient B/c mice resulted in unaltered detrimental disease progression. Thus, it seems either that IL-21R signaling is not essential for *L. major*-driven Th2 responses or that the defect caused by IL-21R deficiency can be overcome by other mechanisms like e.g. continuous TCR triggering. The finding that IgE titers in the sera of IL-21R-deficient mice were comparable to IL-21R-heterozygous littermates and B/c mice would rather support the first point. However, we found a decrease in *L. major* specific IgG1 antibodies, which is probably attributed to the intrinsic B cell defect that has been described before and that was also observed during allergic airway inflammation (135).

Given that a ‘resistant’ phenotype against *L. major* is generally associated with effective Th1 immune responses, the comparable footpad swelling between C57BL/6 and IL-21R-deficient mice after infection with *L. major* suggests IL-21R signaling is not required for effective Th1 mediated-responses. Surprisingly, a significantly higher proportion of lymphocytes isolated from IL-21R-deficient mice, previously infected with *L. major*, produced IFNγ, further supporting the hypothesis that IL-21 can impair Th1 polarization.

However, enhanced IFNγ production did not result in exacerbated Th1-mediated DTH responses after *L. major* infection, although this has been demonstrated after immunization with KLH (134). In contrast, the observed DTH responses were strongly impaired in the absence of IL-21R signaling indicating a reduction in cell recruitment to the site of infection. It is possible that IL-21R-deficient mice have an advantage in parasite clearance, which would result in a lower frequency of IFNγ producing Th1 cells and thus DTH reduction. However, IFNγ production by draining LN cells was comparable, which would further allow the conclusion that this phenotype could also be mediated by other cell types like regulatory T cells, which have been shown to be suppressed in response to IL-21 (46, 56, 141).

Induction of Experimental Autoimmune Myocarditis was also comparable between wild-type and IL-21R-deficient mice. These data suggest that any contribution of Th2 cytokines to the development of this disease must be minimal, or unnecessary, for normal disease development, and further indicate that IL-21 is not required for immune responses driven by IL-17 (53).
Clearly IL-21 exerts pleiotropic effects upon the development of innate and adaptive immune responses, and thus is a potential target for therapy. Our results would indicate that neutralizing IL-21 might be beneficial for the treatment of allergic asthma. However, whilst this could potentially be effective at reducing airways hyperresponsiveness and infiltration of lymphocytes, the resulting high circulating levels of IgE could have direct consequences. In addition, inhibiting the reported role for IL-21 in NK cell effector function is unlikely to be therapeutically beneficial. Administering IL-21 however, might be a more promising alternative. Here, IgE production could be reduced, and potentially NK cell function could be enhanced, although such treatment has been shown to exacerbate EAE (220). The implications these data have on potential therapeutics remains to be seen.

Overall, our data suggests the importance of IL-21R signaling in vivo may be on three distinct levels: (i) the suppression of IFN-γ production; (ii) the direct regulation of B cell IgE isotype switching; and (iii) enhancing T cell survival. The pronounced sensitivity of Th2 responses to the absence of IL-21 signaling in vivo, indicates that this cytokine is key to Th2 rather than Th1 or Th17 inflammation.
4.2. IL-21R signaling is essential for the maintenance of CD8 T cell responses in persistent LCMV infection

Anja Fröhlich¹ and Manfred Kopf¹#

¹Institute of Integrative Biology, Molecular Biomedicine, ETH Zürich, Switzerland

#Address correspondence to: Manfred Kopf, Molecular Biomedicine, Swiss Federal Institute of Technology Zurich, Wagistrasse 27, 8952-Zurich-Schlieren, Switzerland.
Phone: +41-44-633 6470
Email: Manfred.Kopf@ethz.ch
Results

4.2.1. Abstract

Interleukin 21 (IL-21) is a member of the type 1 cytokine family including IL-2, IL-4, IL-7, and IL-15, which are central regulators of T cell development, effector and memory responses. Recent studies suggested that IL-21 mainly acts on CD4 Th subset differentiation and effector responses. However, based on some in vitro studies and in tumor models, it has been proposed that IL-21 is also important for CD8 T cell responses. In this study, we have investigated the role of IL-21R signaling in anti-viral T cell responses using IL-21R-deficient mice. IL-21R signaling was not required for the generation of protective immune responses to acute viral infections such as influenza virus, vaccinia virus, or LCMV WE. Moreover, it was superfluous for the generation, maintenance and secondary expansion of CD8 memory T cells generated upon LCMV WE infection or upon immunization with virus-like particles (VLPs). In contrast, IL-21R signaling was essential for the maintenance of proliferation and effector function of virus-specific CD8 T cells after the peak of the acute response to low dose infection with LCMV DO. In consequence, IL-21R-deficient mice were unable to control viral replication and developed persistent infection. Analysis of mixed bone marrow chimeras (WT/KO → WT) revealed that IL-21R promoted CD8 T cell expansion and function in a cell autonomous manner. Increased PD-1 and IL-10 expression has been associated with chronic LCMV infection. We have demonstrated that impaired CD8 T cell function in IL-21R-deficient mice is not due to deregulation of PD-1 surface expression by virus-specific CD8 T cells or IL-10 production. Interestingly, we found increased frequencies of Foxp3+CD25+CD4 Tregs in IL-21R-deficient mice. However, preliminary data indicate that Treg are not involved in suppression of T cell responses in IL-21R-deficient mice.

Overall, our data show that IL-21R signaling plays a crucial role in sustaining CD8 T cell responses during chronic viral infection, while it is dispensable for resolving acute viral infections and for the generation and maintenance of memory CD8 T cells.
Cytokines are key players in modulating immune responses against foreign and self-antigens. As they have a broad spectrum of activities, they help to ensure appropriate generation and maintenance of innate and adaptive responses. Interleukin-2 (IL-2), IL-4, IL-7, IL-9 and IL-15 belong to the type I cytokine family. Their receptors share the common γc chain and signal via the JAK/STAT pathway (101, 106, 199, 200). These cytokines play crucial roles in polarization, proliferation and generation and maintenance of naïve/memory cells. IL-21 is the most recent described member of this cytokine family (104, 105). The main source of IL-21 is activated CD4 T cells, however, numerous cell types express its receptor. The impact of IL-21 on CD8 T cells has been addressed in different experimental systems. However, the data are controversial. IL-21 has been shown to promote proliferation and effector function of CD8 T cells alone or in context with other cytokines of the γc receptor chain family in vitro (142-144, 209). Several studies support a role for IL-21 in augmenting anti-tumor activity of CD8 T cells by promoting proliferation and effector function (142, 145, 146, 148, 150, 221, 222), maintenance (148) or recruitment to the tumor site (147, 222). Conversely, IL-21 was shown to inhibit IFNγ production (134, 138) and to drive Ag-activated CD8 T cells to apoptosis (145). Recently, it was suggested that IL-21-mediated activation of T cells is associated with suppression of regulatory T cells (223, 224).

CD8 T cells are of great importance not only for tumor defense, but also for the control of viral infections. However, the impact of IL-21 on CD8 T cell during viral infections has only been poorly investigated. IL-21 mRNA was found in CD4 T cells from mice infected with herpes simplex virus 2 (HSV-2) and acute lymphocytic choriomeningitis virus (LCMV) (225). Zeng et al. reported impaired proliferation and effector function of CD8 T cells in IL-21R-deficient mice infected with recombinant vaccinia virus (VV) (142). Using CD8 T cells from HIV-infected patients, IL-21 was shown to augment perforin expression, but not proliferation (152).

To clarify the role of IL-21 during viral infections in vivo, we assessed anti-viral responses in IL-21R-deficient mice. We found that IL-21R was essential to prevent CD8 T cell exhaustion upon chronic LCMV infection, while it was not required for acute CD8 and CD4 T cell responses to a variety of viruses examined.
4.2.3. Material and Methods

Mice, viruses, virus-like particles and peptides

C57BL/6 wild-type mice were purchased from Charles River (Germany). IL-21R-deficient mice were generated and backcrossed as described before (226), SMARTA-2 (Sm2), lymphocytic choriomeningitis virus (LCMV) gp61 TCR transgenic mice (227) were backcrossed onto C57BL/6 background over six generations. IL-21-deficient mice (B6:129S5-IL21tn1Lex) originally obtained from NIH MMRRC (F2 129/SvEvBrd x C57BL6/J) were backcrossed three generations to C57BL/6J at the Garvan Institute, Sydney, Ly 5.1+ C57BL/6 mice were bred in our facility. Bacterial artificial chromosome-transgenic (BAC-transgenic) “depletion of regulatory T cell” mice (DEREG) were described before (228) and kindly provided by Tim Sparwasser (Technical University Munich, Germany) and backcrossed to IL-21R-deficient C57BL/6 mice in our facility. Mice were maintained specific pathogen free at the BioSupport (Zurich) animal facility in isolated ventilated cages. Animals used in experiments were between 8 and 12 weeks of age. Animal experiments were performed according to the regulation of the cantonal veterinary office.

Influenza virus strain PR8 (A/Puerto Rico8/34, H1N1) was originally provided by J. Pavlovic (University Zurich, Zurich, Switzerland). Mice were infected with 50 pfu intranasally (i.n.). The influenza-derived nucleoprotein np366-374 (np34, ASNENMETM) has been ordered from NeoMPS, Inc. (San Diego, CA). The LCMV strains WE and Docile were originally provided by Dr. R. M. Zinkernagel (University Hospital Zurich, Zurich, Switzerland) and Dr. A. Oxenius (Institute of Microbiology, ETH Zurich, Switzerland). They were propagated at low multiplicity on L929 or MDCK cells, respectively. Mice were infected with the indicated doses intravenously (i.v.). The LCMV glycoprotein peptides gp33-41 (gp33 peptide, KAVYNFATM) and gp61-80 (gp61, GLNGPDYIKGVYQFKEFD) have been described previously (229, 230) and were kindly provided by Cytos Biotechnology or purchased from NeoMPS, respectively.

Recombinant vaccinia virus expressing LCMV glycoprotein (VVG2) was originally obtained from Dr. D. H. L. Bishop (Oxford University, Oxford, UK) and grown on BSC40 cells at low
multiplicity of infection; quantification was performed as described (231). Mice were infected with 2x10^6 pfu intraperitoneally (i.p.). Gp33 VLPs, based on peptide coupled to VLPs derived from the bacteriophage Qβ have been described previously (232). Packaging of CpG oligonucleotides (5’-GGGGTCAACGTTGAGGGGG-3’, thioester stabilized) into the gp33-VLPs was performed as described previously (232). Mice were immunized subcutaneously (s.c.) into the flank with 150 µg of gp33-VLPs.

Antibodies and peptide MHC class I tetramers

PE-conjugated peptide-MHC class I tetrameric complexes against gp33 were generated as previously described (233). PE-conjugated tetramers specific for the influenza-derived nucleoprotein np34 were provided by A. Donda, University of Lausanne, Epalinges, Switzerland. The following antibodies were used: PerCp labeled anti-CD8, anti-CD4 and anti-CD11b; FITC-labeled anti-CD45.1, anti-CD62L, anti-PD-1, anti-IFNγ and anti-IL-10; PE-labeled anti-CD25 and anti-TNFα; APC-labeled anti-CD45.2 and anti-CD127, anti-IL-2 and anti-Foxp3 mAb. The PerCp labeled antibodies were purchased from BD Pharmingen, the remaining antibodies from eBioscience.

In vitro restimulation

For peptide-specific cytokine production 2.5x10^6 splenocytes were restimulated with 1µM gp33 peptide or 1µM gp61 peptide in the presence of Monensin (2µg/ml, Sigma Aldrich) for 5 hours at 37°C. For total cytokine production cells were restimulated for 2 hours at 37°C in the presence of PMA (10^{-7}M), Ionomycin (1µg/ml) and another 2 hours with Brefeldin A (5µg/ml).
Immunofluorescent stainings and analysis

For surface stainings single cell suspensions from organs were made by carefully smashing them through sterile 70µm single cell strainers (BD Biosciences) and resuspending them in PBS/2% FCS. For Fcγ-receptor blocking, cells were incubated with anti-CD16/CD32 (clone 2.4G2) antibody for 10 min at 4°C. After washing with PBS/2% FCS, cells were incubated at 4°C with peptide MHC I tetramers (20µg/ml for gp33, 5µg/ml for np34) for 20 min followed by addition of the relevant surface antibodies. After a second incubation (20 min, 4°C), samples were washed once and resuspended in PBS/2% FCS. Whole blood samples were treated with red blood lysis buffer (ACK) for 5 min before surface staining.

For intracellular staining restimulated cells were surface-stained and fixed with PBS/2% formaldehyde at room temperature for 20 min. For Foxp3 staining, unstimulated cells were surface-stained and fixed with BD FACS lysing solution (BD Biosciences). Fixed cells were incubated at 4°C for 20 minutes with permeabilization buffer (PBS, 2% FCS, 0.5% Saponin) containing the respective cytokine antibodies. Cells were washed twice and resuspended in PBS/2% FCS. Samples were analysed by four-colour flow cytometry using a FACSCalibur (Becton Dickinson), data were analysed using FlowJo software (Tree Star, Inc.).

Generation of IL-21R human Fc fusion protein and intracellular staining for IL-21

Recombinant interleukin-21 receptor was produced in the Drosophila melanogaster cell line SL-3. The extra-cellular fragment of the IL-21 receptor, amino acids 1-529, were inserted into the pRmHa3-hG1 expression vector to create a human gamma 1 fusion protein. The fusion protein was purified from supernatants of transfected SL-3 cells by protein A chromatography. Biotin-IL-21R-human Fc fusion protein (bio-IL-21RhuFc) was produced using a biotinylation kit (No. 21217, PIERCE, Rockford, Illinois) and subsequent purification (gel filtration over PD-10 Columns, GE Healthcare, Uppsala) according to the instruction manual. After fixation with PBS/2% formaldehyde, cells were incubated with bio-IL-21RhuFc diluted in permeabilization buffer for 20 min at 4°C. Subsequently, cells were washed twice with permeabilization buffer
and incubated with a mixture of streptavidin-APC and the cytokine antibody (FITC-labeled anti-IFNγ or anti-IL-17A, eBioscience).

