

Mechanisms regulating autoreactive T cell responses in inflammatory heart disease

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1 Summary

Dilated cardiomyopathy is a leading cause of heart failure in young patients in developed countries and often evolves from myocarditis. The mouse model of experimental autoimmune myocarditis (EAM) mirrors many aspects of the physiopathology of human myocarditis. In mice, the time course of myocarditis is biphasic, reflecting an early acute chronic myocarditis followed by a low-grade of inflammation with heart muscle fibrosis and ventricular dilation. Using the EAM model, I elucidated the role of CD4⁺ T helper (Th) cells in inflammatory heart disease. My aim was, on one side, to understand the mechanism that leads to the spontaneous resolution of inflammation after the acute phase of cardiac inflammation, and, on the other side, to find a vaccination strategy to shift the polarization of CD4⁺ Th cells to a biased protective heart-specific cell response.

To answer the first question, I used mice lacking the receptor for IFN- γ (IFN- γ R^{-/-}), because they were extremely susceptible to autoimmune myocarditis. After EAM induction, the number of inflammatory heart-infiltrating cells in IFN- γ R^{-/-} mice was considerably higher compared to wild-type (wt) mice, suggesting that a deeper study of the heart-infiltrating cells was necessary. The origin of those cells was elucidated creating CD45.2 chimeric mice reconstructed with CD45.1 bone marrow. Myocarditis induced in chimeric mice showed that all heart-infiltrating cells were from the bone marrow of donor mice. After providing evidence that Th17 CD4⁺ T cells mediated EAM, the question of a direct involvement of the Th17 cytokine IL-17 arose. In fact, as expected, systemic depletion of IL-17 with blocking antibodies confirmed a direct role of IL-17 in the recruitment of inflammatory cells into the heart. A deeper analysis of the recruited cells showed that CD11b⁺ monocytes were the major heart-infiltrating cell population. A comparison of the anti-inflammatory potential of wt and IFN- γ R^{-/-} CD11b⁺ monocytes revealed that IFN- γ R^{-/-} CD11b⁺ monocytes were not able to inhibit heart-specific CD4⁺ T cells. The relevance of the IFN- γ R signaling pathway for the transcription of anti-inflammatory molecules, like inducible nitric oxide synthase (NOS2), was tested *in vitro*. Indeed, IFN- γ R^{-/-} monocytes showed an impaired capacity to

produce the short living molecule nitric oxide, which is able to reversibly inhibit T cell proliferation. *In vivo*, serial injections of wt CD11b⁺ monocytes into diseased IFN- γ R^{-/-} mice were sufficient to provide a good number of functional CD11b⁺ monocytes to block EAM in high susceptible IFN- γ R^{-/-} mice.

Dendritic cells subsets differently regulate autoimmunity. In fact, bone marrow-derived dendritic cells (bmDC) promoted IL-17-mediated EAM, while splenic CD α ⁺ dendritic cells were slightly pathogenic and induced low levels of EAM. CD8 α ⁺ DC stimulated with LPS- α CD40, producing high levels of IL-1 β and IL-6, induced the development of auto-aggressive IL-17-producing CD4⁺ Th17 cells, while TNF- α -stimulated CD8 α ⁺ DC polarized IFN- γ -producing CD4⁺ Th1 cells. The potential of CD8 α ⁺ DC to induce protective IFN- γ -producing T cells was used to develop a vaccination strategy. Indeed, mice vaccinated with serial injections of self-peptide-loaded and TNF- α -stimulated CD8 α ⁺ DC showed an increased production of IFN- γ by self-peptide-specific CD4⁺ T cells and were protected from EAM.

Taken together, the opposite roles of IFN- γ as protective cytokine and IL-17 as inflammatory cytokine have been further explained in EAM.

2 Aim of the thesis

EAM represents a CD4⁺ T cell-mediated autoimmune heart disease. Autoimmune diseases develop in susceptible animals when a threshold of self-tolerance exceeds. Many autoimmune diseases are linked with preceding microorganism infections that cause damage of tissues, cross-reaction with self-antigens, and activation of the immune system. The activation of the innate immune system through toll-like receptors (TLR) stimulation in concert with the activation of the adoptive immune system is strictly necessary to develop heart failure. Activated dendritic cells (DC) strongly prime naïve T cells, which quickly become heart-specific auto-aggressive T cells. Historically, two major CD4⁺ T cell subsets have been defined according to their cytokine production pattern: IFN- γ -producing Th1 cells, and Th2 cells secreting IL-4. Pathogenicity was mostly attributed to IFN- γ . Recent data, however, demonstrate that mice with an impaired IFN- γ signaling pathway are highly susceptible for EAM development and have a high mortality rate. Moreover, a novel autoreactive CD4⁺ T cell subpopulation characterized by IL-17 production is present at increased levels in IFN- γ ^{-/-} mice with ongoing EAM. A deeper understanding of IL-17 and its inflammatory function in EAM was the first aim of my thesis. Afterward, I focused on the role of IFN- γ in EAM. High susceptible IFN- γ R^{-/-} mice and wt mice showed comparable levels of inflammatory heart-infiltrating cells and IL-17 production at the peak of EAM. Afterwards, however, wt mice drastically reduced the number of heart-infiltrating cells and levels of IL-17, while IFN- γ R^{-/-} mice still presented very high quantities of inflammatory heart-infiltrating cells and IL-17 production. I aimed to find the protective function of IFN- γ in CD4⁺ Th17 cell-mediated EAM.

DC are powerful regulators of the immune system. In the last years, most of the cells used to elucidate and explain autoimmune diseases were DC from bone marrow origin cultivated with GM-CSF. In EAM, it is known that self-peptide-loaded and LPS/ α CD40-stimulated bmDC modulate a strong CD4⁺ Th17 response, which leads to EAM in susceptible mice. However, in contrast to bmDC, other DC subpopulations were more prone to polarize CD4⁺ Th1 or

CD4⁺ Th2 cells rather than CD4⁺ Th17 cells. Some dendritic cells from the spleen, for example, *ex vivo* cultivated with naïve T cells, preferentially induce CD4⁺ T cells to produce IFN- γ . I took advantage from these splenic DC and I focused my work on the development of a vaccination strategy to protect mice and prevent EAM.

Taken together, I aimed my thesis to clarify the opposing roles of IFN- γ and IL-17 in EAM.

3 Introduction

3.1 *Innate and adaptive immunity*

The main role of the immune system is to protect the body from infection. The system is divided into two major branches: the innate immune system and the adaptive immune system. The innate immune system is non-specific, as it fights any type of invading pathogen, and is ready to be mobilized upon the first signs of infection. The adaptive immune system specifically attacks the invading pathogens and requires some time to develop its custom-made response. The adaptive system produces memory cells that are able to remember antigens it has encountered and reacts more quickly and efficiently the next time that antigen is found, yet more slowly than the innate system. Over the past years, research into the causes of autoimmune diseases has largely focused on the adaptive immune system because of its remarkable ability to generate specific immunity to antigens. Recently, however, many scientists are considering the innate immune system as a major player in autoimmune disease. The innate system, though less specific than the adaptive system, is less able to discriminate low from high pathogenic antigens and to generate a potent primary defense against infections. It is becoming clear that autoimmune diseases frequently involve the innate as well as the adaptive immune system.

Activation of the innate immune system is mediated by pattern recognition receptors (PPR) on APC that recognize pathogen-associated molecular patterns (PAMPs) like polysaccharide and polynucleotide (1). The best-characterized signaling PPR are so far the Toll-like receptors (TLRs), which are very able to differentiate between molecular patterns found on pathogens and molecular patterns found on the body's own cells. Indeed, TLR responses are limited and specifically aimed against foreign pathogens and not against self-tissues (2). Whereas TLRs that recognize bacterial and fungal cell wall components are localized to the cell surface, TLRs that recognize viral or microbial nucleic acids are localized to intracellular membranes and are thought to encounter their ligands in phagosomes or endosomes. This localization is thought to be an adaptation ensuring that these receptors

detect nucleic acids released from apoptotic host cells, microbial cells or virions only after phagocytosis and partial digestion of the ingested particles has released the nucleic acids.

The innate response can be enhanced immediately without any delay, but as a consequence there is no memory evolving after an encounter with a specific pathogen. This response will be determined by the binding to one or more of the twelve in mammals known different TLRs. In this way, the TLRs identify the nature of the pathogen and turn on an effector response appropriate for dealing with it. These signaling cascades lead to the expression of genes encoding various cytokines.

The adaptive immune response is the most advanced and specialized arm of immunity and is found in vertebrates only. The components of the adaptive immune system are two major types of immune cells, T and B cells (also called lymphocytes). They display a large variety of cell surface receptors that can recognize and respond to an unlimited number of pathogens. To do that, the adoptive immune system needs to generate large numbers of receptors. To reach a high level of variability, recombination of pre-existing gene segments is necessary to encode B- and T-cell receptor (BCR and TCR, respectively) genes. Variations are created in the antigen-recognition regions of BCR and TCR. The mechanism involves a series of recombination events that fuse variable (V), diversity (D), and joining (J) gene segments in a process called V(D)J recombination. This is achieved by a well-coordinate set of reactions, starting with two lymphocytes-specific proteins called RAG1 and RAG2 that recognize and bind specific conserved recombinant signal sequences (RSSs). This complex cuts the DNA between the rearranging DNA segments and the adjacent RSS motifs. Pre-existing V, D, and J gene segments are rearranged to yield a contiguous V(D)J region, just upstream of another element of the receptor, the constant (C) region. This process is ensured by ubiquitous DNA repair enzymes, which control the functionality of the double-stranded DNA and correctly complete the joining process.

The antigen-recognition regions of BCR and TCR consist of two paired protein segments, the heavy (H) and light (L) chains, and the α and β chains,

respectively. The BCR heavy chain and the TCR β chain consist of V, D, and J segments, while the BCR light and the TCR α chain consist only of V and J segments. As both BCR and TCR are heterodimers, a huge amount of possible rearrangements allow the recognition of an unlimited number of antigens.

Although the adoptive immune system can recognize a lot of different antigens, it responds slower (3-5 days) than the innate immune response, because only a small fraction of cells will have a receptor for a certain antigen and those cells will need to undergo clonal expansion. This specific antigen response comes with one major benefit, as an immunological memory is build up and maintained after an encounter with a specific pathogen, with a fast memory response upon a second challenge with the same antigen or pathogen.

3.2 Self-tolerance

The immune system uses several mechanisms to control and avoid reactions against own organs, tissues and proteins. One hallmark of the vertebrate immune system is self-tolerance, which works to protect tissue antigens from becoming targets of damaging immune responses during clearance of infections. The immune system normally exhibits specificity in distinguishing infectious antigens from self-antigens. The immune response is regulated by opposing immunogenic and tolerogenic signals, and the latter normally prevail for lymphocytes that bind self-antigens. Self-tolerance is induced in the thymus by thymic negative selection of autoreactive T cells and in the periphery by mechanisms inducing cell death and cell anergy, and by suppressor regulatory-T cells. Abrogation of these processes, as well as disturbance in the balance between immunogenic and tolerogenic signals, can lead to autoimmunity.

T-cell development is a highly coordinated process that depends on interactions between thymocytes, thymic epithelium, and bmDC (3). Before entering the peripheral T-cell pool, thymocytes are subject to negative selection, a process that eliminates T cells with high affinity to self-antigens. Thymocytes, which are of bone marrow origin, undergo several maturation

and developmental stages prior to becoming mature T cells.

The thymus is arranged into an outer cortex, which contains immature thymocytes, and an inner medulla, which contains mature thymocytes. Double-negative immature $CD4^-CD8^-$ thymocytes undergo a proliferative expansion that give rise to the main population of double-positive $CD4^+CD8^+$ thymocytes. V(D)J rearrangement of TCR is achieved during this maturation stage. Afterwards, double-positive thymocytes undergo one of three cell fates: death by neglect, positive selection or negative selection. Death by neglect is a passive form of cell death caused by the failure of TCR to engage a peptide-MHC ligand. About 90% of double-positive thymocytes fail to undergo positive selection and die because they do not receive a survival signal. Positive selection occurs when TCR encounters a peptide-MHC ligand with low affinity, resulting in the transduction of a survival and transduction signal. In this process, single-positive cells expressing CD4 but not CD8 develop and recognize foreign peptides in association with class II MHC proteins, while cells expressing CD8 but not CD4 develop and recognize foreign peptides in association with class I MHC proteins. Negative selection occurs when the TCR contacts with a peptide-MHC ligand with high affinity, causing thymocytes to die rapidly via apoptosis. This process destroys T cells with potential autoreactivity, providing a repertoire of peripheral T cells that is largely self-tolerant. Double positive $CD4^+CD8^+$ thymocytes undergoing negative selection react to both ubiquitous expressed antigen and tissue-specific antigens (TSAs). It is not completely clear whether negative selection occurs in the thymic cortex or medulla. However, expression of TSAs in the thymus is mostly a property of medullary thymic epithelial cells (mTECs) (3). In fact, mTECs are sites of promiscuous gene expression and transcribe genes that are normally expressed in peripheral tissues. In this way, medullary epithelial cells might be able to express the entire peptide repertoire of the individual, making the medulla an ideal site for negative selection. The number of mTECs that express any given self-peptide might be relatively small, implying that a thymocyte must scan several of these epithelial APCs to view the entire self-peptide repertoire. An important aspect of the medullary epithelium is the expression of a transcription factor known as autoimmune regulator (AIRE). AIRE is highly expressed in mouse and human mTECs, and

mutations in the AIRE gene are responsible for the rare human autoimmune syndrome called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (4). AIRE-deficient mice also develop multi-organ autoimmune disease. mTECs in AIRE-deficient mice also lack expression of a subset of TSAs linking thymic TSAs expression with T-cell tolerance (5, 6). mTECs expressing high levels of MHCII and CD80 are the subsets of mTECs that express AIRE and the highest number of TSAs (6).

Although negative selection is a very precise mechanism, it is not absolutely perfect. In fact, several auto-aggressive T cells pass the negative selection and are exported. Self-reactive T cells that escape negative selection in the thymus must be inactivated in the periphery. Anergy constitutes one means of imposing peripheral tolerance. Clonal anergy refers to a state of unresponsiveness that is induced in potentially dangerous lymphocytes during antigen recognition. Different mechanisms of anergy include block in TCR signaling, the activation of ubiquitin ligases, and the engagement of inhibitory receptors (7) (8). Co-stimulatory signals between T cells and APC play an important role in T-cell anergy. Both CD28 and CTLA-4 receptors on T cells bind the ligands B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells but exert opposite influences on T-cell activation. CD28 stimulation promotes T-cell activation, whereas CTLA-4 stimulation inhibits T cells. CTLA-4 is a counter-regulatory molecule known to antagonize CD28 both by binding CD80 and CD86 and by inhibiting cell cycle progression. Although the simple absence of co-stimulation is sufficient to induce anergy in effector T cells, CTLA-4 engagement may be necessary to induce anergy in naïve CD4⁺ T cells. In fact, cells lacking CTLA-4 have a reduced potential in tolerization of T cells.