\( ^{51} \text{Cr release assay} \)

\(^{51}\text{Cr release assays were used for the determination of gp33-specific cytotoxicity ex vivo on gp33 peptide-loaded EL-4 target cells (20 ng/ml gp33; 90 min at 37°C)} \) as described previously (231). Secondary CTL responses of influenza virus-infected mice were measured in a conventional \(^{51}\text{Cr release assay essentially as described (234)} \) using np34-peptide pulsed EL-4 target cells (20 ng/ml np34; 90 min at 37°C) and in vitro expanded peribronchial LN cells as effectors. Peribronchial cells (5 x 10^6) were stimulated for 5 days with UV-inactivated influenza virus (2x10^5 pfu) in 2 ml of IMDM containing 10% FCS. In all cases, the starting effector:target (E:T) ratio was adjusted to obtain identical ratios of gp33-specific or np34-specific CD8 T cells to target cells. Spontaneous release is indicated in the respective figure legend.

\( \text{Detection of influenza-specific antibodies} \)

At the indicated time points, serum or BAL fluid (BALF) was measured for virus-specific IgA and IgG Ab isotype levels. 96-well plates (Maxisorp; Nunc) were coated with UV-inactivated influenza virus (PR8) in PBS (2x10^5 pfu/ml) overnight at 4°C. Plates were washed and incubated with PBS/1% BSA for 2 h at room temperature (RT) for blocking. Serum and BAL fluids from individual mice were serially diluted in PBS/0.1% BSA starting with a 1/1 dilution for BAL fluids and a 1/50 dilution for serum, followed by incubation at RT for 2 h. Plates were washed five times and incubated with alkaline-phosphate-labeled goat anti-mouse Abs to IgG1, IgG2a or IgA (Southern Biotech) at a 1/1000 dilution in PBS/0.1% BSA at RT for 2 h. Thereafter, plates were washed five times and substrate p-nitrophenyl phosphate (Sigma-Aldrich) was added. ODs were measured on an ELISA reader (Bucher Biotec) at 405 nm.
Results

**LCMV-specific plaque forming assay**

LCMV-infected mice were bled at the indicated time points, blood was diluted 1/5 with MEM 2% FSC containing heparin. For the termination of viral load in organs the recovered tissue was transferred in 1ml of MEM 2% FCS and smashed by using a tissue lyser (Retsch GmbH, Haan, Germany). Blood and organs were stored at -80°C until the day of analysis. Plaque assays were performed as described previously on MC57 cells (235).

**BrdU incorporation**

Short labeling: starting from day 10 after infection mice were injected with 100µl PBS containing 1mg of BrdU (Sigma) on 4 consecutive days. Long term labeling: mice received BrdU in the drinking water at a concentration of 0.8 mg/ml during 7 days prior to analysis. At the indicated time points spleens were recovered, smashed and stained with PE-labeled anti-CD8 (eBioscience) or gp33 tetramer as described above. For the analysis of bone marrow chimeras, CD8 T cells were purified from the spleen by MACS bead separation following the manufacturers instructions (Miltenyi Biotech) and stained with PE-labeled anti-CD45.1. Cells were processed as described previously (236) and stained with FITC-labeled anti-BrdU (BD Pharmingen). BrdU incorporation was analysed by flow cytometry.

**Ex-vivo proliferation**

CD8 T cells were purified from spleens of infected mice by positive MACS bead separation. Dendritic cells (DCs) were isolated from spleens of naïve mice as described previously (21). For CFSE labeling, CD8 T cells were washed in PBS, CFSE (Molecular Probes, C-1157) was added at a final concentration of 5µM in PBS, cells were rocked gently during a labeling time of 7 minutes at room temperature. CFSE was quenched with pure FCS and washed twice with IMDM/10% FCS. 5x10^4 CD8 T cells were plated with 1x10^4 DCs in the presence of increasing amounts of gp33 peptide. Recombinant IL-21 (rIL-21) was purchased from R&D systems, IL-2
Results

derived from a human IL-2-producing hybridoma (IL-2-t-6 supernatant, dilution 1:200). After 2 days of culture CFSE dilution was assessed by flow cytometry.

Generation of bone marrow chimeras

For the generation of bone marrow chimeras recipient mice were lethally irradiated (9.5 Gy) by using a 60Cobalt source and were injected i.v. with $10^7$ Thy 1 depleted (MACS beads) bone marrow (BM) cells that were collected from tibias and femurs of donor mice. After 6 weeks hematopoietic reconstitution of mice was checked by flow cytometry.

Real-time quantitative PCR

CD4 and CD8 T cells were isolated from spleens of infected mice at the indicated time points by MACS sorting. After washing the cells with PBS, total RNA was isolated using TRI Reagent (Molecular Research Center, Inc.) and treated with DNase (Invitrogen) to avoid genomic DNA contamination, followed by reverse transcription using Super Script III RT (Invitrogen). Quantitative real-time RT-PCR was performed using Brilliant SYBR Green (Stratagene) on an i-Cycler (Bio-Rad Laboratories). Expression was normalized to the house keeping gene β-actin. Primer sequences used: β-actin 5′-TGTCATCCTGCTTTCTTCTC-3′ and 5′-GCACCTTTGAAGCCCTAC-3′; IL-21 5′-CGCCTCTGATTAGACTTCG-3′ and 5′-ATGCTCACAGTGCCCTT-3′.

Statistical analysis:

Student’s $t$ test was used to assess the statistical significance between groups. Significance is depicted as *, $p < 0.05$. 
4.2.4. Results

IL-21R signaling is required for the maintenance of gp33-specific CD8 T cells during persistent LCMV Docile infection

IL-21 has been shown to promote CD8 T cell responses in vitro (142, 144) and in vivo (150). Moreover, IL-21 mRNA is expressed during viral infections like HSV-2 and LCMV (225). Thus, we have addressed immune responses to viral infections in the previously described IL-21R-deficient mice (226). Infection with the fast replicating LCM virus strain Docile (LCMV DO) in mice leads to different disease manifestations dependent of the infectious dose: low dose infections are controlled in wild-type mice, while intermediate and high dose infection lead to viral persistence. The higher the viral burden in the mice the more the virus-specific CD8 T cells suffer from functional exhaustion, which means loss of proliferation, effector cytokine production and cytotoxicity (173). We infected wild-type C57BL/6 and IL-21R-deficient mice with 3 different doses of LCMV DO: 200 pfu (low), 2000 pfu (intermediate) and 2x10^6 pfu (high). We followed the kinetic of virus-specific CD8 T cells in the blood using tetramers loaded with the immunodominant epitope from the glycoprotein gp33 at various days during infection. At day 8 post infection, the percentage of gp33-specific CD8 T cells (gp33+/CD8+) was comparable between wild-type and IL-21R-deficient mice independent of the infectious dose (Figure 4.2.1 A-C). However, from day 14 onward, we observed a strong reduction in percentages of gp33-specific CD8 T cells in the blood of IL-21R-deficient mice infected with low, intermediate and high doses. To find out whether the phenotype we observed in the blood reflects the situation in lymphoid organs, we sacrificed groups of mice at days 8, 15 and 35 after infection and assessed the percentage of gp33-specific CD8 T cells in the spleen (Figure 4.2.1 D-F). In line with the data we obtained from the blood, we found no differences comparing wild-type and IL-21R-deficient mice at day 8. However, virus-specific CD8 T cells were significantly reduced at days 15 and 35 in IL-21R-deficient mice infected with intermediate and high doses (Figure 4.2.1 E, F).
Results

Upon infection with low doses, the reduction of virus-specific CD8 T cells in spleens of IL-21R-deficient mice was not significant (Figure 4.2.1 E, F). These results demonstrate that activation and expansion of CD8 T cells in the acute phase of LCMV DO infection is normal in IL-21R-deficient mice, while maintenance of the response is strikingly impaired in particular with an infection dose that allows establishment of a more persistent infection.

Figure 4.2.1:
IL-21R signaling is required for the maintenance of gp33-specific CD8 T cells during persistent LCMV Docile infection. C57BL/6 wild-type and IL-21R-deficient mice were infected i.v. with 200 (low), 2000 (intermediate) and 2x10^6 pfu of LCMV DO. Blood was taken at the indicated time points after infection and percentages of gp33-specific CD8 T cells were analysed by tetramer staining and flow cytometry. Graphics depict percentages of virus-specific CD8 T cells within the CD8 fraction (gp33+/CD8+) from mice infected with 200 (A), 2000 (B) and 2x10^6 pfu (C). Dots represent the averages of 4 mice, error bars show ± SEM. At days 8 (D), 15 (E), and 35 (F) after infection, mice were sacrificed and splenocytes were analysed for gp33-specific CD8 T cells by tetramer staining and flow cytometry. The graphs show percentages of gp33+/CD8 T cells at indicated infection doses. Dots represent individual mice, lines the averages. * indicates significant differences (p ≤ 0.05). Similar results were obtained in at least 2 independent experiments.
**Results**

**Effector cytokine production by specific T cells is altered in IL-21R-deficient mice upon infection with persistent LCMV Docile**

Chronic viral infection as induced by high dose infection with LCMV DO (or LCMV clone 13) results in deletion of virus specific T cells (Figure 4.2.1) and a decline in effector function including production of IL-2 and IFNγ (179). We have observed a more pronounced decline in gp33-specific CD8 T cells in IL-21R-deficient mice. To address effector function of LCMV specific T cell, C57BL/6 wild-type and IL-21R-deficient mice were infected with various doses of LCMV DO. We sacrificed groups of mice at days 8, 15 and 35 after infection and restimulated CD8 T cells (Figure 4.2.2 A-C) and CD4 T cells (Figure 4.2.2 D-F) with gp33 and gp61 peptide, respectively. At day 8 after infection, IFNγ production by IL-21R-deficient CD8 T cells was comparable to that of wild-type CD8 T cells after low, intermediate and high dose infection (Figure 4.2.2 A). In contrast, in a late stage of infection at day 35, the frequency of IFNγ producing cells was strikingly reduced in IL-21R-deficient mice infected with low or intermediate doses (Figure 4.2.2 C). Expectedly, CD8 T cells from high dose infected wild-type mice displayed an “exhausted” phenotype as they produced less IFNγ compared to CD8 T cells from mice infected with low or intermediate doses. Notably, CD8 T cell exhaustion tended to be even more pronounced in high dose infected IL-21R-deficient mice, although the reduction of IFNγ-producing cells did not reach statistically significant levels (Figure 4.2.2 C). Similar results were observed at day 15, except that IFNγ-producing CD8 T cells were comparable in wild-type and IL-21R-deficient mice infected with low doses.

CD4 T cells have been described as the main producers of IL-21 and are known to confer help to CD8 T cells. Therefore, we asked whether the absence of IL-21R signaling impairs anti-viral cytokine production by CD4 T cells after LCMV DO infection. Indeed, we found that the frequency of gp61-specific IFNγ-producing CD4 T cells was significantly compromised in IL-21R-deficient mice compared to wild-types infected with a range of doses (Figure 4.2.2 D). Surprisingly, no such differences were observed at days 15 or 35 after infection (Figure 4.2.2 E-F).
Figure 4.2.2:
Effector cytokine production by specific T cells is altered in IL-21R-deficient mice upon infection with LCMV Docile. C57BL/6 wild-type and IL-21R-deficient mice were infected i.v. with 200, 2000 and 2x10^6 pfu of LCMV DO. At time points indicated on the left, mice were sacrificed and spleens were recovered. Splenocytes were restimulated in vitro with gp33 and gp61 peptide (1μM each) in the presence of Monensin for 5 hours. The proportion of CD8 and CD4 T cells producing IFNγ was assessed by intracellular cytokine staining and subsequent flow cytometric analysis. Shown is the frequency of IFNγ+ cells of CD8 T cells (A-C) and CD4 T cells (D-F) at indicated infection doses (specified below the graph). The horizontal lines indicate averages, dots represent values of individual mice, (*p ≤ 0.05). Similar results were obtained in at least 2 independent experiments.
Taken together, expansion and effector function of anti-viral CD8 T cells is unaffected in the absence of IL-21R in the acute phase of infection with LCMV DO, while it is essential for the maintenance of effector and memory CD8 T cell responses. In contrast, IL-21R appears critical for efficient anti-viral CD4 T cell responses in the acute effector phase but not in the memory phase of LCMV DO infection.

**Viral clearance upon LCMV Docile infection is strongly impaired in the absence of IL-21R signaling**

CD8 T cells are critical for clearance of LCMV. To assess whether reduced numbers and impaired function of virus specific CD8 T cells in IL-21R-deficient mice affects viral clearance, we determined viral load longitudinally in the blood and in different organs at day 35 after infection. Viral titer was considerably increased in the kidney, liver, lung, and spleen of IL-21R-deficient mice (Figure 4.2.3 D-F). Even with low dose infection, which was cleared in wild-type mice by day 11 (Figure 4.2.3 A), virus was detected in the blood of IL-21R-deficient mice until day 28 (Figure 4.2.3 A-C). Thus, we conclude that the reduced anti-viral CD8 T cell response in IL-21R-deficient mice during the chronic phase of LCMV DO infection is associated with impaired viral control.
Figure 4.2.3: IL-21R-deficient mice exhibit higher viral load in blood and organs during LCMV Docile infection. C57BL/6 wild-type and IL-21R-deficient mice were infected i.v. with 200, 2000 or $2 \times 10^6$ pfu of LCMV DO and blood was taken at the time points indicated. At day 35 post infection, mice were sacrificed and kidney, liver, lung and spleens were removed. Viral titers in blood (pfu/ml blood, A-C) and organs (pfu/organ, D-F) were determined by plaque forming assays, the horizontal line indicates the limit of detection. Dots represent values of individual mice, the lines the averages, (*p ≤ 0.05). Similar results were obtained in another experiment.
Acute immune responses to LCMV Do infection are not altered in IL-21R-deficient mice

The above results suggest that CD8 T cells responses to LCMV DO are normal during the acute phase of response. To confirm these data, we infected C57BL/6 wild-type and IL-21R-deficient mice with low (200 pfu) and high (2x10⁶ pfu) doses of LCMV DO and analysed spleens and organs at day 8 after infection. Percentages of gp33-specific CD8 T cells in spleens were comparable in wild-type and IL-21R-deficient mice with the respective infection dose (Figure 4.2.4 A and D). Moreover, cytolytic activity of CD8 T cells was comparable within the respective infection dose (Figure 4.2.4 B and E). When restimulated with gp33 peptide, CD8 T cells from both strains produced similar amounts of IFNγ and TNFα after low or high dose LCMV DO infection (Figure 4.2.4 C and F). Accordingly, the viral load in organs analysed was similar (Figure 4.2.4 G and H).

Taken together these data demonstrate, that CD8 T cell responses during the acute phase of LCMV DO infection in IL-21R-deficient mice are comparable to wild-type mice at day 8 after infection independent of the initial infectious dose.
**Figure 4.2.4**

**Early immune response to LCMV Docile is not altered in IL-21R-deficient mice.** C57BL/6 wild-type and IL-21R-deficient mice were infected with 200 (low dose) (A-C, G) or 2x10^6 (high dose) pfu of LCMV DO (D-F, H). At day 8 after infection, mice were sacrificed, spleens and organs were recovered. (A, D) Percentages of gp33-specific CD8 T in the spleen were assessed by tetramer staining and flow cytometry. Values show averages ± SD of 3 mice per group. (G, H) Viral titers in organs were determined by plaque forming assay. Dots show values of individuals, except for spleens where dots represent values from a pool of 3. (B, E) CD8 T cells were isolated from spleens and direct ex vivo cytotoxicity was measured in a conventional 51Cr release assay using gp33 peptide pulsed EL-4 target cells. E:T indicates the ratio between CD8 T cells and gp33 peptide pulsed EL-4 target cells. Non-specific lysis of un-pulsed EL-4 cells was below 11%. Dots represent the averages ± SD. (C, F) Splenocytes were restimulated with gp33 peptide (1µM) in the presence of Monensin for 5 hrs. Intracellular cytokine production by CD8 T cells was assessed by flow cytometry. Shown are dot plots of an individual mouse representative for the group, averages of 3 mice ± SD (indicated in the quadrant).
IL-21R signaling in CD8 T cells is responsible for inefficient maintenance of CD8 T cells after chronic LCMV Docile infection

During chronic viral infection, the failure to clear virus is typically associated with CD8 T cell exhaustion (173). Thus enhanced viral titer observed in IL-21R-deficient mice may be responsible for reduced CD8 T cell numbers and function late in infection. To ask whether IL-21R signaling directly regulates CD8 T cell responses independent of viral dose, we generated mixed bone marrow (BM) chimeras by lethal irradiation of wild-type mice and reconstitution with CD45.1+ IL-21R+/+ and CD45.2+ IL-21R−/− bone marrow. We infected the mixed BM chimeras with an intermediate dose of LCMV DO (2000 pfu) and followed percentages of gp33-specific IL-21R+/+ (CD45.1) and IL-21R−/− (CD45.2) CD8 T cells in the blood. Consistent with the data obtained in wild-type and IL-21R-deficient mice, percentages of virus-specific CD8 T cells lacking the IL-21R (in an IL-21R competent host) were strikingly reduced in blood (Figure 4.2.5 A) and spleen (Figure 4.2.5 B) during the late phase of response (i.e. days 18, 25, and 35), while frequencies of IL-21R−/− and IL-21R+/+ virus specific CD8 T cells were comparable in the acute phase (day 7, Figure 4.2.5 A). Moreover, the ability to produce IFNγ was almost completely abrogated in the absence of IL-21R on both virus-specific CD8 and CD4 T cells (Figure 4.2.5 C and D). Our data from mixed BM chimeras demonstrate that maintenance and IFNγ production of IL-21R-deficient virus-specific CD8 T cells is impaired in a wild-type environment. Notably, exhaustion of T cells lacking the IL-21R is even more pronounced in mixed chimeras compared to global IL-21R-deficient mice. These data suggest that IL-21-IL-21R interaction directly regulates maintenance and function of CD8 T cells and not indirectly for instance by another soluble factor with altered expression in the IL-21R-deficient mice.
Figure 4.2.5:

The absence of IL-21R on CD8 T cells is responsible for the inefficient maintenance of CD8 T cells after chronic LCMV Docile infection. C57BL/6 wild-type mice were lethally irradiated and reconstituted with wild-type (CD45.1+) and IL-21R-deficient (CD45.2+) bone marrow cells in the ratio 1:1. (A) Chimera mice were infected with 2000 pfu of LCMV DO, blood was taken at the indicated time points after infection and percentages of gp33-specific CD8 T cells within the CD45.1+ or CD45.2+ CD8 T cell fraction were determined by tetramer staining and flow cytometry. (B) At day 35 after infection, mice were sacrificed, spleens were recovered and percentages of gp33-specific CD8 T cells within CD45.1+ and CD45.2+ CD8 T cell fraction were assessed by tetramer staining and flow cytometry. (C and D) Splenocytes were restimulated with gp33 or gp61 peptide (1μM each) and IFNγ production by CD8 and CD4 T cells was assessed by intracellular staining and flow cytometry. The graphs depict the frequency of IFNγ producing cells within the CD45.1+ or CD45.2+ CD8 or CD4 T cell compartment. Dots represent single mice, the lines the averages (*p ≤ 0.05). Similar results were obtained in at least 2 independent experiments.
IL-21 is mainly produced by CD4 T cells and peaks at day 14 after chronic LCMV Docile infection

So far, our data show that IL-21R signaling on CD8 T cells is essential for their maintenance and function during LCMV Docile infection. To investigate which cells produce IL-21 during acute and chronic LCMV Docile infection, we purified CD8 and CD4 T cells from spleens of mice infected with low, intermediate, and high doses (LCMV Docile) at days 8, 15 and 35 and assessed the level of IL-21 mRNA by quantitative real-time PCR. CD4 T cells clearly expressed more IL-21 mRNA than CD8 T cells at all time points investigated (Figure 4.2.6 A and B, note the different scale). Independent of the infection dose, mRNA levels in CD4 T cells peaked at day 15 after infection (Figure 4.2.6 A), while IL-21 production by CD8 T cells was most prominent at day 8 (Figure 4.2.6 B). The highest level of IL-21 mRNA was found in CD4 T cells after intermediate dose infection. These data indicate that CD4 T cells are the main producers of IL-21 mRNA after LCMV Docile infection and that the production peaks at the time point when CD8 T cell exhaustion in IL-21R-deficient mice was observed first (i.e. day 15). CD8 T cells contain low levels of IL-21R mRNA, which peaks 8 days after infection and therefore follows different kinetics compared to CD4 T cells.

Figure 4.2.6:
IL-21 is mainly produced by CD4 T cells and peaks at day 14 after chronic LCMV Docile infection. Analysis of IL-21 mRNA expression by quantitative real time PCR. C57BL/6 wild-type mice have been infected with low (200 pfu), intermediate (2000 pfu) and high (2x10^6 pfu) doses of LCMV Docile. At the time points indicated, spleens of 4 mice were pooled and T cells were purified by MACS sorting. Expression of IL-21 mRNA was analysed by quantitative real-time PCR in CD4 (A) and CD8 (B) T cells and normalized by the house keeping gene β-actin. Values show averages of duplicates ± SD. Similar results have been obtained in a second independent experiment.
IL-17 production by T cells upon chronic LCMV Docile infection is low and unspecific

So far, our results indicate that CD4 T cells are the main producers of IL-21 upon LCMV DO infection. Next, we were interested whether IL-17 producing T cells emerge upon infection as they have been shown to highly produce IL-21 (56). We infected C57BL/6 wild-type mice with 200, 2000 and $2 \times 10^5$ pfu LCMV DO and assessed IL-17 production by CD8 and CD4 T cells after specific restimulation with gp33 or gp61 peptide, respectively, 8 and 15 days after infection. A very small population (0.5-2%) of IL-17-producing CD8 (Figure 4.2.7 B, C) and CD4 T (Figure 4.2.7 D, E) cells could be detected ex vivo in infected mice, which were not seen in uninfected mice (data not shown). However, antigen-specific restimulation did not further increase the frequency of IL-17-producing cells (Figure 4.2.7 A-E) suggesting that this population may be bystander activated T cells or they are specific for other epitopes of LCMV. Our data indicate that IL-17 production by T cells upon acute and chronic LCMV DO infection is negligible.

Figure 4.2.7:
**IL-17 production upon LCMV Docile infection is low and unspecific.** C57BL/6 wild-type mice were infected with 200, 2000 and $2 \times 10^5$ pfu of LCMV DO. Spleens were recovered at days 8 and 15 after infection and splenocytes were restimulated for 5 hrs with g33 or gp61 peptide (1µM each) in the presence of Monensin. Controls were restimulated without peptide. CD8 and CD4 T cells were stained intracellularly for IL-17 and IFNγ.
Results

and were analysed by flow cytometry. (A) Dot plots of IL-17- and IFNγ-producing CD8 (upper panel) and CD4 T cells (lower panel) with and without peptide restimulation from an individual representative of a group of 3. (B-E) Values show frequencies of IL-17 producing CD8 T cells (B, C) and CD4 T cells (D, E) with and without restimulation at days 8 (B, D) and 15 (C, E) after infection. Dots represent individual mice, the lines indicate averages of groups.

IL-21R-deficient CD8 T cells show impaired proliferation upon chronic LCMV Docile infection

IL-21 has been shown to promote proliferation of CD8 T cells alone or in combination with other cytokines binding to receptors sharing the γ chain (99). Thus, we sought to investigate the proliferation capacity of IL-21R-deficient CD8 T cells after LCMV DO infection. BrdU is a nucleotide analogue and gets incorporated into the DNA during cell division allowing to monitor cell proliferation in vivo. We infected C57BL/6 wild-type and IL-21R-deficient mice with 2000 pfu LCMV DO and fed them with BrdU in the drinking water over a period of 7 days prior to analysis. At day 8 after infection, BrdU incorporation by CD8 T cells from wild-type and IL-21R-deficient mice was similar (Figure 4.2.8 A). At day 15, however, CD8 T cells from IL-21R-deficient mice had incorporated significantly less BrdU (81.15%±3.44 vs. 65.83%±3.26, Figure 4.2.8 A) indicating impaired cell division between days 9-15 post infection. Similar results were obtained in a second experiment, in which mice infected with 2000 pfu LCMV DO were injected i.p. with BrdU daily between days 10-14 on both CD8 and gp33-specific cells (Figure 4.2.8 B and C).

To rule out the influence of viral load on CD8 T cell exhaustion in IL-21R-deficient mice, we compared BrdU-incorporation in wild-type and IL-21R-deficient CD8 T cells isolated from mixed BM chimeras at day 14 after infection. In this situation, IL-21R-deficient CD8 T cells showed a two-fold reduction (53.67% ±4.18 vs. 26.67% ±2.60) in BrdU incorporation (Figure 4.2.8 D). Thus, in a situation when IL-21R-deficient and wild-type CD8 T cells competitively expand and collapse in LCMV infected wild-type mice, the differences in BrdU incorporation between wild-type and knockout T cells is much bigger than comparing CD8 T cell BrdU incorporation in individual wild-type and IL-21R-deficient mice indicating that the increased viral load partially masked the effect of defective T cell responses by stronger T cell stimulation.
Although the reason for decreased BrdU incorporation by IL-21R-deficient CD8 T cells is most likely impairment in proliferation, it was still possible that these cells were more prone to die by apoptosis. To address this possibility, we infected BM chimeras with 2000 pfu of LCMV DO and injected BrdU (i.p.) from day 10 to 13. The frequency of BrdU+ wild-type and IL-21R-deficient CD8 T cells was analysed 6 days later. The results show that frequencies of BrdU+ CD8 T cells are comparably reduced in both wild-type and IL-21R-deficient mice at day 19 compared to day 14 indicating cell death. Frequency of BrdU+ IL-21R-deficient CD8 T cells was still 2-fold lower compared to IL-21R competent CD8 T cells (21.27% ±1.82 vs. 9.67% ±1.12, Figure 4.2.8 E).

Taken together, these results suggest that IL-21R-deficient CD8 T cells show a defect in expansion and not increased cell death in response to LCMV DO infection.

**Figure 4.2.8:**

IL-21R-deficient CD8 T cells show impaired proliferation upon chronic LCMV Docile infection. C57BL/6 wild-type and IL-21R-deficient mice were infected with 2000 pfu of LCMV DO and received BrdU in the drinking water during 7 days prior to analysis. (A) At days 8 and 15 post infection, mice were sacrificed and splenocytes were stained for CD8 and BrdU and analysed by flow cytometry. The graphic depicts the percentage of CD8 T cells that had incorporated BrdU. Values show averages ± SD of 4 mice per group. Wild-type and IL-21R-deficient mice
were infected with 2000 pfu LCMV DO and were injected i.p. with BrdU from day 10 to 13. At day 14, mice were sacrificed, spleens were recovered and splenocytes were stained for CD8 (B) or gp33-specific tetramer (C) in context with BrdU and analysed by flow cytometry. Similar results were obtained in 2 independent experiments.

Bone marrow from CD45.1+ IL-21R+/+ and CD45.2+ IL-21R-/− mice was transferred to irradiated C57BL/6 wild-type mice. Mixed BM chimaeras were infected with 2000 pfu of LCMV DO and injected with BrdU from day 10 to 13. At day 14 (D) and day 19 (E) CD8 T cells were purified from the spleen by MACS sorting and stained for CD45.1 (wild-type). The graph shows the percentage of CD8 T cells that have incorporated BrdU within the CD45.1- (IL-21R-deficient) and the CD45.1+ (wild-type) fraction. Dots represent individual mice, the lines show the averages (* p ≤ 0.05).