Not only molecular components of T-cell signalling are involved in T-cell anergy. Also E3 ubiquitin ligases, important enzymes of the ubiquitin–proteasome system, are an essential component of the T-cell anergy phenotype. Among E3 ubiquitin ligases, there are three which mediate the selective degradation of specific signaling protein: Itch and Cbl-b, whose mutation or deletion in mice is associated with disseminated autoimmune disease, and GRAIL, a transmembrane, endosome-associated protein

whose overexpression blocks IL-2 induction (9). Several different proteins are involved in peripheral T-cell anergy, but many of them are still under investigation.

All together, a failure in one of those auto-regulatory mechanisms may lead to unwanted expansion of self-reactive T cells, to the production of self antibodies, and eventually to autoimmune diseases.

3.3 Autoimmune diseases

Autoimmune diseases represent the third most common category of disease in the United States after cancer and heart disease. They affect approximately 5-8% of the population (about 14–22 million persons). About 80% of them are women (10). Autoimmune diseases can affect virtually every site in the body, including the endocrine system, connective tissue, gastrointestinal tract, heart, skin, and kidneys. At least 15 diseases are known to be the direct result of an autoimmune response, while circumstantial evidence implicates >80 conditions with autoimmunity (11). Autoimmune diseases are divided in two classes by their auto-antigen targets: organ specific and systemic diseases. Organ specific diseases include type I diabetes mellitus, multiple sclerosis, myasthenia gravis, autoimmune thyroiditis diseases, and autoimmune myocarditis, while systemic diseases include systemic lupus erythematosus, rheumatoid arthritis, antiphospholipid syndrome, and scleroderma (12).

Autoimmune disease occurs when T cells that escaped auto-regulatory mechanisms develop a response against self-antigens. Besides T cells, B cells or autoantibodies can induce injury systemically or against a particular organ. Understanding of autoimmune diseases is obstructed by the fact that some level of autoimmunity, in the form of naturally occurring autoantibodies and self-reactive T and B cells, is present in all normal persons (13). Thus, on a proportional basis, developing autoimmune disease is the relatively uncommon consequence of a common autoimmune response. Although an autoimmune response occurs in most persons, clinically relevant autoimmune disease develops only in susceptible persons. A body of circumstantial evidence links many autoimmune diseases with preceding bacterial infections (14, 15). Since infections generally occur before the onset of symptoms of

autoimmune disease, linking a specific microorganism to a particular autoimmune disease is difficult. One possible explanation is the presence of sequence homologies between the pathogen and self-proteins, which results in cross-reaction with self-antigens, and then induction of autoimmunity (16, 17). This mechanism is called “molecular mimicry”. Molecular mimicry, however, has been demonstrated in animal models, but not in human diseases (18-20). Another explanation is that microorganisms expose self-antigens to the immune system by directly damaging tissues during an active infection. This mechanism has been referred to as the “bystander effect” (21). However, whether pathogens mimic self-antigens, release sequestered self-antigens, or both, is difficult to determine.

Other non-specific mechanisms are involved in induction of autoimmunity after infection. In general, a secondary signaling is necessary to trigger autoimmune diseases. The so-called “adjuvant effect” leads the innate immune system to produce restricted responses to particular classes of pathogens through PRR, such as TLR (22, 23). Interaction of the microorganism component of adjuvants with PRR results in activation APCs and up-regulation of molecules essential for antigen presentation and production of pro-inflammatory cytokines. However, usually, adjuvant alone (that means without self-antigens) does not result in autoimmune disease.

In a broader view, microorganisms have to be considered to provide both the adjuvant effect to stimulate the immune response and the damage necessary to make self-antigens available to the immune system, resulting in autoimmune disease.

3.4 Autoimmunity in inflammatory heart diseases

Dilated cardiomyopathy represents the most common cause of heart failure in young patients and often evolves from myocarditis (24-26). Myocarditis is defined by the Dallas criteria as “the presence of an inflammatory infiltrate in the myocardium with necrosis and/or degeneration of adjacent myocytes” (27, 28). Worldwide, infections with the parasitic protozoan *Trypanosoma Cruzi* (Chagas disease), which is endemic in Southern America, are the leading cause of myocarditis. In developed countries, however, viral infections are common triggers of cardiac inflammation (24-26, 29). Besides enteroviruses

or adenoviruses, Parvo-, Hepatitis C-, Human Immunodeficiency- HHV-6, HHV7, Cytomegalo-, Epstein-Barr viruses have been detected in hearts of patients with inflammatory dilated cardiomyopathy (25, 26). Virus infections directly contribute to cardiac tissue destruction by cleaving the cytoskeletal protein dystrophin, leading to a disruption of the dystrophin-glycoprotein complex (30). Moreover, clinical observations and insights from animal models provide evidence that autoimmunity plays a relevant pathogenetic role in most cases of human myocarditis and in many patients with dilated cardiomyopathy (31). Notably, many patients with dilated cardiomyopathy develop heart-specific autoantibody responses (30). Myocytes isolated from patients with diagnosed myocarditis and idiopathic DCM often display increased levels of MHC class I and class II proteins (32), and myocarditis commonly develops in patients with systemic autoimmune disease (25). In addition, immunosuppressive therapy can improve heart function in subgroups of patients, particularly in individuals without evidence for persistence of viral genomes in heart biopsies (33, 34). Such findings have given rise to the hypothesis that while pathogenic attack may be responsible for an acute phase of myocarditis, post-infectious autoimmunity is responsible for chronic inflammation of the myocardium (24).

3.5 The experimental autoimmune myocarditis (EAM) model

In several susceptible mouse strains, infections with the human pathogenic Coxsackie type B3 (CVB3) virus result in chronic myocarditis progressing to heart failure, even after clearance of the virus (35, 36). In mice, chronic myocarditis following CVB3 infection is T cell mediated because adoptive transfer of T cells, but not serum from diseased mice transfers disease in SCID recipients genetically lacking B and T cells (37). Furthermore, peripheral blood lymphocytes from patients with dilated cardiomyopathy transfers disease to SCID mice (38, 39). Inflammatory infiltrates in affected mouse hearts are characterized by the interstitial and patchy accumulation of mononuclear cells and mirror the histologic pattern observed in human myocarditis. The marked reduction of inflammation in infected mice after depletion of CD4⁺ T cells, and the partial reduction of disease severity after depletion of CD8⁺ T cells, suggest that T cells play an important role in the

progression of myocarditis in CVB3 infected mice (37, 40, 41). Taken together, these findings indicate that heart-specific autoimmunity plays an important role in post-viral myocarditis and argue for a critical role of T helper cells in disease development.

Immunization models offer the advantage to study the disease pathogenesis *in vivo* in the absence of infection. Indeed, immunization with alpha-myosin or specific alpha-myosin peptides together with strong immunostimulants, such as CFA, induces heart-specific inflammation (EAM) in susceptible mouse strains, such as BALB/c (H-2^d) and A/J (H-2^a) mice (42, 43). The most pathogenic epitope for BALB/c mice has been identified on the alpha-myosin heavy chain protein (43). Activation of APC, taking up and processing self-antigen, is a prerequisite for T-cell priming. TLRs represent a family of transmembrane receptors that are essential for innate immune responses to pathogens. The different TLRs exhibit different specificities for microbial patterns such as LPS, or double-stranded RNA, as well as for some endogenous products such as stimulatory signals released by dying cells. Recently, it was shown that alpha-myosin-loaded dendritic cells activated through TLR ligands such as LPS or double stranded RNA can induce autoimmune myocarditis in BALB/c mice (44). Disease induction by vaccination with self-antigen-loaded dendritic cells offers an attractive approach to study the APC-effector-cell interactions and priming mechanisms of autoimmune T cells *in vivo*.

EAM can be induced in susceptible mouse strains by immunization with self-peptides derived from the MyHC- α together with a strong adjuvant like CFA (42), or by injection of activated, MyHC- α -loaded dendritic cells (44-46). In BALB/c mice, myocarditis severity peaks 21 days after the first myosin-peptide/CFA immunization or 10-14 days after the first injection of activated, peptide-loaded dendritic cells. Later on, most inflammatory infiltrates resolve spontaneously, but some animals develop fibrosis and dilated cardiomyopathy. In affected mice, impaired cardiac contractility correlates with the percentage of CD4⁺ T cells among heart-infiltrating CD45⁺ cells at the peak of disease. In fact, depletion of CD4⁺ T cells in A/J (H-2^a) mice led to protection from myocarditis after subsequent myosin immunization, whereas depletion of

CD8⁺ T cells reduced severity but not prevalence of disease (37, 40). The decisive role of CD4⁺ T cells in autoimmune myocarditis induction, however, was shown in adoptive transfer experiments. Injection of *in vitro* restimulated CD4⁺ T cells isolated from MyHC- α -immunized diseased mice induced myocarditis, albeit at a low severity grade, in severe combined immunodeficient (SCID) mice or LPS pretreated wild-type mice (37). Importantly, however, adoptive transfer experiments do not work in naïve wild-type mice.

3.6 T helper cells: the changing hypothesis about Th1, Th2, Th17 cells

EAM represents a CD4⁺ T cell-mediated disease. Historically, two major CD4⁺ T cell subsets have been defined according to their cytokine production pattern: IFN- γ -producing Th1 cells, and Th2 cells releasing IL-4. The majority of the heart-infiltrating cells share a monocyte/macrophage phenotype, suggesting that Th1 signals predominate. In addition, signaling through the receptor of the key Th2 cytokine IL-4 is dispensable for EAM development (47, 48). These findings would point to a pathogenic role of Th1 differentiation in EAM. However, loss of either the Th1 effector cytokine IFN- γ or its receptor results in markedly increased disease severity, implying that the key Th1 cytokine IFN- γ is a negative regulator of EAM (47-50). In addition, mice lacking T-bet, a T-box transcription factor essential for Th1 lineage differentiation, show enhanced myocarditis severity (47).

These apparently contradictory findings were finally explained by analysis of gene-targeted mice with mutations in the Th1-driving cytokine IL-12 and its receptor. IL-12p70 binding to the IL-12 receptor represents a heterodimer consisting of an IL-12p40 and an IL-12p35 subunit. The IL-12p70 receptor, on the other hand, consists of an IL-12R β 1- and an IL-12R β 2-subunit, and induces the recruitment of JAK2 and activation of STAT4 upon activation. Experiments with knock out mice revealed that both, the IL-12 β 1 and the IL-12p40 subunit, were essential for myocarditis induction (47, 48, 50). In contrast, IL-12p35-deficient mice developed more severe autoimmune myocarditis compared to wild-type littermate controls (47). These apparent paradoxical findings were finally explained by the discovery that IL-12p40

represents a shared subunit between IL-12p70 and IL-23, another cytokine of the IL-12 family. IL-23 is composed of IL-12p40 and a distinct p19 subunit. Moreover, the IL-23 receptor shares the $\beta 1$ chain with the IL-12p70 receptor. IL-23 has recently been recognized as a key mediator promoting the expansion and survival of another subset of $CD4^+$ T cells characterized by the production of the cytokine IL-17. Several lines of evidence clearly suggest that IL-17-producing $CD4^+$ Th17 cells represent a distinct subset of $CD4^+$ T cells,

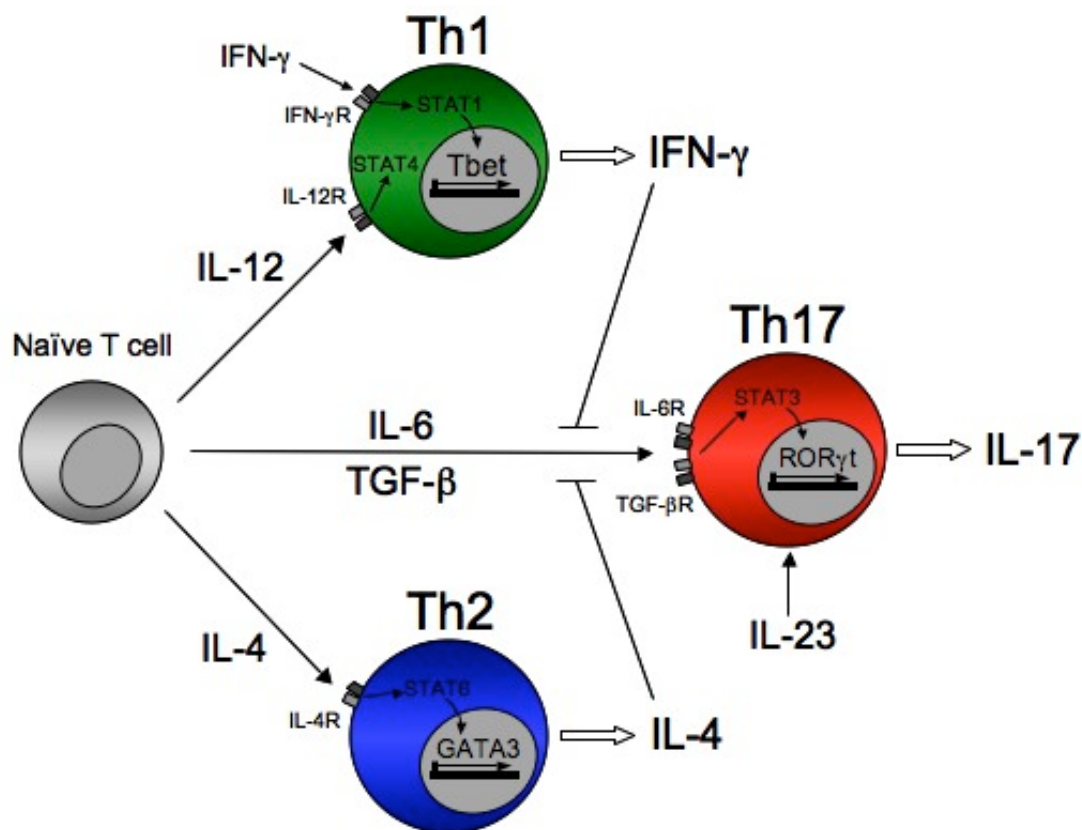


Fig 1.1. Effector differentiation of $CD4^+$ T-cell subsets. Following activation, naïve $CD4^+$ T cells differentiate towards Th1 in the presence of IL-12, which upregulates IFN- γ via Stat4, leading to IFN- γ -mediated Stat1 activation and induction of the Th1 lineage determining transcription factor Tbet. Th2 by contrast differentiates in response to IL-4, which activates Stat6, resulting in induction of GATA3. The Th17 T-cell subset develops in response to IL-6 and TGF- β , and this differentiation step is strongly inhibited by Th1 or Th2 cytokines. Signaling via IL-6 activates Stat3 and the lineage-determining transcription factor ROR γ t. Signaling through TGF- β receptor is also essential for Th17 development, as T cells defective in TGF- β RII signaling cannot differentiate to Th17. (Stockinger B and Veldhoen M, *Curr Opin Immunol*, 2007, modified).