**Impaired CD8 T cell proliferation in the absence of IL-21 receptor signaling late after infection**

Next, we assessed proliferation of C57BL/6 wild-type and IL-21R-deficient CD8 T cells by CFSE dilution at a late time point after infection. Wild-type and IL-21R-deficient mice infected with low (200 pfu) and high (2x10^6 pfu) doses of LCMV DO were sacrificed at day 35, splenic CD8 T cells were isolated, labeled with CFSE and cocultured with naïve splenic DCs in the presence of gp33 peptide. Cell proliferation was assessed by CFSE dilution 3 days later. Wild-type CD8 T cells from low dose infected mice proliferated intensively, while IL-21R-deficient CD8 T cells failed to proliferate (Figure 4.2.9 A). Expectedly, CD8 T cells isolated from high dose infected wild-type and IL-21R-deficient mice did not proliferate upon gp33 peptide restimulation due to exhaustion (Figure 4.2.9 B).
Figure 4.2.9:

**In vitro proliferation of IL-21R-deficient CD8 T cells is impaired after low dose LCMV Docile infection.**

Groups of 4 C57BL/6 wild-type and IL-21R-deficient mice were infected with 200 pfu or 2x10^6 pfu of LCMV DO. CD8 T cells were purified from the spleens 35 days after infection, pooled, labeled with CFSE and cultured with naïve CD11c+ DCs (1x10^4 DCs + 5x10^4 CD8 T cells) in the presence of different gp33 peptide concentrations (indicated above the dot plots). After 3 days, CFSE dilution by CD8 T cells from low (A) and high dose infected mice (B) was assessed by flow cytometry.

Next, CD8 T cells isolated from high dose infected wild-type mice were restimulated with gp33 peptide in the presence and absence of rIL-21 or rIL-2 to investigate whether these cytokines can restore proliferation of exhausted CD8 T cells. Interestingly, IL-2 but not IL-21 restored proliferation of exhausted CD8 T cells (Figure 4.2.10 A). Moreover, combination of IL-2 and IL-21 did not increase proliferation above levels seen with IL-2 only. While these data are preliminary, they indicate that IL-21 does not rescue proliferation of exhausted CD8 T cells after LCMV DO infection.
Results

Figure 4.2.10:
IL-2, but not IL-21, rescues proliferation of exhausted CD8 T cells after LCMV Docile infection. C57BL/6 wild-type and IL-21R-deficient mice were infected with 2x10⁶ pfu of LCMV DO. At day 35 after infection, mice were sacrificed and CD8 T cells were purified from the pooled spleens of wild-type and IL-21R-deficient mice, CFSE-labeled and cultured together with CD11c+ DCs, gp33 peptide and recombinant IL-21 (25ng/ml), IL-2 (500U/ml) or a mixture of both. Proliferation by CFSE dilution was assessed by flow cytometry 3 days later. (A) Proliferation of wild-type (upper panel) and IL-21R-deficient (lower panel) CD8 T cells under the condition indicated above the dot plots in the presence of gp33 peptide (1µM). Similar results were obtained with lower doses of peptide (100, 10 and 1 nM). Dot plots represent one out of a duplicate.

PD-1 expression is unaltered in IL-21R-deficient T cells after LCMV Docile infection

So far, we have shown that CD8 T cell exhaustion is promoted in the absence of IL-21R signaling during chronic LCMV DO infection. PD-1, a negative regulator of T cell responses belonging to the B7 super-family, is highly expressed on exhausted T cells and its blockade restores T cell function (76). Thus, we wanted to investigate whether IL-21R-deficiency is associated with increased PD-1 expression on CD8 T cells. Expectedly, PD-1 expression declined on specific CD8 T cells after low dose infection between days 15 and 35, but remained...
Results

up-regulated after high dose infection of wild-type mice (76). C57BL/6 wild-type and IL-21R-deficient mice showed comparably high expression of PD-1 on gp33-specific CD8 T cells at days 8 and 15 independent of the infection dose (Figure 4.2.11 D and E). However, specific CD8 T cells of IL-21R-deficient mice remained high in contrast to their wild-type counterparts at day 35 after low dose infection (Figure 4.2.11 F). No differences were observed comparing PD-1 expression on all CD8 T cells at days 8, 15 and 35 after infection (Figure 4.2.11 A-C).

Figure 4.2.11:
IL-21R-deficient T cells exhibit no major increase in PD-1 expression compared to wild-type T cells after LCMV Docile infection. C57BL/6 wild-type and IL-21R-deficient mice were infected with 200, 2000 and 2x10^6 pfu of LCMV DO. Mice were sacrificed at the indicated time points and spleens were recovered. Splenic CD8 T cells (A, B, C) and gp33-specific CD8 T cells (D, E, F) were analysed for PD-1 expression by flow cytometry. The graphs depict the percentage of gated cells expressing PD-1. Dots represent individual mice, the lines the averages. Similar results have been obtained in 2 independent experiments.

To compare PD-1 expression on IL-21R-deficient and IL-21R-competent CD8 T cells at the same viral load, we analysed mixed BM chimeras. PD-1 expression was comparably high on both IL-21R-deficient and IL-21R-competent gp33-specific CD8 T cells at days 15 and 35 post infection (Figure 4.2.12 B, D). Surprisingly, PD-1 expression was reduced on total CD8 T cells lacking IL-21R at day 15 and, in particular, at day 35 after infection (Figure 4.2.12 A, C).

Taken together, our data indicate that increased exhaustion in the absence of IL-21R signaling is
not associated with up-regulation of PD-1 expression. Surprisingly, PD-1 expression appeared even to be reduced on entire population of CD8 T cells lacking IL-21R.

Figure 4.2.12:
Analysis of PD-1 expression on CD8 T cells from mixed bone marrow chimeras. C57BL/6 wild-type (CD45.1) and IL-21R-deficient (CD45.2) mixed BM chimeras were infected with 2000 pfu of LCMV DO. At days 15 and 35 after infection, spleens of chimeric mice were recovered and splenocytes were analysed for expression of PD-1, gp33-specific TCR, CD8 and CD45.2 by flow cytometry. The number in the histograms indicate the percentage of PD-1 expressing wild-type (CD45.2-) and IL-21R-deficient (CD45.2+) CD8 T cells (A and C) or gp33-specific CD45.2- and CD45.2+ CD8 T cells (B and D) at the indicated time points. * \( p \leq 0.05 \) between the CD45.2- and CD45.2+ cell subsets. A representative histogram from 1 out of 3 mice is shown in (A) and (B). A representative histogram from 1 out of 9 mice is shown in (C) and (D).
IL-10 production by CD11b+ cells is not increased in IL-21R-deficient mice

Recent reports demonstrated up-regulation of IL-10 production by APCs upon chronic LCMV infection (clone 13) and that blockade of its signaling pathway restored T cell function and enhanced viral elimination (183, 184). Thus we wanted to investigate whether CD8 T cell exhaustion in IL-21R-deficient mice is associated with increased IL-10 production. Intracellular staining of IL-10 production showed that CD11b+ macrophages were the only cells producing IL-10 in C57BL/6 wild-type and IL-21R-deficient mice early and later after infection with 2000 pfu LCMV DO. At days 8 and 15 after infection, percentages of IL-10 producing CD11b+ cells were comparable in wild-type and IL-21R-deficient mice after infection with 2000 pfu of LCMV DO (Figure 4.2.13 A and B). However, late after infection (day 35) percentages of IL-10-producing CD11b+ cells were slightly elevated in IL-21R-deficient mice (Figure 4.2.13 C). These data indicate that impaired CD8 T cell function and virus control in IL-21R-deficient CD8 T cells is independent of IL-10, at least at day 15 after infection. At a late stage of infection (i.e.), we cannot exclude that increased frequency of IL-10+ macrophages contributes to inhibition of CD8 T cells.
Figure 4.2.13:

**IL-10 production by CD11b+ cells is not increased in IL-21R-deficient mice.** C57BL/6 wild-type and IL-21R-deficient mice were infected with 2000 pfu of LCMV DO. At time points indicated, mice were sacrificed, spleens were recovered and splenocytes were incubated in the presence of Monensin and gp33 peptide for 5 hours. Intracellular IL-10 production by CD11b+ cells was assessed by flow cytometry at days 8 (A), 15 (B) and 35 (C) post infection. Dot plots are representative for 1 out of 4 mice, the numbers in the quadrants indicate the group averages ± SD. The graphs show percentages of IL-10+ cells within the CD11b+ cell fraction. Dots represent individual mice, the lines the averages (*p ≤ 0.05).
The role of regulatory T cells (Tregs) in chronic LCMV Docile infection

Percentages of Foxp3+CD25+CD4+ Tregs rises with increased doses of LCMV Docile

Foxp3+CD25+CD4+ regulatory T cells (Tregs) are important immune regulators. During viral infections they can act either beneficial or detrimental on immune responses dependent on the infectious model (237). So far, the role of Tregs during LCMV infection has not been clarified. We infected C57BL/6 mice with 200 (low), 2000 (intermediate) and 2x10^6 (high) pfu of LCMV DO and determined percentages of Foxp3+CD25+CD4+ Tregs in spleens (Figure 4.2.14 A). Infection with high but not low or intermediate dose resulted in an increase in Tregs at day 8 (Figure 4.2.14 B). Intermediate dose infection resulted in postponed Treg expansion peaking at day 15, while Tregs did not expand at all after low dose infection (Figure 4.2.14 C). Until day 35, Treg numbers declined again to base line or close to base line after intermediate and high dose infection, respectively (Figure 4.2.14 D).

These data indicate a correlation between percentages of Foxp3+CD25+CD4+ Tregs and the infection dose. Thus, Tregs may contribute to the phenomenon of T cell exhaustion by suppressing T cell responses especially upon infection with high doses of LCMV DO.
Results

Figure 4.2.14:
Percentages of Foxp3+CD25+CD4+ Tregs rise with increased infection doses of LCMV Docile. C57BL/6 mice were infected with 200, 2000 and 2x10^6 pfu of LCMV DO. (A) Dot plots show surface CD25 and intracellular Foxp3 expression gated on CD4 T cells at day 15 after infection of an individual mouse representative of 4 mice per group. (B-D) Values show percentages Foxp3+CD25+ of CD4 T cells at indicated infection doses and time points after infection of individual mice. Lines indicate the averages (*p ≤ 0.05).

Depletion of Tregs in DEREG mice does not affect virus-specific T cell responses

Next, we investigated T cell responses upon LCMV DO infection in the absence of Tregs. For this purpose, we used BAC-transgenic DEREG mice, which express GFP and a receptor for Diphtheria Toxin (DT) under control of the Foxp3-promotor (228). It has been shown that treatment of those mice with DT selectively depletes GFP+Foxp3+ cells (228).

We infected C57BL/6 wild-type and DEREG mice with either 2000 pfu (intermediate dose) or 2x10^5 pfu (high dose) of LCMV DO and injected DT at days 8, 10, 12 and 14 prior analysis at day 15. This protocol was chosen because the major increase in Tregs was observed between day 8 and 15 after infection (Figure 4.2.14 C). DT treatment of DEREG mice efficiently eliminated all GFP+Foxp3+ cells (Figure 4.2.15 A). However, a considerable population of GFP-Foxp3+CD4 T cells representing up to 40% of all Foxp3+ cells (Figure 4.2.15 C, D) was not depleted. Further characterization of these cells revealed that they were composed of CD25+
Results

and CD25- cells (Figure 4.2.15 B). Next, we assessed whether depletion of GFP+CD4+ Tregs affected percentages of gp33-specific CD8 T cells. As shown in (Figure 4.2.15 D and F), they were comparable within the groups and the infectious doses indicating that partial Treg depletion has no consequences on proliferation or maintenance of virus-specific CD8 T cells.

As observed previously (Figure 4.2.14 C), percentages of Tregs were increased at day 15 after high compared to intermediate infectious doses, although the difference was statistically not significant in this experiment (Figure 4.2.15 C and D).

Figure 4.2.15:
Depletion of Tregs in DEREG mice does not lead to an increase in gp33-specific CD8 T cells after LCMV Docile infection. DEREG and C57BL/6 wild-type control mice were infected with 2000 or 2x10^5 pfu of LCMV DO. Groups of 4 wild-type and DEREG mice were treated with 1µg DT at days 8, 10, 12 and 14 post infection. Mice were sacrificed at day 15 and splenocytes were analysed. Dot plots show expression of Foxp3 versus GFP (A) and Foxp3 versus CD25 (B) gated on CD4 T cells from an untreated (left) and treated (right) DEREG mouse (2000 pfu), representative of groups of 4. (C, D) Shown are percentages of Foxp3+CD4 T cells and gp33+CD8 T cells in DT-treated/untreated mice after infection with 2000 (C, E) or 2x10^5 pfu (D, F). Symbols represent individual mice and lines the averages.

We further investigated whether effector cytokine production by LCMV-specific T cells was influenced by GFP+CD4+ Treg depletion. CD8 and CD4 T cells were restimulated in vitro with gp33 and gp61 peptide, respectively. IFNγ and TNFα production by CD8 T cells from treated and untreated DEREG mice were similar after intermediate dose infection (Figure 4.2.16 A and
Results

B). However, TNFα production was significantly increased in DT treated DEREG mice after high dose infection (Figure 4.2.16 F). Surprisingly, we found also significant differences in TNFα production in untreated wild-type and DEREG mice, which may reflect experimental variations. Effector cytokine production by virus-specific CD4 T cells was comparable in wild-type and DEREG mice, DT-treated or untreated, with both infection doses (Figure 4.2.16 C and D, G and H).

Although these data come from a single experiment and need to be reproduced (in particular the unexpected and inconsistent differences), they suggest that Treg-depletion has no major consequence on the cytokine production by LCMV-specific CD8 and CD4 T cells indicating that Tregs play a minor role during LCMV DO infection.

Figure 4.2.16:
Depletion of Tregs does not influence effector cytokine production by CD8 or CD4 T cells. DEREG and C57BL/6 wild-type control mice were infected with 2000 or 2x10^5 pfu of LCMV DO. Groups of 4 wild-type and DEREG mice were treated with 1μg DT (i.p.) at days 8, 10, 12 and 14 post infection. Mice were sacrificed at day 15 and spleens were recovered. Splenocytes were restimulated for 5 hours in the presence of gp33 or gp61 peptide (1μM each) together with Monensin. Controls were cultured without peptide. Cytokine production was assessed by intracellular staining and flow cytometry. Values in the graphs show frequencies of IFNγ (A, E, C, G) or TNFα (B, D, F, H) producing cells of virus-specific CD8 (A, B, E, F) or CD4 T cells (C, D, G, H) after infection with indicated doses of LCMV DO. Dots represent individual mice, the lines the averages (* p ≤ 0.05).
GFP+ CD4+ Tregs do not produce cytokines upon specific restimulation after LCMV Docile infection

Suppressive function of inducible and natural Tregs has been linked to their ability of antigen-specific IL-10 production (63). Therefore, we asked whether Tregs observed after infection with LCMV were antigen specific IL-10-producers. To this end, we assessed IL-10 production by GFP+CD4+ Tregs in DEREG mice after restimulation with gp61. Expectedly, GFP+CD4+ Tregs did not produce the effector cytokines IFNγ and TNFα (Figure 4.2.17 A and B, upper panel). In line with published data (238), GFP+CD4+ Tregs were also incapable to produce IL-2 (Figure 4.2.17 A and B, lower panel). Similarly GFP+CD4+ Tregs failed to produce IL-10 in response to gp61 stimulation. From these data we conclude that GFP+Tregs that emerge in DEREG mice after chronic LCMV DO infection are not specific for the gp61 peptide.
Results

Figure 4.2.17:
GFP+CD4+ Treg do not produce cytokines after specific restimulation upon LCMV Docile infection.
DEREG mice were infected with 2000 and 2x10^5 pfu of LCMV DO. At day 15 after infection, mice were
sacrificed, spleens were recovered and splenocytes were restimulated with gp61 (1µM) in the presence of Monensin
for 5 hours. GFP+ CD4 T cells were analysed for intracellular IFNγ and TNFα (A and B, upper panel) or IL-10 and
IL-2 production (A and B, lower panel). Controls were cultured without peptide. Representative dot plots from 1
out of 4 mice are shown.
Percentages of Foxp3+CD25+CD4+ Tregs in IL-21R-deficient mice are increased after chronic LCMV Docile infection

Previously, it was demonstrated that IL-21 could inhibit the generation of Foxp3+CD4+ Tregs in vitro (56, 224). Thus, we assessed whether IL-21R-deficiency influences frequencies of Foxp3+CD25+CD4+ Tregs after infection with LCMV DO. Expectedly, Foxp3-expression was only found in CD4, but not in CD8 T cells (Figure 4.2.18 A and B).

At day 8 after infection, percentages of Tregs were comparable in C57BL/6 wild-type and IL-21R-deficient mice (Figure 4.2.18 C). As described above, number of Tregs increased at day 15 and returned to baseline at day 35 in wild-type mice. In comparison, frequencies of Tregs were strikingly increased in IL-21R-deficient mice at days 15 and 35 (Figure 4.2.18 D and E). Thus, our data show that T cell exhaustion and increased viral titer is associated with elevated levels of Tregs in IL-21R-deficient mice.

Figure 4.2.18:
Percentages of Foxp3+CD25+CD4+ Tregs in IL-21R-deficient mice are increased after chronic LCMV Docile infection. C57BL/6 wild-type and IL-21R-deficient mice were infected with 200, 2000 and 2x10⁶ pfu of LCMV DO. Mice were sacrificed at days 8, 15 and 35 after infection. Percentages of Foxp3-expressing CD25+CD4 T cells (A) or CD25+CD8 T cells (B) in spleens were determined by intracellular staining and flow cytometry. Dot plots are representative from 1 out of 4 mice (day 8 p.i., 2000 pfu). Graphs show percentages of Foxp3+CD25+CD4+ Tregs in wild-type and IL-21R-deficient mice after infection with the doses indicated at days 8 (C), 15 (D) and 35 (E) after infection. Dots represent individual mice, the lines the averages (*p ≤ 0.05).
Increased percentages of Foxp3+CD25+CD4+ Tregs in IL-21R-deficient mice is not related to increased viral load

Our previous data strongly suggested a positive correlation between the induction of Foxp3+CD25+CD4+ Tregs and the infection dose. Therefore, we wanted to investigate whether the increase in Tregs observed in IL-21R-deficient mice could be a consequence of their inability to control the virus. To rule out the influence of viral load on Treg-induction, we analysed frequencies of Tregs in mixed wild-type (CD45.1+) and IL-21R-deficient (CD45.2+) BM chimeras infected with 2000 pfu of LCMV DO. At day 35 after infection, percentages of IL-21R-deficient Tregs were clearly increased suggesting that this phenotype was independent of the viral burden, but dependent on IL-21R signaling (Figure 4.2.19 A and B).

**Figure 4.2.19:**
Increased percentages of Tregs in IL-21R-deficient mice do not correlate with viral load. C57BL/6 wild-type (CD45.1+) and IL-21R-deficient (CD45.2+) mixed BM chimeras were infected with 2000 pfu of LCMV DO. At day 35 after infection, spleens were recovered and splenocytes were analysed for percentages of Foxp3+CD25+CD4 T cells within the CD45.1+ and the CD45.2+ compartment by flow cytometry. (A) Dot plots show intracellular staining of Foxp3 in context with CD25 by CD45.1+ CD4 T cells (left panel) and CD45.2+ CD4 T cell (right panel). Dot plots are representative from 1 mouse out of 9. (B) Percentages of Foxp3+CD25+ cells within CD45.1+ and CD45.2+ CD4 T cells are shown. Dots represent individual mice, the lines the averages (*p ≤ 0.05).
Partial depletion of Foxp3+Tregs does not alter T cell responses or viral load in IL-21R-deficient mice

We were now interested whether LCMV DO-mediated induction of Foxp3+CD4 Tregs in IL-21R-deficient mice resulted in suppression of T cell responses and thus contributed to viral persistence. We infected DEREG and DEREGxIL-21R-/-- mice with intermediate doses of LCMV DO and depleted GFP+CD4+ Tregs by DT injection as done previously. As observed in the previous experiment with DEREG mice, Treg depletion was incomplete (Figure 4.2.20 A), as 25-50% of Foxp3+CD4 T cells remained in various organs after DT treatment (Figure 4.2.40 B-E).

Figure 4.2.20:
DT treatment of DEREG and DEREGxIL-21R-/-- mice does not lead to complete depletion of Tregs in the analysed organs. DEREG and DEREGxIL-21R-/-- mice were infected with 2000 pfu of LCMV DO. Mice were left untreated (n=4) or treated (n=4) with 1 µg DT (i.p.) at days 8, 10, 12 and 14 after infection. At day 15, mice were sacrificed and the spleen, liver, kidney, and bronchoalveolar lavage (BAL) was recovered. T cells from liver and kidney were isolated by Percoll gradient. Cells were stained with anti-CD4 and anti-Foxp3 mAbs and analysed by flow cytometry. (A) Shown are dot plots of splenocytes gated on CD4+ cells expressing Foxp3 and GFP from an individual representative for a group of 2-4 mice. (B-E) Values show percentages Foxp3+CD4 T cells in organs indicated. Symbols represent individual mice and the lines indicate the averages.
Comparing frequencies of gp33+CD8 T cells in various organs including spleen, liver, kidney, and BAL we found no significant differences before and after DT treatment suggesting that development and organ recruitment of virus specific CD8 T cells is not regulated by Foxp3+ GFP+ Tregs both in wild-type and IL-21R-deficient mice (Figure 4.2.21 A-E).

**Figure 4.2.21:**
**Depletion of Tregs in DEREGxIL-21R-/− mice does alter percentages of gp33-specific CD8 T cells.** DEREG and DEREGxIL-21R-/− mice were treated and cells of various organs purified as described above (Figure 4.2.20). (A) Shown are dot plots of splenocytes after staining with gp33 tetramers and anti-CD8 mAb and flow cytometry. Data are representative of 1 out of 2-4 mice per group. The graphs show percentages of gp33-specific CD8 T cells in the spleen (B), liver (C), kidney (D) and BAL (E). Symbols represent individual mice, the horizontal line the averages (*p ≤ 0.05).

Next, we analysed cytokine production by short-term (5h) restimulation of splenic CD8 and CD4 T cells with gp33 and gp61 peptide, respectively. Surprisingly, frequencies of IFNγ and TNFα producing CD8 T cells were considerably increased in IL-21R+/+DEREG mice but remained unaltered IL-21R-/−DEREG mice upon DT treatment (Figure 4.2.22 A-C). Cytokine production by CD4 T cells was not influenced by DT treatment in both groups of mice (Figure 4.2.22 D and E).
Figure 4.2.22:
Depletion of Foxp3+CD4+ Tregs does not lead to an increase in effector cytokine production by IL-21R-deficient T cells after LCMV Docile infection. DEREG and DEREGxIL-21R-/- mice were treated as described in Figure 4.2.20. Mice were sacrificed at day 15 after infection and spleens were recovered. CD8 and CD4 T cells were restimulated with gp33 or gp61 peptide (1µM each) in the presence of Monensin for 5 hrs. Controls were re-stimulated in the presence of Monensin without peptide. IFNγ and TNFα production by virus-specific CD4 and CD8 T cells was assessed by intracellular staining and flow cytometry. (A) Dot plots show intracellular IFNγ and TNFα staining of CD8 T cells. Data are representative of 1 out of 2-4 mice per group. The graphs depict the percentages of IFNγ (B) and TNFα (C) producing CD8 T cells within the indicated groups. Similarly, IFNγ and TNFα production by CD4 T cells is shown (D, E). Dots represent individual mice, the lines the averages (*p ≤ 0.05).

We further assessed whether Treg-depletion had an impact on viral load. Viral titers in liver, kidney, lung, and brain were comparable in treated and untreated DEREGxIL-21R-/- mice (Figure 4.2.23 A-D). In contrast, virus clearance appeared to be more efficient in peripheral organs of DEREG mice treated with DT (Figure 4.2.23 A-D).

In summary, these data demonstrate that depletion of Foxp3+CD4+ Tregs in IL-21R-deficient mice does not influence number of virus specific T cells, their function, or viral clearance. In this experiment, however, Treg depletion in wild-type mice results in improved CD8 T cell function and viral clearance. It should be noted that this experiment has been performed only once and the results are therefore preliminary. The role of Treg in LCMV infection is under further investigation in the group of M. Kopf.
Figure 4.2.23:
Depletion of Tregs in IL-21R-deficient mice does not lead to a reduction in viral titers. DEREG and DEREGxIL-21R−/− mice were treated as described previously (Figure 4.2.20). Mice were sacrificed at day 15 after infection, liver, kidney, lung, and brain were recovered and homogenized. Viral titers were determined by plaque forming assays. The graphs depict the viral load in pfu/organ in (A) liver, (B) kidney, (C) lung and (D) brain. The horizontal line at 10^2 pfu indicates the limit of detection in this assay. Dots represent individual mice, the lines the averages (*p ≤ 0.05).
Results

Percentage of CD62L+CD127+ CD8 T cells in the spleen is increased in IL-21R-deficient mice after LCMV Docile infection

Expression of CCR7, CD62L and CD127 has been used to characterize central versus effector memory CD8 T cells (88). These markers have also been used to discriminate effector cells (T_E) effector memory cells (T_EM) or central memory cells (T_CM) after LCMV infection (239, 240). However, virus-specific CD8 T cells expressing characteristics of T_CM do not arise after chronic LCMV DO infection (240).

We addressed whether IL-21R deficiency influences the expression of CD62L and CD127 on emerging gp33-specific CD8 T cells after LCMV DO infection. C57BL/6 wild-type and IL-21R-deficient mice were infected with 2000 pfu of LCMV DO and the kinetics of CD62L and CD127 expression on gp33-specific CD8 T cells was assessed in the blood. At day 8 after infection, the majority of virus-specific CD8 T cells displayed a T_E phenotype (CD62L-CD127-) and percentages were comparable between the groups (Figure 4.2.24 A, left). However, at days 15 and 21, we observed a considerable reduction in T_E population (CD62L-CD127-) in IL-21R-deficient mice consistent with reduced effector function and proliferation described above (Figure 4.2.24 A, middle and right). In addition, we observed an increase in the CD62L+CD127+ population, which has been previously defined as T_CM (86, 240). However, definition of classical T_CM may not apply to LCMV infection (241). A similar pattern of CD62L and CD127 expression was found on the total CD8 T cell population (Figure 4.2.24 B).
Figure 4.2.24: Percentage of CD62L+CD127+ CD8 T cells is increased in IL-21R-deficient mice after LCMV Docile infection. C57BL/6 wild-type and IL-21R-deficient mice were infected with 2000 pfu of LCMV DO and blood was taken at indicated time points for analysis of CD62L and CD127 expression on gp33+CD8 T cells (A) and global CD8 T cells (B) by flow cytometry. Dot plots show samples of individual mice representative for groups of 3.
Immune response to acute LCMV WE is not altered in IL-21R-deficient mice

Next, we were interested whether IL-21R-deficient mice show a similar defect upon infection with LCMV WE, which usually does not induce chronic infection in immunocompetent mice. Interestingly, no differences were observed in frequencies of gp33-specific CD8 T cells in the blood of C57BL/6 wild-type and IL-21R-deficient mice upon infection with 2000 pfu of LCMV WE during the entire observation time until day 45 (Figure 4.2.25 A). Evaluation of the viral titers showed that both groups were similarly able to clear the virus by day 11 (Figure 4.2.25 B). Spleen analysis at day 35 after infection revealed similar frequencies of virus-specific CD8 T cells (Figure 4.2.25 C) and normal effector cytokine production upon specific restimulation (Figure 4.2.25 D). Furthermore, CD8 T cells from IL-21R-deficient mice were not impaired in proliferation (Figure 4.2.25 F) or showed any differences in CD62L and CD127 expression (Figure 4.2.25 E).