developing as a Th1 and Th2 cell independent lineage (51-54). Th17 cells play a key role in the development of various models of chronic and autoimmune inflammation, such as experimental autoimmune encephalomyelitis (54), graft-versus-host disease (55), and allergic asthma (56). Recent data provided evidence that autoimmune myocarditis development critically depends on the IL-23-STAT4 axis (50) and on the expansion of heart-specific Th17 CD4⁺ T cells (47, 57-59). Notably, the direct pathogenic role of Th17 cells was illustrated in adoptive transfer experiments. Immunization of mice with MyHC- α /CFA results in relatively low numbers of heart-specific Th17 cells in peripheral lymphatic organs. This observation explains why adoptive transfer with *in vitro* re-stimulated CD4⁺ T cells isolated from draining lymph nodes or spleens of diseased mice results in minimal cardiac inflammation only. Selective *in vitro* expansion of MyHC- α -specific IL-17-producing CD4⁺ Th17 cells in the presence of recombinant IL-23 and anti-IL-12p70 antibodies, however, yields heart-specific Th17 cell lines which are highly pathogenic after transfer in wild-type mice (59). Based on these findings, the question arises whether IL-17 is rather a surrogate marker of a pathogenic T helper cell subset or a disease promoting cytokine by itself. The observations that systematic depletion of IL-17 in MyHC- α /CFA-immunized mice by an anti-IL-17 antibody (59) or by active vaccination (57) both ameliorate disease severity and the recruitment of inflammatory cells, suggest a direct pathogenic role of IL-17 in EAM.

3.7 Cytokines promoting Th17 lineage commitment

Activation of antigen presenting dendritic cells through Toll-like receptors results in up-regulation of MHC class II as well as co-stimulatory molecules. Activated dendritic cells produce high amounts of pro-inflammatory cytokines, which exert auto- and paracrine effects on both, antigen-presenting cells as well as T cells. Major pro-inflammatory cytokines released by TLR activated dendritic cells include IL-12p40, IL-1 β , IL-6, and TNF- α , which are all critical for EAM induction and progression (44, 46, 47, 60). Gene-targeted mice lacking the TNFRp55receptor, for example, are protected from disease, and it had been hypothesized that TNF- α mediates the activation of heart resident

dendritic cells as well as adhesion molecules on the vascular endothelium (61).

Interaction of different cytokines is necessary for the development of the Th17 lineage. Th17 differentiation is initiated by IL-6 and TGF- β (Fig. 1.1) (62, 63). Moreover, also IL-1 is considered necessary for Th17 initiation (64). In fact, mice lacking IL-1, IL-6, or TGF- β , present an impaired Th17 development and an increased production of Th1 and Th2 (64, 65). As mentioned before, IL-23 is a critical mediator for the expansion and survival for the pathogenic Th17 subset (51-53). Th17 cells produce IL-17, IL-17F, and IL-22, all of which regulate inflammatory responses (66, 67). IL-21 is considered as an autocrine factor induced by IL-6 to regulate Th17 differentiation (68, 69). Cytokines that usually trigger differentiation of Th1 and Th2 cells, as IFN- γ and IL-4, negatively regulate Th17 differentiation (51, 53).

Analogous to STAT4 and STAT1 in Th1 and STAT6 in Th2 differentiation, STAT3 mediates Th17 differentiation (70, 71). Overexpression of STAT3 enhances Th17 differentiation, whereas STAT3 deficiency impairs Th17 differentiation *in vitro* (70, 71) and *in vivo* (72). The precise biochemical function of STAT3 is not completely clear. Although STAT3 has been shown to bind to the IL-17 gene promoter (73), STAT3 appears to control more than just IL-17 gene expression (71). Similarly to STAT1 and STAT6, STAT3 seems to be involved in regulation of lineage-specific master-transcription-factor expression. One such Th17-specific transcription factor is a retinoid-related orphan nuclear receptor, ROR γ . The specific isoform ROR γ t is the transcription factor to be selectively expressed in Th17 cells (74) and it is regulated by STAT3 (70, 71). Overexpression of ROR γ t promotes Th17 differentiation when Th1 and Th2 development is inhibited (74). On the contrary, lacking of ROR γ t results in profound Th17 deficiency and protection from autoimmune diseases. However, ROR γ t deficiency does not completely abolish Th17 differentiation, suggesting the involvement of other factors. Overexpression of another orphan nuclear receptor, ROR α , which is induced by TGF- β and IL-6 in a STAT3-dependent manner (75), promotes Th17 differentiation up-regulating IL-17. Lacking of ROR α results in reduced IL-17 expression *in vitro* and *in vivo*, while double deficiencies in ROR α and ROR γ

entirely impairs Th17 generation *in vitro* and completely inhibits autoimmune diseases (75).

3.8 Interferon gamma (IFN- γ) and nitric oxide (NO)

IFN- γ , a type II IFN, is considered a pro-inflammatory cytokine. In a broader view, IFN- γ is one of the first cytokines involved in host defense when bacteria attack the body. Invading pathogens soon encounter macrophages, which activation is one of the first actions to occur in innate immunity. When activated, macrophages acquire microbicidal effector functions, secrete pro-inflammatory cytokines, and respond to a range of different cell products and proteins during innate and adoptive immune responses. IFN- γ , originally called macrophage-activating factor, is one of the most important cytokine that activates macrophages. The importance of IFN- γ for macrophages is connected with the transcription of inducible nitric oxide synthase (NOS2), a crucial enzyme for the production of the short living radical gas nitric oxide (NO) (76, 77). NOS2 induction is regulated at transcriptional level by two discrete regulatory regions: region I contains LPS-related responsive elements, region II contains motifs for binding IFN- γ -related transcription factors (78, 79). Single activation of one those regions only slightly increases NOS2 transcription, whereas simultaneous cooperation of both regions highly increases NOS2 expression (78, 79). NOS2 has a relevant function in host defense and in the cardiovascular system, whereas other nitric oxide synthases like endothelial nitric oxide synthase (eNOS) and neural nitric oxide synthase (nNOS) seem not to be involved in these two systems.

Nitric oxide is considered one of the most potent mediators for T-cell inhibition and suppression. Activated NO-producing macrophages are extremely efficient T-cell suppressors (77). Especially inflammatory T cells are inhibited by macrophage-derived NO, which reversibly dephosphorylates intracellular signaling molecules involved in the control of T cell proliferation, like STAT5 and Jak3 (24, 80). NO is an important regulator in autoimmune diseases. This is proven in mice lacking NOS2, which are high susceptible to develop auto-aggressive T cells attacking self-antigens (81, 82).

3.9 Dendritic cells: key players in EAM regulation

Dendritic cells are potent antigen presenting cells that induce primary T cell responses (83-86). Dendritic cells modulate peripheral tolerance, contributing to the expansion and differentiation of T cells that, in turn, regulate or suppress other T cells (87). These immunomodulatory functions are supported by several stimulatory signals, which determine the maturation stage of dendritic cells and the release of different levels of proinflammatory cytokines (87, 88). The efficient priming of heart-specific, autoreactive T cells requires co-operation between the innate and adaptive activation signals beyond an individual and genetically defined threshold level. Dendritic cells are sentinels of the immune system scavenging foreign pathogens as well as cell debris and necrotic tissues. Non-specific activation of dendritic cells through Toll-like receptors is supposed to be critical for autoreactive T cell priming. Once activated, CD40 ligand expressing autoreactive T cells further increase the priming efficacy of the dendritic cells by a positive feedback loop that promotes production of pro-inflammatory cytokines and survival of activated dendritic cells via CD40-CD40L interaction (45).

Several lines of evidence point towards a critical role of dendritic cells in inflammatory heart diseases. Clinically total peripheral blood dendritic cells are elevated in patients with end stage heart failure (61). In mice, a specific subset of interstitial cells expressing the dendritic cell marker CD11c had been identified in perivascular heart tissue. Interestingly, these cells constitutively express cardiac self-antigens on MHC class II molecules even in the healthy heart (89). The number of these cells and up-regulation of their MHC class II expression parallels the development of cardiac inflammation (43, 90). The precise role of these heart resident dendritic cells is not clear.

As mentioned above, dendritic cell activation is largely mediated by TLRs. Importantly, different TLR exhibit different specificities for microbial patterns such as LPS, or double stranded RNA, as well as for some endogenous products such as heat-shock proteins and other stimulatory signals released by dying cells (91). Accordingly, injection of mice with MyHC- α -loaded dendritic cells activated through TLR ligands such as LPS or CpG triggers autoimmune myocarditis and heart failure in susceptible BALB/c mice (45).

Furthermore, mice lacking the common adaptor molecule MyD88 for different Toll like receptors are protected from autoimmune myocarditis (60).

Antigenic mimicry can put the organism at risk for autoimmune heart disease. An immune system that was exposed to symptomatic or sub clinical infections with any microorganisms containing self-antigen like structures could be more susceptible to boost an autoreactive T cell response after a second hit that releases self-antigen on the background of a non-specific inflammatory response (24). In fact, some patients develop heart-specific autoimmunity after non-infectious tissue damage, for example after cardiac surgery or myocardial infarction (92, 93). It is conceivable that tissue damage of any cause results in uptake of self-antigens by dendritic cells. If dendritic cells, which uptakes self-antigens, become activated, might initiate an autoimmune response depending on the genetic susceptibility of the affected individual (94, 95). It seems that activation of self-antigen-loaded dendritic cells boost autoreactive T cells, aggravating cardiac inflammation.

Dendritic cells are not only essential for the induction of autoimmune T cell responses and diseases. Dendritic cells stimulated with the inflammatory cytokine TNF- α in the absence of a strong TLR stimulant become semi-mature dendritic cells, while exposure TLR stimulants, especially in the presence of CD40 ligation, rather results in mature activated dendritic cells (96, 97). Interestingly, bone marrow-derived semi-mature, i.e. TNF- α -stimulated dendritic cells (bmDC), expressing CD11c⁺ and CD11b⁺, but not CD8 α ⁺, confer antigen-specific protection from autoimmunity in the mouse models of experimental autoimmune encephalomyelitis (EAE) (98, 99), collagen-induced arthritis (100), and experimental autoimmune thyroiditis (EAT) (101). LPS plus α CD40 antibodies-stimulated bmDC, on the other hand, do not have any significant effect on the disease course (98-100). In contrast, as previously described, self-peptide-loaded bmDC stimulated with LPS plus α CD40 antibodies induce experimental autoimmune myocarditis (EAM) (44-46, 60) and experimental autoimmune uveitis (EAU) (102).

Several lines of evidence indicate that other dendritic cells subsets have the potential to modulate various T cell responses. For example, in the spleen reside up to five subpopulations of dendritic cells with different capacity to

stimulate the proliferation of T cells (103). Indeed, some splenic dendritic cell subpopulations are able to modulate T cell differentiation promoting the production of anti-inflammatory cytokines (104-106). Splenic DC from lymphoid origin, which express $CD8\alpha^+$, can expand IFN- γ -producing Th1 cells, while splenic DC from myeloid origin, lacking $CD8\alpha$ expression, promote IL-4-producing Th2 cells (104-107). Phenotypically, $CD8\alpha^+$ DC express high levels of the typical DC-marker $CD11c$, but not $CD11b$, while $CD8\alpha^-$ DC express high levels of $CD11c$, as well, but also the myeloid marker $CD11b$ (106).

As the amount of splenic DC expressing $CD11c$ is less than 1% of all splenocytes (108), several cytokines are used to increase the number of these cells. One of the most used cytokine is FLT3L, a hematopoietic growth factor that stimulates the proliferation and differentiation of hematopoietic stem and progenitor cells (103). Mice treated with serial injections of FLT3L significantly accumulate DC in the spleen, lymph nodes, bone marrow, and liver (103, 109). Another important cytokine that expands DC is GM-CSF. Comparing the two cytokines, GM-CSF induces a biased myeloid lineage of dendritic cells which express high levels of $CD11c$ and $CD11b$, but extremely low levels of $CD8\alpha^+$, whereas FLT3L induces DC of both lymphoid and myeloid origin (110). Recently, GM-CSF has been shown to be crucial for the development of pathogenic $CD4^+$ Th17 cells in autoimmune myocarditis (65), suggesting that GM-CSF mostly acts as a pro-inflammatory cytokine, FLT3L, on the other hand, might act preferentially as a tolerogenic cytokine.

4 Material and methods

4.1 In vivo experiments

4.1.1 MICE

Mice were used at 6-8 weeks of age. Rag2^{-/-}, IFN- γ ^{-/-}, and DO11.10 mice (all BALB/c background) were obtained from The Jackson Laboratory. IFN- γ R^{-/-} mice have been backcrossed on BALB/c background by Prof. Urs Eriksson more than 10 times. CD45.1 BALB/c mice were obtained from Prof. Antonius Rolink. All mice were housed in an optimized hygienic area with SPF (specific pathogen-free) rooms (in accordance with the guidelines of the Department of Biomedicine at the University Hospital Basel) and received acidified (pH 2.5) water and sterile food. Newborn male and female mice were separated 3 weeks after birth in sterilized cages covered by a special protecting filter. No more than 5 mice were put together into each cage.

4.1.2 INJECTIONS

- Intravenous injection (i.v.): intravenous injections are given into one of the two tail veins. After putting mice under a heat lamp to increase blood flow, suspensions of at most 200 μ l were injected into the lateral tail vein with a 29G syringe (BD Micro-Fine, 0.5 ml, U-100 insulin) (Fig. 4.1). Quantities bigger than 200 μ l were usually not injected, because they cause acute fluid overload and death.