Our data demonstrate, that IL-21R signaling is not required to resolve an acute LCMV WE infection.
Figure 4.2.25:

Immune response to acute LCMV WE is not altered in IL-21R-deficient mice. C57BL/6 wild-type and IL-21R-deficient mice were infected with 2000 pfu of LCMV WE. Blood was taken at time points indicated to determine (A) percentages of gp33-specific CD8 T cells by tetramer staining and (B) viral titers (the horizontal line indicates the limit of detection). (E) CD62L and CD127 expression on gp33-specific cells was assessed by flow cytometry, circles indicate the average of 3 mice ± SD. At day 35 post infection, mice were sacrificed and frequencies of splenic gp33-specific CD8 T cells were assessed by flow cytometry (C). CD8 T cells were restimulated with gp33 peptide (1µM) in the presence of Monensin for 5 hours. IFNγ and TNFα production was assessed by intracellular staining and flow cytometry (D). Symbols represent single mice and horizontal lines indicate averages. (F) Splenocytes of each group were pooled and CD8 T cells were isolated. CD11c+ DCs were isolated from a naïve wild-type mouse and co-cultured with wild-type or with IL-21R-deficient CD8 T cells (1x10^5 DCs, 5x10^4 CD8 T cells) in the presence of the indicated gp33 peptide concentrations for 3 days. CFSE dilution was assessed by flow cytometry. Dot plots are representative from duplicates of each group.
Intracellular detection of IL-21 after LCMV WE infection

Next we aimed at identification and quantification IL-21-producing cells. Unfortunately, detection of intracellular IL-21 with the available IL-21 mAb is impossible. To circumvent this problem, colleagues in our group (J. Kisielow, J. Weber) generated an IL-21R-Fc fusion protein. As Th17 cells are the most potent producers of IL-21, transgenic Sm2 CD4 T cells were polarized under Th17 conditions (56) and IL-21 production was assessed by detection with the biotinylated form of the IL-21R-Fc protein (Figure 4.2.26 A). The data demonstrate that under these conditions, a considerable proportion of IL-21+IL17+ and IL-21+IL-17- CD4 T cells could be measured. We then measured IL-21 production by T cells after LCMV WE infection using T cells from IL-21-deficient mice as controls. CD4 T cells producing IL-21 were detectable after stimulation with gp61 and PMA/Ionomycin (Figure 4.2.26 B, upper and lower panel, respectively).
**Figure 4.2.26:**

**Intracellular detection of IL-21.** Purified transgenic Sm2 CD4 T cells were cocultured with DCs and gp61 peptide (1μM) in the presence of TGFβ (5ng/ml) and IL-6 (20ng/ml) for 3 days. (A) Dot plots show intracellular staining of: FITC-labeled anti-IL-17 with streptavidin-APC to monitor staining background (left panel), biotinylated α-γδ T cell receptor antibody followed by staining with anti-IL-17 FITC/streptavidin-APC to assess unspecific antibody binding (middle panel) and with biotinylated IL-21R-Fc fusion protein/anti-IL-17 FITC/streptavidin-APC (right panel). C57BL/6 wild-type and IL-21-deficient mice (control) have been infected with 2000 pfu of LCMV WE. At day 8 after infection, spleens were recovered and splenocytes were restimulated with either gp61 (1μM) and Monensin for 5 hrs or with PMA/Ionomycin for 4 hours (Brefeldin A was added after 2 hours). (B) The upper panel shows representative dot plots of intracellular staining with IFNγ and biotinylated IL-21R Fc fusion protein/streptavidin-APC in wild-type (left) and IL-21-deficient (right) CD4 T cells after gp61 peptide restimulation. The histogram shows the IL-21 expression of IFNγ-producing wild-type and IL-21-deficient CD4 T cells. The lower panel shows in a similar arrangement IL-21 production in CD4 T cells restimulated with PMA/Ionomycin/Brefeldin A. Dot plots and histograms are representative of 1 mouse out of 3.
Next, we assessed the kinetics of IL-21 production by T cells after LCMV WE infection. At days 8 and 15 after LCMV WE infection, splenic CD8 and CD4 T cells were restimulated with the specific peptides and IL-21 production was assessed. At both time points, IL-21 was detectable in a population of CD4 T cells (which coordinately produced IFNγ) (Figure 4.2.27 B), while it was absent in CD8 T cells (Figure 4.2.27 A).

These data demonstrate that IL-2R-Fc is a new tool for quantification of IL-21-producing cells in vitro and in vivo and that CD4 T cells are the main producers of IL-21 after LCMV WE infection. We are currently running a similar analysis after LCMV DO infection.

Figure 4.2.27:
**Kinetic of IL-21 production in T cells upon LCMV WE infection.** C57BL/6 wild-type and IL-21-deficient mice (control) were infected with 2000 pfu of LCMV DO. At days 8 and 15 after infection, splenocytes were re-stimulated with gp33 or gp61 peptide (1μM each) in the presence of Monensin for 5 hours. Cells have been stained intracellularly with anti-IFNγ and IL-21R-Fc as described above. Shown are histograms gated on (A) IFNγ+CD8 and (B) IFNγ+CD4 T cells at indicated times. Cells from an IL-21-deficient mouse served as negative control. Shown is an individual mouse representative for 3.
Immune responses to infection with influenza virus are not altered in IL-21R-deficient mice

Next, we used the well-established model of influenza infection to assess immune response to an acute cytopathic virus in IL-21R-deficient mice. The virus infects lung epithelia and induces rapid CTL and antibody responses. The latter are essential for viral elimination (188). Analysis of BAL recovered from C57BL/6 wild-type and IL-21R-deficient mice at day 10 after infection, which is the peak of lymphocyte infiltration, showed no differences in numbers of total leucocytes or influenza-specific np34+CD8 T cells (Figure 4.2.28 A and B). Similarly, production of IFNγ and TNFα by BAL CD8 T cells restimulated with PMA/Ionomycin was comparable (Figure 4.2.28 C). Furthermore, CD8 T cells from lung draining LNs exhibited unimpaired Ag-specific cytotoxicity (Figure 4.2.28 D). Determination of influenza-specific IgM (Figure 4.2.28 E and H) and IgG2a (Figure 4.2.28 F and I) in the serum and the BAL-fluid revealed no differences. However, influenza-specific IgG1 was slightly reduced in IL-21R-deficient mice (Figure 4.2.28 G and J).

Taken together, these data demonstrate unimpaired T and B cell responses upon influenza infection in the absence of IL-21R signaling. The observed impairment in IgG1 class switching has been observed previously in other model systems (135).
Figure 4.2.28:

Immune responses to infection with influenza virus are not altered in IL-21R-deficient mice. C57BL/6 wild-type and IL-21R-deficient mice were infected i.n. with 50 pfu of influenza virus (PR8). At day 10 after infection, mice were sacrificed, the bronchoalveolar lavage (BAL) was performed, lung-draining LNs were recovered and blood was taken. Total BAL cells were counted, (A) the percentage of np34-specific CD8 T cells was assessed by tetramer staining and flow cytometry, total numbers were calculated according to the total BAL cells (B). Dots represent individual mice, the lines the averages. (C) BAL cells were restimulated with PMA/Ionomycin, IFNγ and TNFα production was assessed by intracellular staining and flow cytometry. The bars show the mean of 4-5 mice per group + SD. Lung draining LN cells from wild-type and IL-21R-deficient mice were expanded over 5 days in the presence of UV-inactivated influenza virus. Cells were added to 51Cr-labeled np34 peptide-pulsed EL-4 cells at the ratios indicated, and incubated for 5 h before measurement of 51Cr release (D). Nonspecific lysis of unpulsed EL-4 cells was below 10%. Dots represent the averages of 4-5 mice per group + SD. Levels of influenza-specific Ab isotypes in serum and BALF were measured on virus-coated plates by ELISA. IgM, IgG2a and IgG1Ab isotypes are shown from sera (E, F and G) and the BALF (H, I and J). Dots represent the averages of 4-5 mice per group + SD. Similar results were obtained in at least 2 independent experiments.
Primary immune response to vaccinia (VVG2) infection is not altered in IL-21R deficient mice

We further assessed the outcome of acute infection with vaccinia virus (VV), where the acute CD8 T cell response is dependent on CD4 T cell help (A. Oxenius, personal communication) (196, 197). Infection with a VV variant carrying the glycoprotein of LCMV (VVG2) allowed monitoring of virus-specific CD8 T cells using gp33 tetramers. C57BL/6 wild-type and IL-21R-deficient mice were infected with 2x10^6 pfu and analysis of spleen and ovaries was performed 7 days later. In both organs, percentages of gp33-specific were comparable (Figure 4.2.29 A and B, left). In line with this, virus-specific CD8 T cells were not altered in their effector cytokine production after specific restimulation (Figure 4.2.29 A and B, middle and right). Similarly, CD4 T cells from ovaries produced similar amounts of effector cytokines after PMA/Ionomycin restimulation (Figure 4.2.29 C).

Thus, our data indicate that IL-21R-deficient mice mount normal T cell responses to infection with VVG2.

Figure 4.2.29:
Primary immune response to vaccinia (VVG2) infection is not altered in IL-21R deficient mice. C57BL/6 wild-type and IL-21R-deficient mice were infected with 2x10^6 pfu of VVG2 (i.p.). At day 7 after infection, percentages of gp33-specific CD8 T cells in the ovaries and spleens were determined by tetramer staining and flow cytometry (A, B, left). Ovary cells and splenocytes were restimulated with gp33 peptide (1µM) in the presence of Monensin for 5 hours and the percentage of CD8 T cells producing IFNγ and TNFα was assessed by intracellular cytokine staining and flow cytometry (A, B, middle/right). Ovary cells were restimulated with PMA/Ionomycin for 4 hours, Brefeldin A was added during the last 2 hours. IFNγ and TNFα production by CD4 T cells was assessed by flow cytometry (C). Dots represent single mice, the lines the averages.
Generation and recall potential of memory CD8 T cells is intact in IL-21R-deficient mice

Previous reports suggested that IL-21 and IL-15 synergize in regulation of proliferation of naïve and memory CD8 T cells (142). We therefore sought to investigate whether IL-21R signaling is involved in generation and maintenance of CD8 T cell memory. Immunization with replication-incompetent virus-like particles (VLPs) carrying the gp33 peptide of LCMV together with CpGs leads to the generation of a protective gp33-specific memory CD8 T cell population (232). C56BL/6 wild-type and IL-21R-deficient mice were immunized with gp33-VLPs repeatedly every 7 days until day 21. Analysis of gp33-specific CD8 T cells showed a strong increase in gp33-specific CD8 T cells after the 1st and 2nd challenge in the blood of wild-type mice. A similar increase was observed in the blood of IL-21R-deficient mice (Figure 4.2.30 A).

At day 42 after the first immunization, the mice were challenged with vaccinia virus VVG2 and the immune response at day 7 after infection was analysed. Percentages of virus-specific CD8 T cells in the ovaries were comparable (Figure 4.2.30 B). Restimulation of ovary CD8 and CD4 T cells with PMA/Ionomycin resulted in comparable production of IFNγ and IL-2 (Figure 4.2.30 C and D).
Figure 4.2.30:

Generation of CD8 T cell memory by VLP-immunization is intact in IL-21R-deficient mice. C57BL/6 wild-type and IL-21R-deficient mice were immunized s.c. at days 0, 7 and 14 with 150 µg of gp33-VLPs. (A) Seven days after each immunization, blood was taken and percentages of gp33-specific CD8 T cells were assessed by tetramer staining and flow cytometry. At day 42, mice were challenged with 2x10^6 pfu of VVG2. Seven days after infection, ovaries were recovered and frequencies of gp33+CD8 T cells was determined (B). Ovary cells were restimulated in the presence of PMA/Ionomycin for 4 hrs, Brefeldin A was added during the last 2 hours. IFNγ and IL-2 production by CD8 (C) and CD4 (D) T cells was assessed by intracellular staining and flow cytometry. Symbols represent individual mice and horizontal lines the averages. Similar results have been obtained in 2 independent experiments.

We have shown above that development of virus-specific effector and memory CD8 T cells is unaffected in IL-21R-deficient mice following acute and resolved LCMV WE infection. To assess the function of these memory cells, groups of LCMV WE infected mice were challenged with high doses of LCMV DO and the expansion of virus-specific memory CD8 T cells was monitored during the first week after challenge (96). As shown in (Figure 4.2.31 A) IL-21R-deficient mice showed a slight delay in expansion of gp33-specific CD8 T cells at day 3 but no
differences were observed at days 5 and 7. Moreover, splenic CD8 T cells produced similar amounts of IFNγ after specific restimulation at day 7 after challenge (Figure 4.2.31 B).

Figure 4.2.31:
Memory CD8 T cells generated after LCMV WE infection expand normally after LCMV DO infection in IL-21R-deficient mice. C57BL/6 wild-type and IL-21R-deficient mice were infected with 2000 pfu of LCMV WE. At day 50 after primary infection, mice were challenged with 2x10⁶ pfu of LCMV DO. (A) At the indicated time points, blood was taken and the percentages of gp33-specific CD8 T cells in the blood were assessed by tetramer staining and flow cytometry. (B) At day 7 after challenge, mice were sacrificed, spleens were recovered and splenocytes were re-stimulated in the presence of gp33 peptide (1µM) and Monensin for 5 hrs. Cells were stained for CD8 and intracellularly for IFNγ. Percentages of cytokine-producing cells were assessed by flow cytometry. Dot plots represent single mice, the line the averages.

From these data we conclude that IL-21R signaling is dispensable for the generation and maintenance of CD8 T cell memory. Moreover it seems to play no critical role regarding secondary expansion and migration of memory T cells.

**IL-21-deficient mice mount a normal immune response to LCMV Docile**

So far, our data indicate a pivotal role of IL-21R in maintaining CD8 and CD4 T cell responses upon chronic LCMV DO infection. To confirm the importance of IL-21, we studied LCMV DO infection in mice lacking IL-21, the only known ligand of IL-21R. Surprisingly, in contrast to IL-21R-deficient mice, no differences were found in frequencies of gp33-specific CD8 T cells in the blood of IL-21-deficient during the course of infection with 2000 pfu (Figure 4.2.32 A).
Spleen analysis 35 days after infection further confirmed that percentages of virus-specific CD8 T cells were comparable (Figure 4.2.32 B) and cytokine production by CD8 and CD4 T cells was unimpaired (Figure 4.2.32 C and D). Moreover, percentages of Foxp3+CD25+CD4+ Tregs were unaltered (Figure 4.2.32 E) in IL-21-deficient mice.

Although this experiment is preliminary, it appears that impaired T cell function observed in IL-21R-deficient is not mediated by IL-21-IL-21R interaction. This finding sheds a new light on IL-21R-signaling indicating that another cytokine may signal through this receptor.

**Figure 4.2.32:**

**IL-21-deficient mice are not altered in their immune response to LCMV DO.** C57BL/6 wild-type and IL-21-deficient mice were infected with 2000 pfu LCMV DO. (A) At the time points indicated, blood was taken and percentages of gp33-specific CD8 T cells were assessed by tetramer staining and flow cytometry. (B) At day 35 after infection, mice were sacrificed, spleens were taken and splenocytes were analysed for the percentage of gp33-specific CD8 T cells by flow cytometry. Splenocytes were restimulated in the presence of gp33 or gp61 peptide (1µM each) and Monensin. Percentages of IFNγ and TNFα producing CD8 T cells (C) or CD4 T cells (D) were determined by intracellular staining and flow cytometry. (E) Percentages of Foxp3+CD25+CD4+ Tregs in the spleens were evaluated by flow cytometry. Symbols represent individual mice; horizontal lines indicate the averages.
4.2.5. Discussion

While IL-21 has been thought to mainly regulate CD4 T cell responses, a number of reports also suggest a beneficial effect of IL-21 for CD8 T cell responses. In several tumor models it has been shown that supplementation of IL-21 enhances CD8 proliferation and killing capacity leading to tumor regression (142, 146, 149). Recent studies in humans demonstrate that therapeutic treatment with IL-21 leads to the enhancement of CD8 T cell function (150). These findings underline the not yet fully discovered potency of IL-21 in therapeutic treatment of tumors.

Until today the available data on the role of IL-21R signaling on CD4 and CD8 T cell in acute and chronic viral responses are limited. Holm et al. demonstrated the expression of IL-21 mRNA in activated CD4 T cells during HSV-2 and acute LCMV infection (225). Moreover, frequencies and function of CD8 T cells were shown to be reduced in IL-21R-deficient mice infected with vaccinia virus (142). Exposure of human CD8 T cells isolated from HIV-infected patients to IL-21 in vitro induced an increase in perforin mRNA transcription, but had no effect on proliferation (152).

To better characterize the role of IL-21 during adaptive immune response to viruses we investigated CD8 and CD4 T cell responses in IL-21R-deficient mice infected with cytopathic and non-cytopathic viruses representing models for acute, resolved and chronic viral infection. We first assessed immune responses to chronic LCMV DO infection. Initial CD8 T cell priming and expansion after LCMV DO infection was not dependent on IL-21R signaling as frequencies of virus-specific T cells, their cytokine production and cytotoxicity were not altered. This was further supported by comparable viral titers in the analysed organs. In contrast, IL-21R-deficient virus-specific CD4 T cells were impaired in IFNγ production at day 8 after LCMV DO infection, indicating that early IL-21R signaling supported anti-viral effector function by CD4 T cells.

During the further course of LCMV DO infection, percentages of virus-specific CD8 T cells decreased together with their ability to produce IFNγ indicating that IL-21R signaling on CD8 T cells is essential during the chronic phase of LCMV DO infection to sustain their expansion and/or survival. The loss of CD8 T cell function resulted in elevated viral titers in blood and
Results

organs of IL-21R-deficient mice. Interestingly, CD8 T cell exhaustion by IL-21R-deficient CD8 T cells was much more pronounced in C57BL/6 wild-type chimeras reconstituted with a mixture of bone marrow from IL-21R-deficient and IL-21R-competent mice than by comparing CD8 T cell function in individual IL-21R-deficient and IL-21R-competent mice. This indicates that increased viral load in IL-21R-deficient mice (compared to wild-type or mixed BM chimeras) partially compensates the defect of IL-21R-deficient CD8 T cells by triggering expansion/survival and effector function.

While CD4 T cell cytokine production appeared to be relatively normal in IL-21R-deficient mice late after infection, the analysis of mixed BM chimeras revealed defective cytokine production by CD4 T cell cytokine lacking the IL-21R at day 35. Similar to the situation with CD8 T cells, the differences between IL-21R-deficient CD4 T cells in the global IL-21R-deficient and the mixed BM chimeras are probably due to different antigen loads in the two types of mice. Thus, the absence of IL-21R signaling also affected the CD4 T cell population. Interestingly, mice devoid of CD4 T cell help show a similar phenotype including loss of CTLs, cytotoxicity and viral persistence upon infection with LCMV DO or LCMV clone 13 (91, 174) demonstrating that CD4 T cell help is critical to maintain CD8 T cell responses. However, how CD4 T cells provide help to sustain CD8 T cell responses remains poorly understood. A recent study demonstrated dramatically increased exhaustion of IL-2Rα-deficient CD8 T cells, similar to IL-21R-deficient CD8 T cells. But it was questioned that IL-2 production by CD4 T cells was responsible for CD8 T cell exhaustion, because CD4 T cells produced very little if any IL-2 after chronic LCMV DO infection (242). In the case of IL-21, our real time data identified CD4 T cells as the major source of IL-21. In addition, IL-21 was also detectable to a small amount in CD8 T cells. Whether IL-21 finally acts on CD8 T cells in an autocrine or paracrine manner via CD4 T cells or other cell types (132, 133) remains to be investigated. Therefore, IL-21 could be one of the factors by which help is conferred to CD8 T cells during chronic LCMV infection. IL-21 alone or in combination with IL-7 and IL-15 has been shown to promote proliferation of CD8 T cells alone (143, 105, 142, 146-148). Thus, we assessed proliferation of IL-21R-deficient CD8 T cells after LCMV DO infection by measurement of BrdU incorporation in vivo or upon antigen-specific restimulation in vitro. Progressing exhaustion of CD8 T cells in IL-21R-deficient mice (between day 8-15) was associated with reduced BrdU up-take indicating impaired proliferation of anti-viral CD8 T cells. The difference was even more dramatic in mixed BM chimeras arguing again that strong TRC stimulation could compensate for IL-21R
Results

signaling to a certain extend. However, reduced BrdU up-take could also be a consequence of impaired cell survival. In fact, IL-21-mediated protection from apoptosis has been previously reported (148, 133). To clarify this issue we performed a pulse-chase experiment and compared BrdU up-take by CD8 T cells in mixed bone marrow chimeras 1 and 6 days after uptake. The decline in frequencies of BrdU+ cells between days 1 and 6 after incorporation was comparable in wild-type and IL-21R-deficient CD8 T cells. Thus, in this model IL-21R signaling is essential to maintain CD8 T cell proliferation and probably plays a minor role in promoting T cell survival.

Next, we characterized virus-specific CD8 T cells by their expression of CD127 and CD62L. CD127 (IL-7Rα) is expressed on naïve as well as long-lived memory cells (86, 87). CD62L (L-selectin) can be similarly found on naïve and memory T cell and is responsible for T cell homing to secondary lymphoid organs (85, 243). Both CD62L and CD127 have been used to characterize effector T cells (T_E, CD62L-CD127-), effector memory T cells (T_EM, CD62L-CD127+) and central memory T cells (T_CM, CD62L+CD127+) (86, 240). However, after chronic LCMV infection it was shown that most of the long-lived virus-specific CD8 T cells are of the T_E phenotype while the other subsets are barely detectable (240). This is caused by constant antigen exposure, which does not lead to the generation of “conventional” memory CD8 T cells as they are normally generated after acute LCMV infection (241, 244).

We followed expression of CD62L and CD127 on virus-specific CD8 T cells in the blood. In the acute phase (day 8), percentages of virus-specific CD8 T cells were similar and their majority expressed T_E features confirming our previous observations that early response to LCMV DO is unimpaired in the absence of IL-21R signaling. During disease progression (day 15 and 21) the overall percentages of virus-specific CD8 T cells decreased in the absence of IL-21R signaling and so did the fraction of T_E expressing cells. In parallel, we noticed an increase in cells expressing the T_CM phenotype. However, these cells are unlikely to be memory cells for afore mentioned reasons. We rather suppose that they are less activated and therefore re-express CD62L and CD127. This would correlate with the reduction of virus-specific CD8 T cells expressing the T_E phenotype and the previously observed impaired proliferation and effector function. Our considerations can also be applied to the total CD8 T cell population. In this case however, it is also conceivable that there is an enhanced recruitment of recent thymic emigrants in the absence of IL-21R signaling (245). As these cells are naïve, they would express both CD62L and CD127.
Several molecules have been shown to be implicated in chronic LCMV infections. PD-1 delivers inhibitory signals to activated T cells and is up-regulated on exhausted CD8 T cells upon chronic LCMV infection. Its blockade restores their effector functions and leads to viral control (76). Although virus-specific IL-21R-deficient CD8 T cells showed increased exhaustion after LCMV DO infection, PD-1 surface expression was comparable to wild-type cells indicating that PD-1 is not responsible for exhaustion of IL-21R-deficient CD8 T cells. Surprisingly, even a smaller percentage of all CD8 T cells in IL-21R-deficient mice were positive for PD-1. As PD-1 expression is also induced by T cell activation, this result probably indicates impaired CD8 T cell activation in IL-21R-deficient mice. Recent studies demonstrating that IL-10 promoted exhaustion of T cells after chronic LCMV (clone 13) infection (183, 184) prompted us to analyse whether IL-21R-deficiency was associated with altered IL-10 production. We found that CD11b+ macrophages were the main source of IL-10 in wild-type mice. In fact, IL-10 was undetectable in CD11c+ DC and T cells. IL-10 appears not to be responsible for exhaustion of CD8 T cells in IL-21R-deficient mice, as no differences in IL-10 production was observed at day 15 of infection. Nevertheless, we observed elevated IL-10 production in IL-21R-deficient mice late after infection (i.e. at day 35), which may be driven by viral persistence. Analysis of IL-10 production in mixed BM chimeras is underway to address this question.

Regulatory T cells are important modulators of immune responses. However, the role of this T cell subset in chronic viral infections is not understood and may depend on the infection model (237). We also wanted to address the role of Tregs upon chronic LCMV DO infection. Initially, we could observe that frequencies of Tregs in wild-type mice increased with the infectious dose. This could indicate on the one hand that Tregs promote viral persistence by impairment of viral immune responses as shown before in human hepatitis B infection (HBV) (246) or in a murine model of herpes simplex virus (HSV) (247). On the other hand, Tregs could also be beneficial for the host, as they have been shown to contribute to the dampening of chronic immune activation and protection from tissue damage (248) (249).

To clarify this issue, we depleted Foxp3+ Tregs during the period when their numbers increased and peaked (between day 8-15) by injection of Diphtheria Toxin (DT) using the previously described DEREG mouse (228). We further compared the roles of Tregs upon intermediate and high dose infection in wild-type mice. Depletion of Tregs during high dose infection did not show any effect on virus-specific T cell responses including numbers and effector cytokine production. Depletion during intermediate dose infection had no effect in a first experiment, but
Results

led to increased effector cytokine production and viral clearance in a second experiment. One possibility could be the efficiency of depletion as DT treatment only removed GFP+Foxp3+CD4+ Tregs, but not GFP-Foxp3+CD4+ Tregs. In the first experiment, only 50% of GFP-Foxp3+CD4 T cells were depleted, while in the second experiment around 75% of GFP-Foxp3+CD4 T cells were depleted. The reason for these differences is unclear as the treatment scheme was comparable in both experiments. Currently, it is also not clear why these GFP-Foxp3+CD4 T cells do not express GFP and whether they are functional Tregs. In conclusion, further experiments are needed to clarify the contribution of Tregs to chronic LCMV DO infection. Also the available literature about the role of Tregs in LCMV infection is scarce and conflicting: a review mentioned a minor role for Tregs (237), while a recent report stated an essential role for Tregs in migration of virus-specific T cells to peripheral organs and therefore their contribution to virus elimination (250). However, migration of virus-specific CD8 T cells to peripheral organs after Treg depletion was not impaired in our setup. Further experiments with better depletion rates (e.g. combination of DT and anti-CD25 Treatment) may clarify this issue.

As shown before, antigen-specific Tregs induced by hepatitis C virus (HCV) or Epstein-Barr Virus (EBV) secrete IL-10 (251) (252). However, this population was negative for Foxp3 expression. Nevertheless, we assessed cytokine production by GFP+CD4+ Tregs in DEREG mice. Independent of the infection dose, these cells did not produce IL-10.

A previous report showed that IL-21 differentially regulates Th17 and iTreg development (56) (224). Therefore, we wanted to address whether Treg development is altered in IL-21R-deficient mice infected with LCMV DO. Indeed, we found a remarkable increase in Foxp3+CD4+ Tregs. This observation was independent of viral burden as mixed BM chimeras also showed increased numbers of IL-21R-deficient Foxp3+CD4+ Tregs. Importantly, numbers of natural Tregs were normal in naïve IL-21R-deficient mice (data not shown). These results demonstrate that absence of IL-21R on CD4 T cells promoted expansion of Tregs upon LCMV DO infection. However, preliminary data show that depletion of Tregs in DEREGxIL-21R-/- mice did neither restore CD8 T cell number and function nor viral control arguing that increased numbers of Tregs were not responsible for exhaustion of CD8 T cells in IL-21R-deficient mice. However, this question is currently under investigation.
To support our data that IL-21R is not essential for the expansion of anti-viral CD8 T cells during acute infection, we studied other models of acute viral infection including non-cytopathic LCMV WE and cytopathic vaccinia and influenza viruses. They differ in their requirement for CTLs, CD4 T cell help and antibodies to resolve the infection.