Figure 4.1. Intravenous injection. (Experimental Critical Care Medicine Lab, Valaperti and Germano)



Figure 4.2. Intraperitoneal injection. (Experimental Critical Care Medicine Lab, Valaperti and Germano)

- Intraperitoneal injection (i.p.): using a 1 ml syringe with a 26G needle, the skin and the abdominal muscles are pierced to inject the solution into the intraperitoneal cavity. Usually, 200 μ l were injected into one mouse. The mouse is restrained and tilt so that the head is facing downward and its abdomen is exposed. The needle is inserted cranially into the abdomen at a 30-45 degree angle caudal to the umbilicus and lateral to the midline (Fig. 4.2). To test whether internal organs have been damaged, the syringe's plunger should be retracted before injecting the suspension: if vacuum has been created in the peritoneum, nothing has been damaged, but if a yellow fluid has been sucked into the syringe, the gut or other organs of the gastrointestinal tract have been damaged and the contaminated suspension has to be discarded.

- Subcutaneous injection (s.c.): s.c. injections were usually performed in two different body regions: in the inguinal area and in the footpad.

Inguinal s.c injections were done to immunize mice for EAM induction (see "Emulsion preparation for mice immunization"). A 26G needle connected with a special Omnifix syringe (B. Braun, Melsungen, Germany) was inserted at the tail base through the skin, taking care to not break the peritoneum. After verifying that a vacuum was created retracting the syringe's plunger, the emulsion was carefully injected and the needle was retracted only some seconds after the whole emulsion was injected. A subcutaneous pouch was visible in the inguinal region of the mouse.



Figure 4.3. Footpad injection in anesthetized mouse (Experimental Critical Care Medicine Lab, Valaperti and Germano)



Figure 4.4. The direction of the needle relative to the foot (Lovik et al., 2007)

Footpad injections were performed to test the potential of different DC subsets to induce T cell differentiation *in vivo*. The advantage of this method is that foot's draining lymph nodes are located in the popliteal region (Fig. 4.7), and almost all injected cells reach the popliteal LN (pLN). Only a small amount of footpad-injected cells bypasses the pLN and reaches other lymphnodes. For this reason we took advantage from the footpad injection to study T cell development in a well defined peripheral region. To perform footpad injection, suspensions were resuspended in a maximum volume of 50 μ l, because higher volumes leak out of the foot. Mice were anesthetized with isoflurane and were provided with anesthesia during injection (Fig. 4.5). The cells were injected with a 29G needle inserted from the heel in the direction of the toes (Fig. 4.4). After injection, a subcutaneous bubble is visible in the footpad.

- Isoflurane inhalation anesthesia: Mice were placed in a plastic container connected with a tube providing an anesthetic gas mixture. A vaporizer provided standardized gas concentration to the outlet tube. Isoflurane was administered in a mixture of N_2O/O_2 . Anesthesia was induced by 2.5% vaporized isoflurane until loss of righting reflex (Fig. 4.5). After putting the animal on a stable support for surgical operation (Fig. 4.3), anesthesia was maintained by direct inhalation of iso-vaporized isoflurane at a concentration of 1.5% during injection. During injection, treated mice did not move, did not have any reaction to pain stimuli, and their body temperature was kept constant at 37°C. After injection, mice were kept under a warm UV lamp till their waking up.



Figure 4.5 Equipment for isoflurane anesthesia. (Experimental Critical Care Medicine Lab, Valaperti and Germano)

- Emulsion preparation for mice immunization: each mouse received 150 μg of peptide derived from α -myosin heavy chain (MyHC- α , sequence 614-634 Ac-RSLKLMATLFSTYASADR, CASLO, Denmark) emulsified in 100 μl of Complete Freund Adjuvant (CFA, 1 mg/mL, H37Ra, Difco Laboratories). Because MyHC- α -peptides are high static, they are weighted in a closed analytical balance (Mettler AE-260). Well-shaken CFA was pipetted directly on the weighted peptides in the balance with a 1-ml pipette, which is also used to properly resuspend peptides in CFA. After filling the mixed suspension into a syringe with a thread (Ominfix 2 ml, B. Braun, Germany) through an 18G needle, another syringe filled with the same amount of sterile phosphate saline buffer (PBS) is joined to the first one by fixing them in to a three ways stopcock (BD Connecta, Sweden). PBS is first pressed into the peptide/CFA suspension, and the emulsion is ready for use as soon as the resistance in the syringes is increased. Pouring some drops into water checks the quality of the suspension: if the suspension is correctly done, the drops maintain their shape and consistence, floating on the water's surface.

Mice were subcutaneously immunized at 6 to 8 weeks of age on day 0 at one side of the tail base and boosted on day 7 on the other side (Fig. 4.6). No deaths were observed after immunization.

4.1.3 LYMPH NODE ISOLATION

Proper lymph node (LN) isolation is a prerogative to perform experiments with high

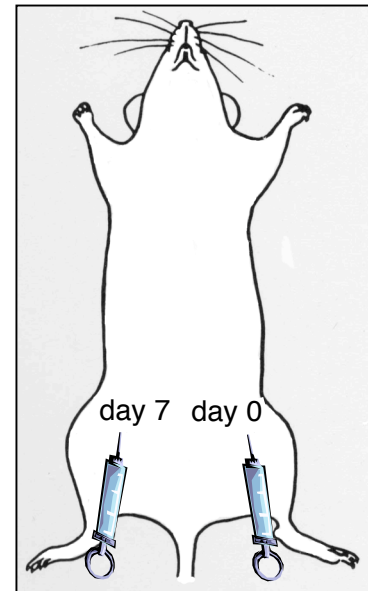


Figure 4.6. schematic view of the inguinal region where mice are immunized for EAM induction.

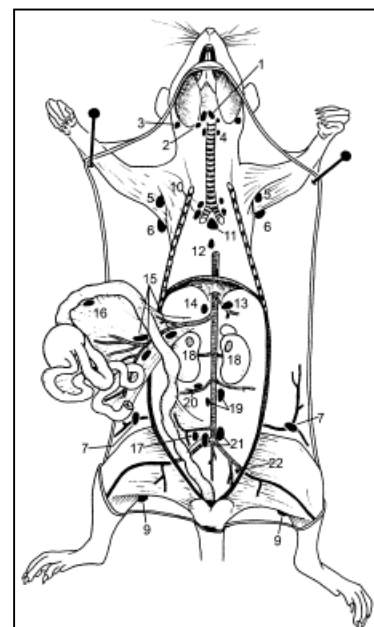


Figure 4.7. Overview of all LN. Nr. 10: mLN; Nr. 7: iLN; Nr. 9: pLN. (Van den Boek et al., 2006)

quality material. Organs should not be contaminated by other organs (for example parts of pancreas joined to the isolated spleen contain several enzymes that can interfere with the isolation of splenocytes), should not be damaged or broken, and surrounding organs should be intact (for example during mediastinal LN isolation, damaging the thymus can cause thymocytes contamination on the isolating mediastinal LN). Incorrectly isolated organs can compromise the validity of the results.

- Mediastinal LN (mLN): mLN are located in the intrathoracic region. Two LN (bilaterally) are located lateral to the thymus along the internal thoracic artery and vein (Fig. 4.7). To extract them, the mouse is observed from the lateral side. Using tweezers, the lung and the thymus are carefully turned over to the opposite side (Fig. 4.8). Two LN surrounded by some fat are visible. With little curved tweezers, the two LN are gently extracted, taking care to not damage the thymus, which can contaminate the tweezers with high amounts of thymocytes. If the thymus is accidentally involved, extracted mLN and tweezers are cleaned in sterile PBS and resuspended in fresh sterile PBS.

- Inguinal LN (iLN): iLN are located in the fold of the flank, near the deep circumflex of the iliac artery and vein. It is relatively easy to find them, because they are situated closed to the bifurcation of the superficial epigastric vein (Fig. 4.5). Sectioning the mice with a sagittal cut in the abdomen, continuing cutting toward the arms and the legs of the mice, it is possible to turn over the skin and see the iLN, which are carefully extracted with a little curved tweezers, avoiding to collect too much surrounding fat.

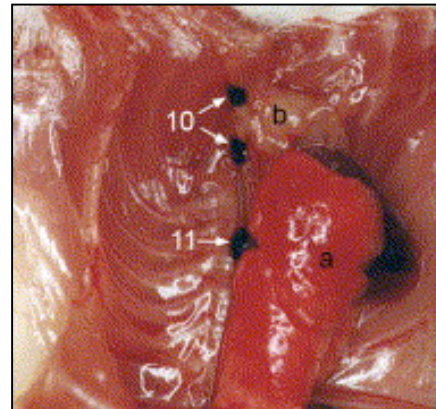


Figure 4.8. Localization of one of the two pairs of mLN in the thoracic cavity. Nr. 10: mLN; Nr. 11: tracheobronchial LN; a: lung (turned over); b: thymus (turned over). (Van den Broek et al, 2006).

- Popliteal LN (pLN): pLN are located in the popliteal fossa in the backside of the knee between the muscle biceps femuri and the muscle semitendinosus (Fig. 4.9). To harvest the pLN, a slit is made in the skin below the knee with scissors, and the skin is thereafter pulled upwards towards the thigh. With normal tweezers in one hand, the biceps femuri is lightly lifted up, while a little curved tweezers in the other hand is used to carefully extract the pLN. This step is really important,

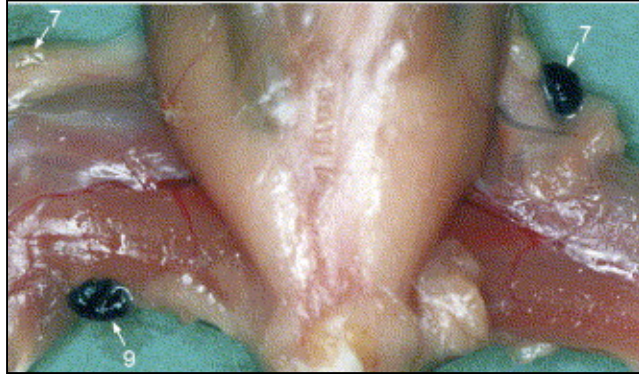


Figure 4.9. Localization of iLN (Nr. 7) and pLN (Nr. 9) in the hind limb region. (Van den Broek et al., 2006).

because the pLN are surrounded by high amounts of fat components that are almost round-shaped and misleading. In contrast to the white transparent color of the soft fat tissue, however, pLN have grey color and firm consistence, and a very constant location in the fork of a large branching artery. Properly extracted pLN sink when placed in medium, while fat tissue floats. This control is valid for all kind of extracted LN.

- Spleen: with its length of approximately 1.5 cm, it is the easiest lymph organ to extract. It is localized in the left superior abdominal quadrant and has a lengthened, oval, and slightly curved shape. For extraction, the body of the spleen is held with tweezers and the hilum together with the gastrosplenic ligament is cut. Intact spleens can be kept on ice for several hours before meshing them to yield splenocytes.

4.1.4 BLOOD COLLECTION

As for humans, the total blood volume of the mouse makes up about 6-8% of its body weight (the body weight of a 8-12 weeks old mouse is between 25 and 30 gram, meaning about 2 ml of blood). The blood was usually collected immediately after the mice have been killed from the inferior vena cava, using a 26G needle and a 1 ml syringe (Luer tuberculin, Primo Primo, Denmark). About 0.5 ml mouse blood was collected with this method. Mice should be quickly dissected, to avoid blood clotting, but carefully handled, to not break

any organ, which can bleed and reduce the yield. Mouse blood was collected and centrifuged for ten minutes at 10000 g in little tubes containing a gel (SST Tubes, BD Microtainer), which was of optimal density to separate blood cells from plasma.

4.1.5 CHIMERA

6 to 8 weeks old BALB/c CD45.2 mice were lethally irradiated with two doses of 6.5 Gy using a Gammatron (^{60}Co) system, with a break of four hours between the two irradiations. Irradiated mice were reconstituted with a total of 2×10^7 donor bone marrow cells, which were isolated from the bones of BALB/c CD45.1 mice. Femur and tibia were dissected, cleaned from all muscles, washed for one minute in 70% ethanol (only if no part of the bone was broken, because ethanol entering into the damaged bone could destroy many marrow cells), and put in PBS. The two extremities of each bone were cut and, with the help of a PBS-filled 10 ml syringe joined to a 26G needle, the bone marrow was flushed out of the bones. Marrow cells were filtered with a 70 μl cell sieve, centrifuged, treated for two minutes with 2 ml/spleen of warm ACK to lyse red blood cells, washed with PBS, centrifuged, and resuspended in PBS at a concentration of 10^8 cells/ml. Donor BALB/c CD45.1 bone marrow cells were injected i.v. into the lateral tail vein. Chimeric mice were housed in a specific pathogen free environment and received prophylactic antibiotics (0.2% Bactrim, Roche) in drinking water. Chimeric mice were used 2 month after reconstitution.

4.2 Ex vivo cell cultivation

4.2.1 BONE MARROW-DERIVED MACROPHAGES

Bone marrow was isolated from 4-8 weeks old mice and prepared as described for chimera reconstruction. Six millions cells resuspended in 6 ml RPMI 1640 medium/10% FBS supplemented with 50 ng/ml macrophage colony stimulating factor (rmM-CSF, PeproTech) were seeded in 10 cm bacterial dishes (Ten-twenty-nine, Petri Dish, Falcon, BD) and cultured for 5 days in an humidified incubator at 37°C containing 5% CO_2 . At day 1 and day 3, 3 ml of medium were added. At day 5, macrophages surface markers were

analyzed by FACS. Mature bone marrow-derived macrophages (BMM) expressed CD11b, F4/80, CXCR4, CD11c, CD80 and MHC II. For *in vivo* experiments, BMM were stimulated for three hours with 0.1 µg/ml lipopolysaccharides (LPS) and 1 µg/ml recombinant mouse (rm) IFN-γ (PeproTech). After washing, stimulated BMM were resuspended in PBS at a density of 10^7 cells/ml, and 2×10^6 cells/mouse were injected i.v. into the lateral tail vein.

4.2.2 BONE MARROW-DERIVED DENDRITIC CELLS

As for BMM, bone marrow was isolated from 4-8 old mice and prepared as described for chimera reconstruction. 2×10^6 bone marrow-derived dendritic cells (bmDC) were resuspended in 10 ml RPMI 1640 medium/10% FBS supplemented with 200 ng/ml granulocyte macrophage colony stimulating factor (M-CSF, PeproTech), seeded in 10 cm bacterial dishes (Ten-twenty-nine, Petri Dish, Falcon, BD), and cultured at least for 8 days in an humidified incubator at 37°C containing 5% CO₂. At day 3, 5 ml of medium containing GM-CSF are added, while at day 6 and day 8, 5 ml of supernatant were removed, centrifuged, resuspended in fresh medium, and added back into the dish. At day 8, non-adherent cells were collected by gently pipetting with cold PBS, resuspended in fresh medium containing GM-CSF, plated into a new dish at a density of 1.5×10^6 cells/ml, loaded with 10 µg/ml MyHC-α-peptide for 1 hour, and stimulated for two additional hours with 0.1 µg/ml LPS and 5 µg/ml anti-CD40 antibodies. PBS was used to detach and wash adherent bmDC, which were then resuspended at a density of 2.5×10^6 cells/ml for myocarditis induction. Aliquots of stimulated and non-stimulated bmDC were analyzed for the expression of surface markers such as CD11c, CD1b, MHC II, CD40, and CD80. Half a million of loaded and stimulated bmDC per mouse were injected i.p. three times at day 0, day 2, and day 4. Myocarditis severity scores peaked ten days after the first immunization.

4.2.3 ISOLATION OF SPLENIC CD8α⁺ DENDRITIC CELL

Because the percentage of dendritic cells (DC) in the mouse spleen is lower than 1%, mice were daily treated for ten days with 10 µg/mouse of

recombinant FLT3 ligand (FLT3L), promoting DC expansion. Treatment resulted in an increase of the percentage of DC up to 15-20% of all splenocytes. After FLT3L treatment, the spleen was dissected with sterile instruments and put in a 6 ml Petri Dish (Falcon, BD) containing 5 ml of Collagenase buffer (Collagenase buffer for 50 ml ddH₂O: 0.119 g of 10mM Hepes-NaOH pH7; 0.4383 g of 150 mM NaCl; 0.018 g of 5 mM KCl; 0.047 g of 1 mM MgCl₂; 0.00998 g of 1.8 mM CaCl₂) supplemented with 185.2 µl of collagenase type IV (stock concentration: 27 mg/ml, Worthington, Lakewood, NJ). Using a 29G needle insulin syringe (0.5 ml, U-100 insulin, BD Micro-Fine) filled with collagenase solution, the spleen was injected twice in both extremities to properly digest all stroma cells. The spleen was then cut in small pieces with two sterile surgical blades (Swann-Morton, Sheffield, England) and incubated for 40 minutes at 37°C. Immediately after incubation, PBS supplemented with 1% FBS and 2 mM EDTA was added to block the digestion. The dish was properly washed with cold PBS to collect all cells, and, if necessary, also scratched with a cell scraper (16 cm, Sarstedt, Newton, NC) to detach adherent DC. Cell suspension was mashed up through a 70 µm cell strainer (BD Falcon) with the plunger of a 1 ml tuberculin syringe. After centrifugation, red blood cells were lysed with 2 ml of warm ACK for two minutes. Splenocytes were resuspended in PBS, centrifuged, filtered through a 40 µm cells strainer (BD Falcon), centrifuged again, and resuspended in 200 µl MACS buffer (PBS supplemented with 1% FBS and 2 mM EDTA, sterile filtered) for each 10⁸ splenocytes. This is the optimal amount of buffer for Microbeads labeling. Splenocytes were incubated for ten minutes on ice with a biotin-antibody cocktail staining for B cells (CD45R), T cells (CD90), and natural killer cells (CD49b) (CD8⁺ Dendritic Cell Isolation Kit, mouse, MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). This staining allows a depletion of unwanted cells, increasing the frequency of DC among the splenocytes. 150 µl of MACS buffer for each 10⁸ cells were added, and immediately 100 µl of anti-biotin Microbeads were mixed together. After 15 minutes incubation on ice, cells were washed, centrifuged, and resuspended in 500 µl of MACS buffer for each 10⁸ cells. The cell suspension was ready for depletion using an autoMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany), which was rinsed and cleaned according to

the provider. Selecting the depletion program “Depl025” and waiting for about twenty minutes, a more pure DC population was collected from the negative-depleted outlet port. After quick washing, negative-depleted cells were resuspended in 400 μ l of MACS buffer for each 10^8 initial cells together with 100 μ l of CD8 α^+ Microbeads and incubated for 30 minutes on ice. After washing, the cells were resuspended in 500 μ l for each 10^8 initial cells and positively sorted selecting the program “Posseld2” of the autoMACS Separator. Sorted CD8 α^+ DC were quickly washed with MACS buffer and resuspended in RPMI 1640 medium/1% NMS for cell counting. CD8 α^+ DC were seeded in 6 ml Petri Dish (Falcon BD) at a concentration of 1.5×10^6 cells/ml, loaded with 2 μ g/ml MyHC- α -peptide and stimulated with either 50 ng/ml tumor necrosis factor alpha (TNF- α , PeproTech) or 0.1 μ g/ml LPS plus 5 μ g/ml anti-CD40 antibodies for 4 hours. After incubation, DC were washed with cold PBS, if necessary scratched with a cell scraper to detach adherent cells, centrifuged, and resuspended in PBS at a concentration of 2.5×10^6 /ml. Each vaccinated mouse received 0.5 Mio loaded and stimulated CD8 α^+ DC by i.v. injection three times, that is every second day.

4.2.4 MYHC- α -SPECIFIC CD4 $^+$ T-CELL LINES

CD4 $^+$ T cells were sorted and purified from the spleens of diseased mice digested as described in “Isolation of splenic DC”. Splenocytes suspension was resuspended in 0.5 ml MACS buffer and incubated with 50 μ l anti-CD4 Microbeads (Miltenyi, Bergisch Gladbach, Germany) for 15 minutes at 4°C. After incubation, stained cells were washed, centrifuged, and resuspended in 0.5 ml MACS buffer for cell sorting. Choosing the program “Posseld2” in the autoMACS Separator, five minutes later CD4-labelled splenocytes were separated and collected from the “pos2” outlet port. Purified CD4 $^+$ T cells were quickly washed, centrifuged, resuspended in 5 ml RPMI 1640/10% FBS, and cultured in a 6 well plate (Falcon, BD) with irradiated (25 Gy) splenocytes at a 1:2 ratio in the presence of 2 μ g/ml MyHC- α for 7 days. Cells were then washed and rested in the presence of 20 ng/ml IL-2 (PeproTech) and either 10ng/ml IL-12p70 (PeproTech) and 1 μ g/ml anti-IL-4 (self-produced), or 10 ng/ml IL-23 (eBiosciences), 1 μ g/ml anti-IL-4, and 0.1 μ g/ml anti-IFN-

γ (PeproTech) for another 7 days. We used either recombinant mouse cytokines or anti-murine antibodies. This pulse/rest cycle was repeated at least three times. After three cycles, CD4⁺ T cell clones were stimulated for 4 hours with 20 ng/ml phorbol myristate acetate (PMA), 1 μ M ionomycin (IO), and 10 μ g/ml Brefeldin A (BFA) (all Sigma) for analysis of intracellular cytokines production by FACS. Production of IFN- γ , IL-4, and IL-17 were parameters used to characterize CD4⁺ Th1, Th2, and Th17 cell lines, respectively. Finally, CD4⁺ T cells were re-stimulated on MyHC- α pulsed APC for 4 days before i.p. injection of 10⁷ cells into syngeneic wild-type or RAG2^{-/-} recipient mice. Myocarditis develops after 10-15 days.

4.2.5 ISOLATION OF HEART INFILTRATING CELLS

Hearts were gently perfused with 10 ml of fresh PBS using a 26G needle and a 10 ml syringe, dissected with scissors, placed in a 6 ml Petri Dish containing 20 μ g/ml Liberase Blendzyme (Roche), cut in small pieces with two sterile surgical blades, and digested for 45 minutes at 37°C. Digested hearts were resuspended in PBS supplemented with 1% FBS and 2 mM EDTA to block the enzymes, mashed up on a 70 μ m cell strainer (BD Falcon) with the help of the plunger of a 1 ml syringe, centrifuged at 50 g for two minutes to pellet unwanted cardiomyocytes, passed again through a 40 μ m cell strainer, centrifuged, and finally passed through a 15 μ m self-assembled strainer (Sefar AG). Cell suspension was resuspended in 1 ml/heart of FACS buffer (PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA, filtered) for analysis of surface markers.

4.3 *In vitro* experiments

4.3.1 PROLIFERATION ASSAY

CD4⁺ T cells were purified as described in “MyHC- α -specific CD4⁺ T-cell lines”. 5x10⁴ sorted CD4⁺ T cells were resuspended in 200 μ l RPMI 1640/10% FBS and re-stimulated in a 96 well plate (U-Bottom, Falcon, BD) on 10⁵ irradiated (25 Gy) syngenic splenocytes pulsed with 2 μ g/ml MyHC- α -peptide for 48 hours. For peptide-unspecific stimulation, 200 μ l anti-CD3 and anti-

CD28 (both 1 mg/ml, BD Biosciences) resuspended in PBS were incubated for 2 hours at 37°C into 96 well plate. After washing and centrifuging, 5×10^4 CD4⁺ T cells were incubated for 48 hours. Titrating amounts of BMM were co-cultured with peptide-specific or peptide-unspecific stimulated CD4⁺ T cells. Eight hours before the incubation is finished, 0.5 μ Ci [³H]-thymidine (PerkinElmer, Boston, MA) were added into each well. The cells were then passed through special 96 well filters (UniFilter-96, GF/C, PerkinElmer), washed with 70% ethanol, treated with a scintillation buffer (Microscint 20, PerkinElmer), covered with an adhesive sealing film (TopSeal-A, 96-well microplates, PerkinElmer), and analyzed for fluorescence in a TopCount-NXT (Packard). Incorporation was measured as a read-out for proliferation responses.

4.3.2 CELL LABELING

Cells were labeled to monitor migration and proliferation of cells injected into mice. The most stable intracellular fluorescent dye was carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes), used at an optimized final concentration of 1 μ M for BMM. Mature BMM were resuspended in PBS supplemented with 5% FBS and 1X penicillin/streptomycin at a concentration of 2×10^6 cells/ml. 110 μ l of 10 μ M CFSE were rapidly added to each ml of BMM and quickly vortexed to uniformly distribute the dye to all cells. After five minutes incubation at room temperature, the cell suspension was washed three times with 10 volumes of PBS/5% FBS and centrifuged at room temperature. Labeled BMM were injected intravenously at 2×10^6 cells per mouse on days 7, 9, and 11 after the first immunization.

For IL-17 depletion, which was performed in the same experiments, neutralizing antibodies against IL-17 (R&D Systems), or its isotype control antibody (rat IgG_{2A}) were injected i.p. every second day at a dosage of 50 μ g per mouse. Mice were killed at day 14 and hearts were analyzed for infiltrating CFSE-labeled BMM.

4.4 Cells analysis

4.4.1 FACS

Cell suspensions were stained using fluorochrome-conjugated mouse-specific antibodies. Following fluorochromes were used: Pacific Blue™, excited by the UV-laser light (405 nm), 455 nm emission maximum; Fluorescein isothiocyanate (FITC), excited by the argon laser light (488 nm), 525 nm emission maximum; Phycoerythrin (PE), excited by the argon laser light, 575 nm emission maximum; Peridinin Chlorophyll Protein (PerCP), excited by the argon laser light, 675 nm emission maximum; Phycoerythrin/Cyanine 5.5 (PE/Cy5.5), a tandem conjugate dye, excited by the argon laser light, 690 nm emission maximum; Peridinin Chlorophyll Protein/Cyanine 5.5 (PerCP/Cy5.5), a tandem conjugate dye, excited by the argon laser light, 690 nm emission maximum; Phycoerythrin/Cyanine 7 (PE/Cy7), a tandem conjugate dye, excited by the argon laser light, 774 nm emission maximum; 7-amino-actinomycin D (7AAD), a viability dye and ready-to-use reagent combining between bases of the DNA, excited by the argon laser light, 647 nm emission maximum; Allophycocyanin (APC), excited by the helium/neon (He/Ne, 633/635 nm) laser light, 660 nm emission maximum.

Following antibodies were titrated and used at the corresponding concentration (optimized for CyAN ADP Flow Cytometer, Dako):

Surface staining: CD3 ϵ -FITC, 1:400; CD3 ϵ -PE/Cy7, 1:1200; CD4-APC, 1:600; CD11b-APC, 1:600; CD11c-APC, 1:600; CD11c-PE, 1:300; CD14-biotinylated, 1:300; CD19-APC, 1:600; CD25-PE, 1:1000; CD31-FITC, 1:300; CD40-FITC, 1:300; CD44-FITC, 1:300; CD45.1-FITC, 1:400; CD45.2-FITC, 1:400; CD45.2-PerCP, 1:300; CD45-PE, 1:300; CD49b-APC, 1:1200; CD62L-PE, 1:300; CD69-PE, 1:300; CD80-FITC, 1:300; CD86-FITC, 1:300; MHC II (I-A^d)-FITC, 1:1200; CXCR4-FITC, 1:400; F4/80-PE, 1:400; GITR-PE/Cy7, 1:800; Gr-1 (Ly6G/C)-FITC, 1:400; AnnexinV-PE, 1:200; 7AAD, 1:200; Streptavidin-PerCPCy5.5, 1:400; Streptavidin-APC, 1:600.

Round-bottom 96 well plates were used to perform both surface and intracellular staining. Staining for FACS analysis starts with the labeling of surface markers. 5×10^5 cells/well were resuspended in 200 μ l/well FACS buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA), centrifuged at 2000 rpm (Heraeus, Multifuge 4kr), resuspended in 200 μ l/well of FACS buffer supplemented with 1:50 NMS to block unspecific bindings, and incubated for 15 minutes at 4°C. In the meantime, antibodies were properly diluted in FACS

buffer. After centrifugation, cells were resuspended in 50 μ l/well of prepared antibodies according to the protocol, incubated for 15 minutes at 4°C, washed three times with 200 μ l/well, and resuspended in either 400 μ l FACS buffer for acquisition or 200 μ l 4% paraformaldehyde for intracellular staining.

Antibodies for intracellular staining (concentrations optimized for CyAN ADP Flow Cytometer, Dako): IFN- γ -PacificBlue, 1:400; IFN- γ -PE, 1:1000; IL-4-PE, 1:400; IL-6-FITC, 1:600; IL-6-PE, 1:300; IL-10-PE, 1:300; IL-12p70-PE, 1:300; IL-12p70-PECy5.5, 1:700; IL-17A-FITC, 1:800; IL-17A-PE, 1:400; TNF- α -FITC, 1:1000; FoxP3-FITC, 1:50, NOS2, 1:75; chicken anti-rabbit-Alexa488, 1:100. Antibodies were purchased from BD Pharmingen, eBiosciences, or BioLegend.