Control of influenza virus critically depends on IgM and IgG antibody responses. CD4 T cells are required for B cell help and development of memory but not primary CD8 T cells (189, 190, 253). We found normal development, lung recruitment, and effector function of anti-viral CD8 and CD4 T cells in IL-21R-deficient mice. It has been shown previously that IL-21 is required for efficient Th dependent B cell responses (135, 136) mainly due to its fundamental role for the generation of T follicular help (T(FH)) cells (254, 255). Interestingly, we found normal titers of influenza-specific IgG2a antibodies in sera and BALFs of IL-21R-deficient mice, while anti-IgG1 antibodies were reduced. These results may indicate that anti-viral IgG2 responses are not dependent on T(FH). Although CD8 T cells are not required for control of acute vaccinia virus (VVG2) infection (195), their expansion during primary and secondary challenge requires CD4 T cell help (197). Similar to infection with influenza virus, we found normal numbers and effector function of anti-viral CD8 T cells in the spleen and in the ovaries, the site of VV replication, indicating normal CD8 expansion and recruitment, respectively. While this result is in agreement with our other data showing normal acute CD8 T cell responses to several viruses in IL-21R-deficient mice, it is in discrepancy to another report showing impaired CTL activity to vaccinia virus (142) for reasons we cannot explain.

IL-21R-deficient mice also displayed normal expansion, effector function, and contraction of CD8 T cells to acute infection with LCMV WE. Moreover, in contrast to LCMV DO, frequencies of virus-specific CD8 T cells did not drop and viral replication was controlled over long periods indicating normal CD8 and CD4 T cell memory responses. Infection with LCMV DO several months after infection with LCMV WE resulted in rapid expansion and cytokine production by memory CD8 T cells in IL-21R-deficient mice demonstrating intact quality of memory CD8 T cells in a recall response to LCMV. Furthermore, immunization of IL-21R-deficient mice with replication-incompetent virus-like particles carrying the gp33 peptide of LCMV (gp33-VLPs) resulted in effective generation of specific effector and memory CD8 T cells (256), which were able to mount protective responses to recall infection with vaccinia virus expressing the LCMV glycoprotein (VVG2).
Taken together these data demonstrate IL-21R signaling is dispensable for normal effector and memory CD8 T cell responses to acute and resolved infection, while it plays a key role to prevent CD8 T cell exhaustion and chronic infection with the fast replicating LCMV DO.

The question why IL-21R signaling is essential for chronic, but not for acute LCMV infections still remains open. It is very likely that chronic viruses induce a different T cell differentiation and maintenance program, which depends on IL-21R signaling. Thus, it would be of interest to analyse other chronic viral models in mice (such as infection with herpes virus and MCMV).

As discussed above, absence of IL-21R and IL-2Rα resulted in a similar defect in CD8 T cell function in chronic LCMV DO infection (242). This indicates an overlapping but non-redundant activity of IL-2 and IL-21, which may be explained by common and unique signaling pathways of the two cytokines. While IL-21 induces preferentially phosphorylation of STAT 1 and STAT 3 and more transiently STAT 5, IL-2 activates primarily STAT 5 and weakly STAT 1 and STAT 3. However, both cytokines activate the MAPK and PI3K pathways (127, 257), which have been shown be involved in proliferation. To better understand overlapping and unique roles of these two cytokines during chronic LCMV infection, we assessed whether CD8 T cell function could be restored by addition of IL-2 and IL-21 to exhausted CD8 T cells. Proliferation of both wild-type and IL-21R-deficient CD8 T cells was promoted by addition of IL-2, but not by IL-21. Moreover, IL-21 did not cooperate with IL-2 in restoring proliferation of exhausted CD8 T cells from wild-type mice. The ability of IL-2 to restore CD8 T cell proliferation in IL-21R-deficient CD8 T cells and the failure to of IL-21 to promote proliferation of exhausted wild-type T cells may suggest that IL-21R-deficient mice have a defect in IL-2 production. Arguing against this possibility, increased exhaustion of IL-21R-deficient CD8 T cells has been observed in mixed BM chimeras, which should have normal levels of IL-2. Intriguingly, preliminary studies indicated that mice lacking IL-21 are unimpaired in long-term CD8 T cell responses and control of intermediate dose infection with LCMV DO, in contrast to IL-21R-deficient mice. While these data need to be confirmed, they may indicate that a cytokine other than IL-21 requires IL-21R (possibly in combination with another cytokine receptor) for signaling and prevention of chronic viral infection. Possible candidates would be cytokines that require the common γ chain for signaling. Further studies are warranted to clarify this aspect.
5. Appendix

5.1. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAM</td>
<td>Alternative activated macrophage</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation-induced cell death</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CCR7</td>
<td>Chemokine (C-C motif) receptor 7</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy DNA</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxylfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ds</td>
<td>Double-stranded</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EAM</td>
<td>Experimental Autoimmune Myocarditis</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>Eomes</td>
<td>Eomesodermin</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>HA/A</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Hp</td>
<td><em>Heligmosomoides polygyrus</em></td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-21R</td>
<td>IL-21 receptor</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>iTreg</td>
<td>Inducible regulatory T cell</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JUN</td>
<td>C-Jun-N-terminal kinase</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LCMV DO</td>
<td>LCMV strain Docile</td>
</tr>
<tr>
<td>LCMV WE</td>
<td>LCMV strain WE</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
</tr>
<tr>
<td>MCMV</td>
<td>Murine cytomegalovirus</td>
</tr>
<tr>
<td>mIgM</td>
<td>Membrane-bound immunoglobulin M</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
</tr>
<tr>
<td>NA/N</td>
<td>Neuramidase</td>
</tr>
<tr>
<td>Nb</td>
<td><em>Nippostrongylus brasiliensis</em></td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domaine</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>np</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
</tbody>
</table>
PD-1  Programmed death-1
PD-1L  Programmed death-1 ligand
pfu  Plaque forming unit
PRR  Pattern recognition receptor
RNA  Ribonucleic acid
ROI  Reactive oxygen intermediates
s.c.  Subcutaneous
Sm2  Smarta-2
ss  Single-stranded
STAT  Signal transducer and activator of transcription
T_{CM}  Central memory T cells
T(FH)  Follicular T helper cells
TCR  T cell receptor
T_{E}  Effector T cells
T_{EM}  Effector memory T cells
TGF  Tumor growth factor
Th1  T helper 1
Th17  T helper 17
Th2  T helper 2
TNF  Tumor necrosis factor
TLR  Toll-like receptor
TRAF  TNF receptor-associated factor
Treg  Regulatory T cell
VLP  Virus-like particle
VV  Vaccinia virus
VVG2  Recombinant VV expressing the LCMV gp
5.2. Acknowledgements

I would like to thank all the people who contributed to this work:

Prof. Manfred Kopf for the great opportunity to perform my PhD thesis in his lab and for giving me the chance to learn all the various models and methods. Thank you for your supervision, for your support, for the scientific and non-scientific discussions and for your patience.

Prof. Annette Oxenius for being co-examiner at the PhD defense and for the great input and discussions, especially regarding the LCMV project.

Giandomenica Iezzi for being a motherly supervisor during the first period of the thesis, for teaching me the secrets of Leishmania infection and FACS analysis and for the ice cream support.

Benjamin Marsland for adopting me as a step-student for a while, for teaching me the Th2 models, for the insightful scientific discussions and for proofreading the thesis. Thank you for your support and patience.

Prof. Nicola Harris for providing her help and expertise regarding the helminth infections and for the scientific input.

The Biosupport team Paul, Therese and Hans-Ruedi for mouse maintenance and for always taking great efforts to make last-minute experiments possible.

Jan Kisielow for the great effort to design and generate the Fc fusion proteins. Furthermore for his overwhelming scientific discussions, I was very rarely able to cope with them. But at least we always agreed that the The Cure concert was great…
Bettina Ernst for FACS sorting, for initial help with the accommodation in Switzerland, for organization and that you always had an open ear for problems.

Special thanks to Jacqueline Weber for the production and purification of the Fc fusion proteins and for helping me with experiments and methods. Moreover for teaching me how to deal with the frustration of not working monster plaque assays. Besides that, you were always an irreplaceable mental support, lab neighbour and shopping partner.

E un ringraziamento molto speciale va a Chiara Nembrini. Senza di te avrei riso e pianto molto di meno. Senza di te non sarei mai riuscita ad arrivare fino in fondo. Grazie per il tuo grande sostegno e per essere una grande amica. Grazie Schatzi!

Alex “Junior” Heer for technical influenza expertise, IT support, for proofreading the thesis and for being always ready for a good…ok sometimes bad…joke (especially Monday and Friday mornings via E-mail…).

Luigi Tortola for helping me with cloning and for his permanent willingness to sing particular Romanian songs with me.

The PhD students and technicians of the Molecular Biomedicine and the Environmental Biomedicine groups for their support during work and for entertaining coffee and lunch breaks. Thanks to Koshika Yadava, Joanna Massacand, Corinne Schär, Rebecca Stettler, Tina Herbst, Anke Sichelstiel and Regina Reissmann.

Franziska Ampenberger for her help with the maintenance of *Leishmania* parasites and the complicated four-hand ear injections.

Michael Kurrer for his patho-logical and sometimes entertaining non-logical expertise.

Ivo Sonderegger for help with the asthma experiments, for his great contribution to this project and for fruitful discussions.
Stefan Freigang for LCMV maintenance, expertise and discussions.

The former members of the lab Japar Shamshiev and Brian Abel for the “very interesting” findings, discussions and nicknames.

The semester and diploma students who brought new inspiration into the group. Special thanks to Reto “Knuspi” Meier, Iwana ”will be announced” Schmitz and Christoph “The Rock” Schneider.

People from Cytos Biotechnology for their great collaboration, especially Katrin Schwarz and Martin Bachmann.

Mira Fischer, Wolf-Joachim von Rosenstiel, Claudia Führling, Ahmed Hegazy und Martina Ruppe for irreplaceable mental balance, fun and support outside of the lab.

Ich möchte mich vor allem bei meinen Eltern Hildegard und Karlheinz Fröhlich bedanken, die mich immer bei allen Dingen (und mit allen Dingen!) tatkräftig unterstützt haben.
5.3. References


Appendix


Appendix


Curriculum Vitae

Name: Anja Stefanie Fröhlich
Date of birth: 21. Juni 1975
Place of birth: Nürnberg, Germany
Marital status: single
Current address: Murhaldenweg 10, CH-8057 Zürich, Switzerland

Education:

1981-1985  Primary school in Nürnberg
1985-1994  Grammar school (Wilhelm-Löhe Schule, Nürnberg), high school diploma

Vocational education:

1994-1997  Technical training for laboratory assistant at the Landesgewerbeanstalt (LGA) in Nürnberg, completed in July 1997

University education:

WS 1997/98-WS 2001/02  Studies in teaching profession for grammar schools, subject biology and chemistry, at the Friedrich-Alexander University in Erlangen
SS 2002-SS 2003  Studies in biology at the Friedrich-Alexander University in Erlangen; diploma thesis at the Chair of Zoology 1, supervisor Prof. Lutz-Thilo Wasserthal;
Subject: Funktionsmorphologie der thorakalen Stigmen von Calliphora vicina (Functional morphology of the thoracic spiracles from Calliphora vicina, Calliphoridae)
Diploma examination: 1.4

Postgradual education:

July 2003-February 2004  Member of the graduated college „Immunemodulation“ at the University of Würzburg; doctorate thesis at the Institute of Molecular Infectious Biology (IMIB), Infectious Disease Unit
Subject: The role of dendrite cells in immune regulation of Leishmaniasis; supervisor Prof. Heidrun Moll
Since April 2004
Doctorate Thesis at the Institute of Molecular Biomedicine, ETH Zürich
Subjects: The role of inflammatory and costimulatory molecules in dendritic cell migration; adaptive immune responses in the absence of IL-21R signaling; supervisor: Prof. Manfred Kopf

Spoken languages
English, French, Italian (basics)

Publications:


3) Ramirez-Pineda JR, Fröhlich A, Berberich C, Moll H. Dendritic cells (DC) activated by CpG DNA ex vivo are potent inducers of host resistance to an intracellular pathogen that is independent of IL-12 derived from the immunizing DC. J Immunol. 2004, 172(10), 6281-9

4) Fröhlich A, Kopf M. IL-21R signaling is essential for the maintenance of CD8 T cell responses after persistent LCMV infection. Manuscript in preparation

Presentations:
1) XVII. Meeting of the Swiss Immunology Ph.D. students at Schloss Wolfsberg, poster presentation, 29-31 March 2005. Title: The role of IL-1 in migration of skin-derived DC subsets in response to skin irritation and infection with Leishmania major

2) XVIII. Meeting of the Swiss Immunology Ph.D. students at Schloss Wolfsberg, oral presentation, 20-22. March 2006. Title: The role of IL-21R signaling during Th2 responses

3) Schweizer Gesellschaft für Allergologie und Immunologie (SGAI), Annual meeting 2006, poster presentation, 30-31. March 2006, Zürich. Title: IL-21 plays a central role in development on Th2 responses and may contribute to counter-regulation of cytotoxic CD8 T cells
4) XIX. Meeting of the Swiss Immunology Ph.D. students at Schloss Wolfsberg, oral presentation, 26-28. March 2007. Title: *IL-21R signaling is essential for the maintenance of CD8 T cell responses after persistent LCMV Docile infection*

5) 13th International Congress of Immunology, oral and poster presentation, 21-25. August 2007, Rio de Janero. Title: *IL-21R signaling is integral for the development of Th2 responses in vivo*

6) Schweizer Gesellschaft für Allergologie und Immunologie (SGAI), Annual meeting 2008, oral presentation, 17-18. April 2008. Title: *IL-21R signaling is essential for the maintenance of virus-specific CD8 T cells in persistent LCMV infection*