Prior to intracellular staining, cells were re-stimulated for 4 hours with 20 ng/ml PMA, 1 μ M ionomycin, and 10 μ g/ml Brefeldin A (all Sigma). After surface staining, cells were fixed with 4% paraformaldehyde for 20 minutes at 4°C, washed twice with 200 μ l 0.5% saponin buffer (FACS buffer supplemented with 0.5% saponin (Sigma)), and resuspended with 200 μ l 0.5% saponin buffer for 15 minutes at 4°C. In the meantime, antibodies were properly diluted in 0.5% saponin buffer. After centrifugation, cells were resuspended in 50 μ l/well of prepared antibodies according to the protocol, incubated for 30 minutes at 4°C, washed three times with 200 μ l/well 0.5% saponin buffer, washed for the last time with FACS buffer to close the cell membranes, and resuspended in 400 μ l FACS buffer for acquisition.

Samples were acquired with a CyAN ADP Flow Cytometer (Dako) until at least 200000 events, collected from a live gate using forward/side scatter plots, and then analyzed using FlowJo (TreeStar) software.

4.4.2 ELISA AND GRIESS REACTION

Supernatants of various cultivated cells were collected, centrifuged for 10 minutes at 3 g in a table centrifuge (Eppendorf), and either quickly frozen at -80°C or directly used for ELISA. Cytokine levels were measured using commercially available ELISA kits for IL-1 β (PeproTech), IL-6 (PeproTech), IL-17 (Quantikine, R&D Systems), IL-23 (eBiosciences), IFN- γ (Quantikine, R&D Systems), TGF- β (Quantikine, R&D Systems), and TNF- α (Quantikine,

R&D Systems). In general, ELISA-plates were incubated overnight with coating antibodies, washed several times with washing buffer, blocked with blocking buffer supplemented with FBS or BSA, and washed again at least four times. Serial dilutions were performed for the standard curve and supernatants of interest were usually diluted 1:2 to 1:10. After incubation of 2 hours, the plate was washed several times and avidin-loaded detecting antibodies were incubated for 1 hour. The plates were washed again several times and horseradish peroxidase reacting with the avidin was incubated for 30 minutes. After washing, a substrate was added, incubated for 30 minutes and blocked for plate reading.

Nitrite (NO_2^-) levels reflecting NO production in culture supernatants were assessed using a colorimetric assay based on the Griess reaction (Promega). 50 μl supernatant reacted with 50 μl sulfanilamide solution for 10 minutes. After a second reaction with 50 μl NED (N-1-naphthylethylenediamine dihydrochloride) developed a purple-magenta color, the plates were ready for plate reader with a filter between 520 and 550 nm.

4.4.3 QUANTITATIVE RT-PCR

Hearts were dissected and put without previous perfusion in a 15 ml tube (non-pyrogenic, Sarstedt) filled with 1 ml Trizol™ reagent (Invitrogen). With a sonicator, heart tissue was homogenized after 5 cycles of 6 seconds, with a break of 10 seconds between each cycle. Importantly, sonication was performed on ice, as this homogenization process produced high quantities of heat. RNA was then isolated adding 0.1 ml bromochloropropane and centrifuging at 12000 g for 15 minutes at 4°C, precipitated adding isopropanol and vigorously mixing, washed using 75% ethanol, and resuspended adding 100 μl ddH₂O. 2 μg RNA were used to perform a reverse transcriptase reaction to produce cDNA. RNA was mixed with 1 μl oligo dT primer (Invitrogen), incubated for 5 minutes at 70°C, put on ice for 4 minutes, and spun down. 7.5 μl of a master mix containing dNTPs, reverse transcriptase, ribonuclease inhibitors, and a buffer, were added and transferred in little PCR tubes for the reaction.

After cDNA synthesis, real-time PCR was performed with SYBR Green (Applied Biosystems), using a 7500 Fast Real time PCR System (Applied

Biosystems) with GAPDH as an internal control. The following primers were used: SDF-1 Fw 5'-CGT GAG GCC AGG GAA GAG-3' Rv 5'-TGA TGA GCA TGG TGG GTT GA-3', MCP-1 Fw 5'-CAT CAC TGA AGC CAG CTC TCT CT-3' Rv 5'-GCA GGC CCA GAA GCA TGA-3', MIP-1a Fw 5'-TTT TGA AAC CAG CAG CCT TTG-3' Rv 5'-TCT TTG GAG TCA GCG CAG ATC-3', GAPDH Fw 5'-CCT GCA CCA CCA ACT GCT TA-3' Rv 5'-TCA TGA GCC CTT CCA CCA TG-3'.

4.5 Statistics

The Mann-Whitney *U* test was used for the evaluation of severity scores. Dichotomous data were analyzed using Fisher's exact test. Normally distributed data, such as proliferation responses and cytokine levels were compared using ANOVA and Student's *t*-test. Statistical analysis was conducted using Prism 4 software (GraphPad Software).

5 Results

5.1 *Th17, but not Th1 cells, are pathogenic in the EAM model*

Experimental autoimmune myocarditis is a CD4⁺ T cell-mediated disease (17). Nevertheless, adoptive transfer of *in vitro* re-stimulated CD4⁺ T cells isolated from diseased mice usually results in low disease scores in SCID, RAG2^{-/-} or LPS pretreated wild-type recipients (111, 112). Based on the hypothesis that CD4⁺ Th17 cells represent the pathogenic subpopulation of heart-specific CD4⁺ Th cells, we isolated CD4⁺ Th cells from draining lymph nodes of diseased mice and expanded them *in vitro* by repetitive and alternating rounds of re-stimulation on MyHC- α -loaded irradiated antigen-presenting cells followed by a resting phase in either IL-12p70/anti-IL-4 or IL-23/anti-IL-4/anti-IFN- γ containing medium. Depending on these culture conditions, we generated MyHC- α -specific CD4⁺ Th cell lines with either a Th1, or Th17 phenotype (Fig. 5.1A). Importantly, both Th1 and Th17 cell lines showed comparable *in vitro* proliferation responses after 72 hours of re-stimulation with the MyHC- α antigen on irradiated APC (Fig. 5.1B).

To compare the capacity of the MyHC- α -specific Th1 and Th17 T cell lines to

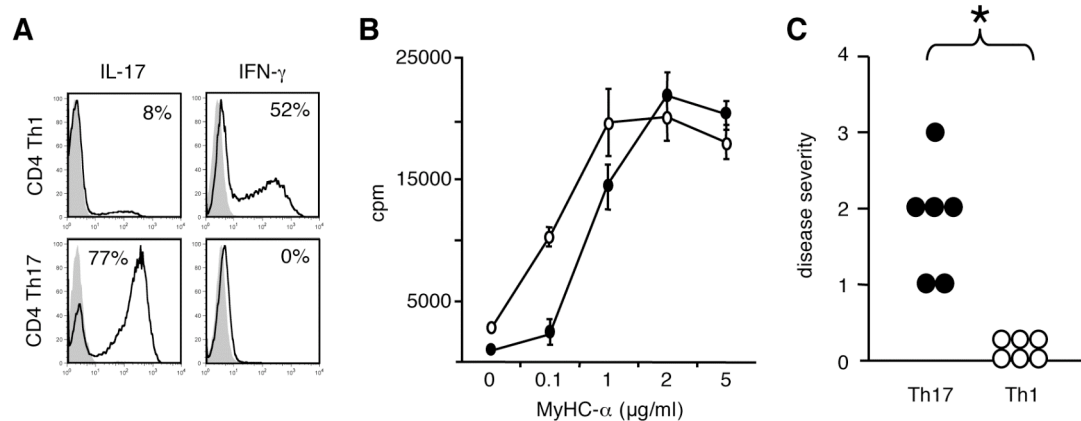


Figure 5.1. CD4⁺ Th17 cells, but not CD4⁺ Th1 cells, induce EAM. A, Characterization of MyHC- α -specific Th17 and Th1 cell lines. B, MyHC- α -specific Th17 (●) and Th1 (○) cell lines show comparable *in vitro* proliferation on irradiated MyHC- α -pulsed splenocytes. Proliferation was assessed by measurement of ³H-thymidine incorporation. C, Disease severity scores of individual mice adoptively transferred with 10⁷ MyHC- α -specific Th17 cells (●) vs. MyHC- α -specific Th1 cells (○). *p = 0.0022.

induce autoimmune myocarditis, groups of age and sex matched wild-type BALB/c mice were injected with 10^7 CD4⁺ T cells/mouse. As illustrated in Fig. 5.1C, only CD4⁺ Th17 cells, but not CD4⁺ Th1 cells, induced autoimmune myocarditis.

Of note, increasing the numbers of the transferred Th17 cells increased average myocarditis scores (figure not shown). In contrast, Th1 cell treated mice were protected from myocarditis (Fig. 5.1C) and transfer of up to 10^8 CD4⁺ Th1 cells per mouse induced only minimal pericarditis, and some non-specific increase in the numbers of perivascular mononuclear cells.

Taken together these findings illustrate the direct pathogenic role of MyHC- α -specific CD4⁺ Th17 cells. In contrast, MyHC- α -specific CD4⁺ Th1 cells do not mediate autoimmune myocarditis.

5.2 IL-17 is critical for the recruitment of CD11b⁺ monocytes to the heart

Inflammatory infiltrates in EAM mainly consist of monocytes, granulocytes and some activated CD4⁺ T cells (113). As illustrated in Fig. 5.2, MyHC- α /CFA immunization resulted in a 3 to 4 fold increase of CD45⁺ cells in hearts of diseased mice, compared to control hearts of CFA injected control mice. Analysis of immunized CD45.1/CD45.2 chimeras confirmed that heart-infiltrating CD45⁺ cells were from host bone marrow (Fig. 5.2). Importantly, the

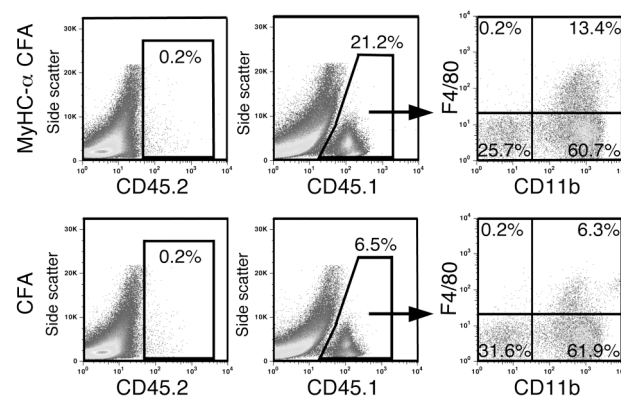


Figure 5.2. CD11b⁺ monocytes are bone marrow-derived and reflect the major population of heart-infiltrating leukocytes in EAM. Heart-infiltrating cells were isolated from hearts of CD45.1/CD45.2 chimeric mice 21 days after either MyHC- α /CFA or CFA immunization, stained for CD45.1, CD45.2, CD11b, and F4/80, and analyzed by flow cytometry.

vast majority of heart-infiltrating leukocytes expressed CD11b⁺ and included a minority of CD11b⁺F4/80⁺ macrophages (Fig. 5.2), some CD11b⁺Gr-1^{high} granulocytes, and CD11b⁺CD11c⁺ dendritic cells (not shown). Based on reports that IL-17 promotes the recruitment of blood monocytes to inflamed organs (22), we therefore decided to specifically address the effect of IL-17 on the recruitment of CD11b⁺ monocytes to the inflamed heart in the EAM model. As illustrated in Fig. 5.3A, we first compared the release of IL-17 from *in vitro* re-stimulated, heart-infiltrating T cells of immunized wild-type BALB/c mice at the peak of disease at day 21, and at day 30 after resolution of most of the inflammatory infiltrates. As shown in Fig. 5.3B, histological disease severity scores parallel IL-17 release from heart-infiltrating T cells. Next, we treated groups of MyHC- α /CFA immunized mice every second day for 21 days with either an IL-17 blocking antibody, or an isotype control Ig. As illustrated in Fig. 5.3C, anti-IL-17 treatment markedly reduced the gross disease severity scores, confirming a direct pathogenic role of IL-17 in EAM.

To directly assess the relevance of IL-17 in the recruitment of CD11b⁺ monocytes to the inflamed heart *in vivo*, we generated immature, CD11b⁺CXCR4⁺Gr-1^{low} co-expressing monocytes (Fig. 5.3D) from bone marrow, and labeled the cells with CFSE. 2x10⁶ CFSE-labeled CD11b⁺ monocytes were then injected intravenously at day 14 post immunization in groups of mice with autoimmune myocarditis, 24 hours after application of

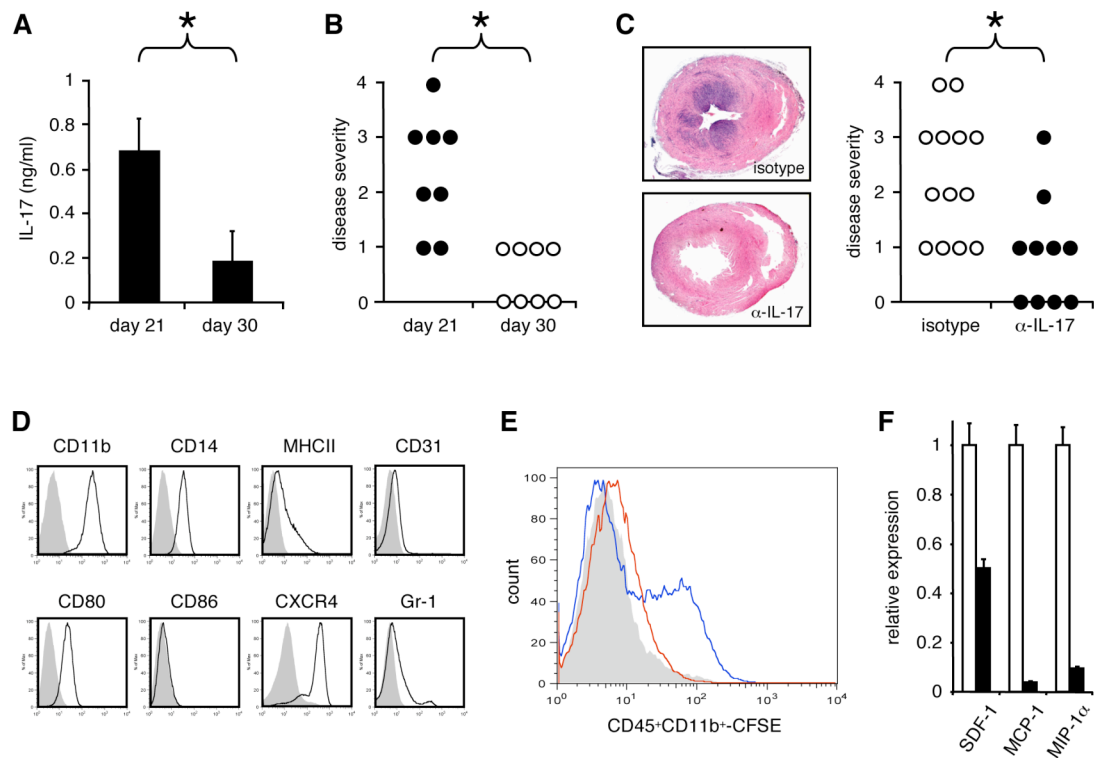


Figure 5.3. A, IL-17 production of heart-infiltrating T cells. 10^5 /ml of infiltrating CD3⁺ cells were isolated from diseased hearts of wild-type mice, 21 or 30 days after MyHC- α immunization, and re-stimulated with anti-CD3/anti-CD28 for 24 hours. IL-17 was measured in supernatants using commercially available ELISA kits. * $p = 0.0071$.

B, Disease severity scores of individual, MyHC- α /CFA-immunized wild-type mice at days 21 and 30. * $p = 0.0023$.

C, Anti-IL-17 treatment reduces myocarditis severity in MyHC- α /CFA immunized wild-type mice. Mice were immunized with 150 μ g MyHC- α /CFA and injected with either 50 μ g anti-mouse IL-17 or isotype antibody every second day. Mice were sacrificed on day 21. Individual disease scores (* $p = 0.0092$), X25 and X200 original magnifications are shown.

D, Surface molecule expression patterns of bone marrow-derived CD11b⁺ monocytes representing an immature population.

E, Anti-IL-17 treatment blocks recruitment of CFSE-labeled CD45⁺CD11b⁺ cells to the heart. Heart-infiltrating cells were isolated at day 15 from immunized wild-type mice injected with CFSE-labeled wild-type CD11b⁺ monocytes, 24 hours after either anti-IL-17 injection (red line) or isotype antibody injection (blue line). The grey area represents CD45⁺CD11b⁺ cells of untreated immunized wild-type mice injected with unlabeled wild-type CD11b⁺ cells. The histogram is gated on CD11b and CD45 expressing cells.

F, Anti-IL-17 treatment suppresses SDF-1, MCP-1, and MIP-1 α expression in heart tissues of immunized wild-type mice. Relative expression of SDF-1, MCP-1, and MIP-1 α mRNA in hearts of immunized and anti-IL-17 (black bars) vs. isotype (white bars) treated control mice are shown.

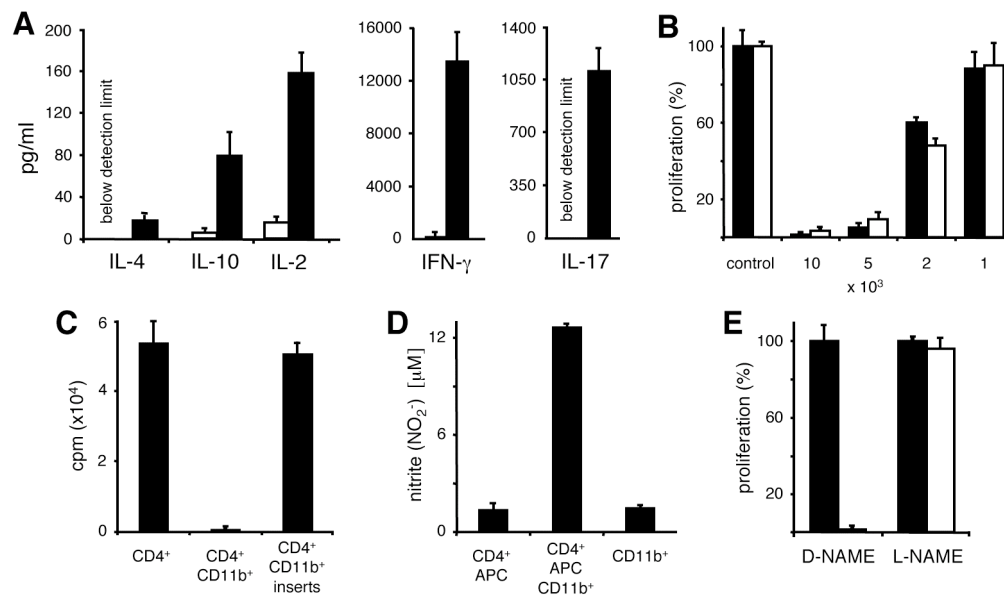


Figure 5.4. A, Cytokine production profile of MyHC- α specific CD4⁺ T cells. CD4⁺ cells were isolated from lymphnodes of diseased wild-type mice, and re-stimulated on irradiated splenocytes in the presence (black bars) or absence (white bars) of 2 μ g/ml of MyHC- α for 48 hours. The indicated cytokines were measured using supernatant ELISA. * $p < 0.05$.

B, CD11b⁺ monocytes suppress proliferation of autoreactive CD4⁺ T cells. CD4⁺ cells from diseased wild-type mice were re-stimulated on irradiated splenocytes pulsed with either 2 μ g/ml of MyHC- α (black bars) or anti-CD3/anti-CD28 (white bars) in the presence of titrating amounts of bone marrow derived wild-type CD11b⁺ monocytes. Proliferation responses are expressed as percentages (mean \pm SD from 5 culture wells) compared to an average standard value representing proliferation in the absence of CD11b⁺ monocytes.

C, The suppressive effects of CD11b⁺ monocytes require close contact to APCs and T cells. Wild-type CD4⁺ T cells were MyHC- α re-stimulated on irradiated APC in the presence of 2.5 $\times 10^3$ wild-type CD11b⁺ monocytes, which were either in contact with the CD4⁺ T cells/APC or separated by culture well inserts.

D, Nitric oxide release parallels the reduced proliferation of CD4⁺ T cells in the presence of CD11b⁺ monocytes. CD4⁺ T cells from diseased wild-type mice were MyHC- α re-stimulated on irradiated APC in the presence of 2.5 $\times 10^3$ CD11b⁺ monocytes. Nitrite (NO₂⁻) levels reflecting NO production in culture supernatants were assessed using a colorimetric assay based on the Griess reaction.

E, Addition of the NOS inhibitor L-NAME, but not its inactive enantiomer D-NAME, restores proliferation in the presence of CD11b⁺ monocytes. CD4⁺ T cells from diseased wild-type mice were MyHC- α re-stimulated on irradiated APC in the presence (white bars) or absence (black bars) of 2.5 $\times 10^3$ CD11b⁺ monocytes.

either an IL-17-blocking antibody or an isotype control antibody. Twenty-four hours later, the relative numbers of CFSE-labeled CD11b⁺ cells were analyzed in hearts of treated mice. FACS analysis of heart infiltrating cells from anti-IL-17-treated mice showed no CFSE-labeled CD11b⁺ monocytes within the CD45⁺CD11b⁺ gate. In contrast, CFSE⁺ cells were detected in suspensions of heart infiltrating cells isolated from isotype treated controls (Fig. 5.3E).

To address the question how anti-IL-17 treatment affects the recruitment of CD11b⁺ monocytes we compared mRNA expression of two cytokines, known to mediate monocyte/macrophage recruitment in EAM (114). As illustrated in Fig. 5.3F, anti-IL-17 treatment markedly reduced MIP-1 α and MCP-1 mRNA expression in hearts of immunized mice.

Taken together, our findings confirm the critical role for IL-17 in the recruitment of CD11b⁺ bone marrow-derived monocytes, the predominant heart-infiltrating cell population, to the heart in the EAM model.

5.3 *CD11b⁺ monocytes suppress CD4⁺ T cell proliferation*

As mentioned previously, we consistently observed spontaneous disease regression in diseased wild-type mice after a peak of histological severity scores at day 21 after immunization. Given the fact that most of the infiltrating cells belong to the CD11b⁺ monocyte fraction (Fig. 5.2), we next asked how these cells interact with heart-specific, autoreactive T cells. Accordingly, we isolated CD4⁺ T cells from lymph nodes and spleens of mice with autoimmune myocarditis, and re-stimulated for 72 hours on irradiated splenocytes pulsed with the MyHC- α self-antigen. Of note, *in vitro* MyHC- α re-stimulated CD4⁺ T cells from diseased mice comprise a heterogeneous population of IL-4, IFN- γ , IL-2, IL-10 and IL-17 producing cells (Fig. 5.4A). As illustrated in Fig. 5.4B, the *in vitro* proliferation of CD4⁺ T cells isolated from mice with autoimmune myocarditis was markedly reduced on MyHC- α -pulsed, irradiated antigen-presenting cells, if titrated numbers of syngenic CD11b⁺ monocytes were added to cell culture wells. As illustrated in Fig. 5.4C, close contact between CD11b⁺ monocytic cells and T cells was critical for the observed inhibition of T cell proliferation, as culture well inserts separating T cells/antigen-presenting

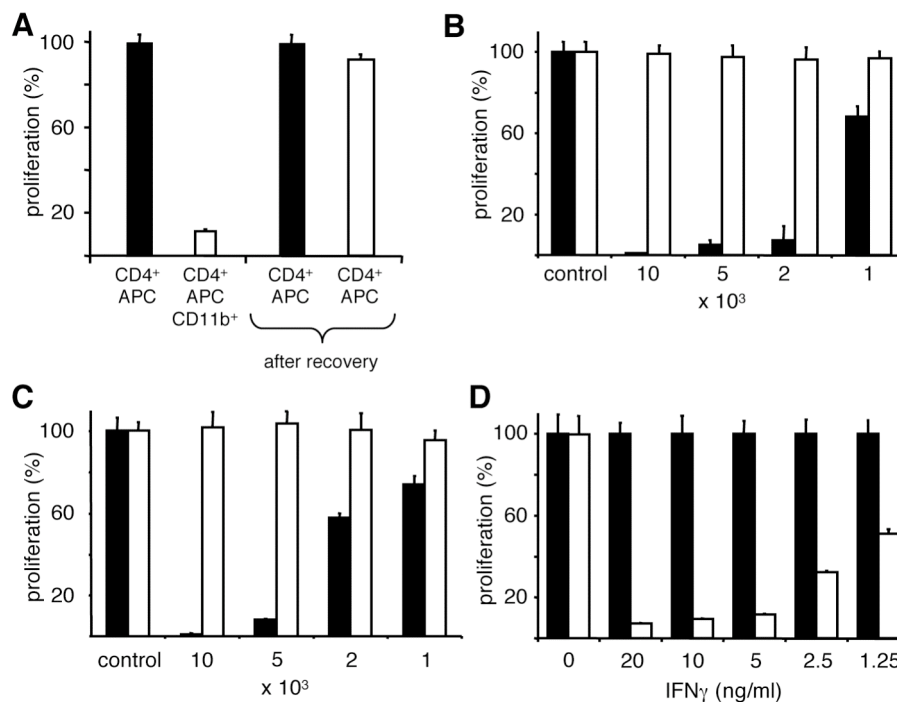


Figure 5.5. A, CD11b⁺ monocytes suppress CD4⁺ T cell responses reversibly. Wild-type CD4⁺ cells were re-stimulated on irradiated splenocytes pulsed with 2 μg/ml of MyHC-α in the presence (white bar, left) or absence (black bar, left) of 2.5 × 10³ CD11b⁺ monocytes. After 48 hours, CD4⁺ T cells were isolated, washed, and recovered for 4 days in fresh medium containing 20 U/ml IL-2, before re-stimulation for another 48 hours on MyHC-α-pulsed APC in the absence of CD11b⁺ monocytes (black and white bars, right). Proliferation responses are expressed as percentages compared to an average standard value representing proliferation of CD4⁺ T cells on APC only.

B, CD11b⁺ monocytes lacking the IFN-γ receptor do not suppress CD4⁺ T cell proliferation. CD4⁺ T cells from diseased wild-type mice were re-stimulated on irradiated APC in the presence of either IFN-γR^{-/-} monocytes (white bars) or IFN-γR^{+/+} monocytes (black bars). Proliferation responses are expressed as percentages compared to an average standard value representing the proliferation of CD4⁺ T cells on APC in the absence of monocytes.

C, CD11b⁺ monocytes suppress proliferation of MyHC-α-specific Th1 cell lines but not Th17 cell lines. 5 × 10⁴ MyHC-α-specific CD4⁺ Th1 (black bars) and Th17 (white bars) cells were re-stimulated on irradiated APC pulsed with 2 μg/ml MyHC-α in the presence of titrating amounts of CD11b⁺ monocytes for 72 hours. Proliferation responses are expressed as percentages compared to an average standard value representing proliferation of CD4⁺ Th17 cells on APC only.

D, IFN-γ triggers the suppressive effects of IFN-γR^{+/+} but not IFN-γR^{-/-} CD11b⁺ monocytes on CD4⁺ Th17 cell proliferation. MyHC-α specific Th17 T cell lines were re-stimulated on irradiated MyHC-α-pulsed APC in the presence of 2.5 × 10³ either IFN-γR^{+/+} (white bars) or IFN-γR^{-/-} (black bars) CD11b⁺ monocytes and titrating amounts of recombinant mouse IFN-γ.

cells and CD11b⁺ monocytic cells prevented the suppression of the proliferation response. These results show that the CD11b⁺ monocyte-mediated effect requires close contact of CD11b⁺ monocytes to T cells. Similar results were obtained if CD4⁺ T cells were re-stimulated with plate bound anti-CD3/anti-CD28 (Fig. 5.4B). The latter findings largely exclude a relevant role of the antigen-presenting cells in the CD11b⁺ monocyte-mediated T cell suppression mechanism.

5.4 CD11b⁺ monocytes release nitric oxide suppressing T cell proliferation

As shown previously, the CD11b⁺ monocyte-mediated effects require close contact of CD11b⁺ monocytes to T cells. This observation argues for either a short-lived soluble mediator or direct cell-cell interaction. We first looked for a short-lived, soluble mediator and analyzed nitrite levels reflecting nitric oxide production in supernatants of APC/T cell/ CD11b⁺ monocyte co-cultures. Nitric oxide had been shown to mediate suppression of T cells (49). As illustrated in

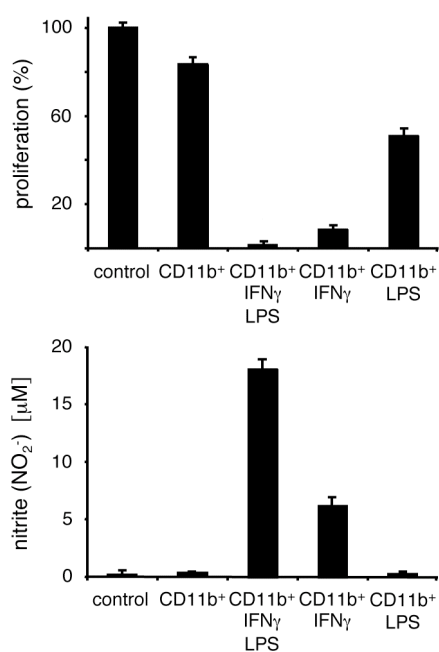


Figure 5.6. IFN- γ enhances nitric oxide release from CD11b⁺ monocytes mediating Th17 T cell suppression. MyHC- α -specific CD4⁺ Th17 cell lines were re-stimulated on irradiated, MyHC- α -pulsed APC in the presence or absence of 2.5×10^3 CD11b⁺ monocytes together with either 5 ng/ml of recombinant mouse IFN- γ , 0.1 μ g/ml LPS, or IFN- γ /LPS. Proliferation responses are expressed as percentages compared with an average standard value representing proliferation of CD4⁺ T cells on APC only. Nitrite (NO₂⁻) levels reflecting NO production in culture supernatants were assessed using a colorimetric assay based on the Griess reaction.

Fig. 5.4D, the addition of CD11b⁺ monocytes to T cell/APC cultures indeed

resulted in greatly enhanced nitric oxide release. The decisive role of nitric oxide in suppressing activated CD4⁺ T cells was finally confirmed by the observation that the T cell suppressing effects of the CD11b⁺ monocytes disappeared in the presence of the non-specific NOS inhibitor L-NAME. In addition, adding titrating numbers of paraformaldehyde fixed CD11b⁺ cells to T-cell/APC cultures did not affect T cell proliferation (not shown). Together with the complete reversibility of the suppressive effect of the CD11b⁺ monocytes on the T cell proliferation in the presence of the NOS inhibitor L-NAME, we conclude that release of nitric oxide explains the suppressive effects of the CD11b⁺ monocytes in our proliferation assays.

To analyze the reversibility of the nitric oxide-mediated T cell suppression, we recovered T cells from monocyte/APC/T cell co-cultures and expanded them in fresh medium in the presence of IL-2. As illustrated in Fig. 5.5A, recovered and purified CD4⁺ T cells again proliferated, if MyHC- α loaded APC were added in the absence of CD11b⁺ monocytes, confirming the reversibility of the suppressive effect.

5.5 IFN- γ triggers NO-mediated T cell suppression by CD11b⁺ monocytes

IFN- γ has been recognized as a major NOS2 inducer in macrophages. Accordingly, CD11b⁺ monocytes lacking the interferon gamma receptor did not suppress T cell proliferation (Fig. 5.5B). The question therefore arises whether the CD11b⁺ monocyte mediated T cell suppression also affects CD4⁺ Th17 cells, the major pathogenic T cell population in EAM. In fact, and as illustrated in Fig. 5.5C, the proliferation of CD4⁺ Th17 cells was not affected by the addition of titrating numbers of wild-type CD11b⁺ monocytes on IFN- γ R^{-/-} antigen-presenting cells. In contrast, the proliferation of CD4⁺ Th1 cells was markedly reduced. Adding IFN- γ , however, promoted the release of nitric oxide in culture supernatants and inhibited CD4⁺ Th17 cell proliferation (Fig. 5.5, B & D, Fig. 5.6). Of note, this effect was independent of the presence of the IFN- γ R on the CD4⁺ Th17 cell line (not shown). Taken together these findings show, that IFN- γ directly acts on the CD11b⁺ monocytes and triggers suppression of both, CD4⁺ Th1 and CD4⁺ Th17 cells.

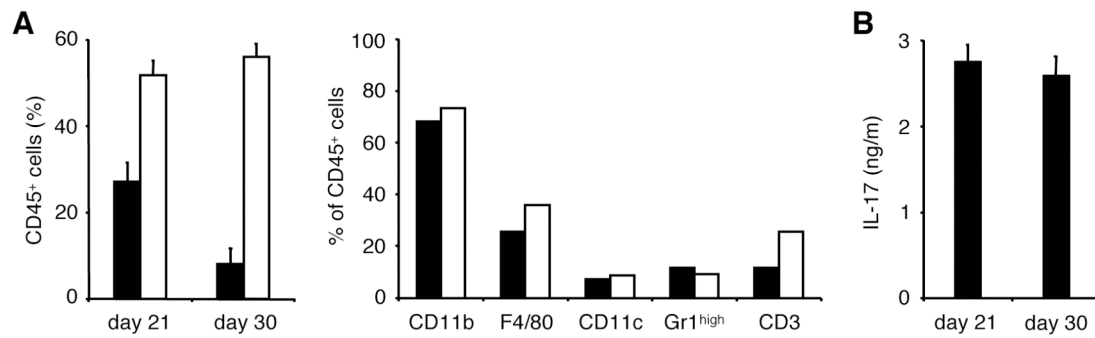


Figure 5.7. A, Patterns of heart-infiltrating CD45⁺ cells in diseased IFN- γ R^{+/+} (black bars) and IFN- γ R^{-/-} (white bars) mice. Heart-infiltrating cells were isolated from groups of mice 21 and 30 days after MyHC- α /CFA immunization, stained for CD45, and either CD11b, F4/80, CD11c, Gr-1, or CD3 and analyzed by flow cytometry. The left graph illustrates CD45⁺ cells as % of total cells on day 21 and day 30. The right graph shows the pattern of infiltrating cells as % of CD45⁺ cells at day 21 in wild-type vs. IFN- γ R^{-/-} hearts.

B, Comparable IL-17 production of heart-infiltrating T cells at days 21 and 30 in immunized IFN- γ R^{-/-} mice. 10⁵/ml of infiltrating CD3⁺ cells were isolated from diseased hearts and re-stimulated with anti-CD3/anti-CD28 for 24 hours. $p = 0.21$.

In wild-type mice, numbers of heart-infiltrating CD11b⁺ monocytes peak 21 days after immunization, and slowly resolve thereafter. In contrast, autoimmune myocarditis shows a progressive and severe disease course in IFN- γ ^{-/-} or IFN- γ R^{-/-} mice (48, 49). Together with the *in vitro* findings described above, we hypothesized that the progressive disease course in IFN- γ deficient mice reflects a defective, IFN- γ -dependent, CD11b⁺ monocyte-mediated negative feedback loop.

As illustrated in Fig. 5.7A, diseased IFN- γ R^{-/-} mice show similar patterns of cardiac infiltrations as wild-type mice with myocarditis, except the relative numbers of heart infiltrating T cells, which are consistently higher in IFN- γ R^{-/-} hearts. Furthermore, and in contrast to wild-type mice (Fig. 5.3A), IL-17 production of heart-infiltrating T cells did not differ between days 21 and 30 in IFN- γ R^{-/-} mice (Fig. 5.7B).

To specifically address the role of heart-infiltrating CD11b⁺ monocytes as key players in an IFN- γ -dependent negative feedback loop *in vivo*, we took advantage of the IFN- γ producing capacity of autoreactive T cells in immunized mice lacking the IFN- γ receptor (47). Accordingly, we injected

groups of immunized $\text{IFN-}\gamma\text{R}^{-/-}$ mice with either $\text{IFN-}\gamma\text{R}^{-/-}$ or $\text{IFN-}\gamma\text{R}^{+/+}$ CD11b^+ monocytes, and analyzed myocarditis severity. As illustrated in Fig. 5.8 A & B, $\text{IFN-}\gamma\text{R}^{-/-}$ mice injected with $\text{IFN-}\gamma\text{R}^{-/-}$ monocytes exhibited severe myocarditis at day 21, whereas myocarditis largely resolved in $\text{IFN-}\gamma\text{R}^{+/+}$ monocyte treated animals. In parallel, proliferation responses of *in vitro* MyHC- α re-stimulated CD4^+ T cells were markedly reduced in animals treated with $\text{IFN-}\gamma\text{R}^{+/+}$ monocytes (Fig. 5.8C). Of note, $\text{IFN-}\gamma\text{R}^{+/+}$ monocytes were not effective in $\text{IFN-}\gamma\text{R}^{-/-}$ mice, confirming the idea that $\text{IFN-}\gamma$ directly acts on injected monocytes. These *in vivo* findings illustrate, that heart-infiltrating monocytes are the cellular key player in an $\text{IFN-}\gamma$ -signaling-dependent negative feedback loop in autoimmune heart disease.

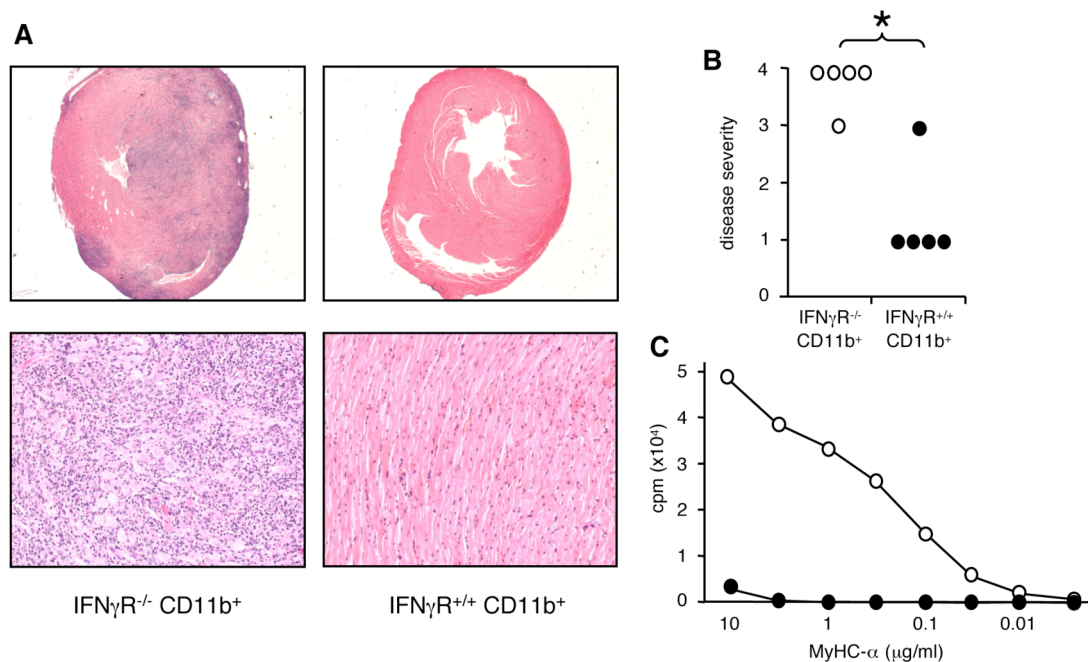


Figure 5.8. A, Reduced disease severity in $\text{IFN-}\gamma\text{R}^{-/-}$ mice treated with $\text{IFN-}\gamma\text{R}^{+/+}$ CD11b^+ monocytes. MyHC- α /CFA-immunized $\text{IFN-}\gamma\text{R}^{-/-}$ mice were injected on days 7, 9, and 11, with either $\text{IFN-}\gamma\text{R}^{+/+}$ or $\text{IFN-}\gamma\text{R}^{-/-}$ CD11b^+ monocytes and sacrificed on day 21. X25 and X200 original magnifications are shown.

B, $\text{IFN-}\gamma\text{R}^{+/+}$ CD11b^+ monocytes abrogate chronic myocarditis in $\text{IFN-}\gamma\text{R}^{-/-}$ mice. Individual $\text{IFN-}\gamma\text{R}^{-/-}$ mice were treated with either $\text{IFN-}\gamma\text{R}^{-/-}$ (○) or $\text{IFN-}\gamma\text{R}^{+/+}$ (●) CD11b^+ as indicated above. * $p = 0.016$.

C, CD11b^+ monocyte treatment suppresses proliferation of CD4^+ T cells in $\text{IFN-}\gamma\text{R}^{-/-}$ mice. CD4^+ T cells were isolated from either $\text{IFN-}\gamma\text{R}^{-/-}$ (○) or $\text{IFN-}\gamma\text{R}^{+/+}$ (●) CD11b^+ monocyte treated $\text{IFN-}\gamma\text{R}^{-/-}$ mice and re-stimulated on irradiated MyHC- α -pulsed APC.

5.6 MyHC- α -specific Th1 T cells protect from Th17-induced myocarditis

So far, we provided *in vitro* and *in vivo* evidence for an IFN- γ -dependent negative feedback mechanism mediated by CD11b⁺ monocytes. Immunization of wild-type mice, however, results in the expansion of a mixed population of MyHC- α -specific CD4⁺ T cells. So far, it is not clear whether heart-specific CD4⁺ Th1 cells are part of this organ specific feedback loop, or whether they simply act as one out of several “bystanders” providing IFN- γ . To address this question *in vivo*, we generated MyHC- α -specific CD4⁺ Th17 cell lines and co-injected 0.5×10^7 cells/mouse together with either 0.5×10^7 activated MyHC- α -specific CD4⁺ Th1 cells or OVA-specific DO11.10 transgenic CD4⁺ Th1 cells in groups of RAG2^{-/-} mice lacking functional B and T cells. As illustrated in the table, we found that co-transfer of MyHC- α -specific Th1 CD4⁺ T cells but not OVA-specific DO11.10-transgenic CD4⁺ Th1 cells protected from myocarditis development after adoptive transfer of autoreactive, heart-specific CD4⁺ Th17 cells. These findings suggest that heart-specific CD4⁺ Th1 cell responses are a prerequisite for the initiation of an IFN- γ -dependent and CD11b⁺ monocyte-mediated negative feedback mechanism confining heart-specific autoimmunity and myocarditis.

Co-transfer of MyHC- α -specific CD4⁺ Th17 cell lines together with OVA-specific vs. MyHC- α -specific CD4⁺ Th1 cell lines

Th17 T cell lines [numbers/specificity]	Th1 T cell lines [numbers/specificity]	Disease prevalence day 12 [# diseased/# treated]	Severity grade [median -range]
0.5×10^7 / MyHC- α	0.5×10^7 / MyHC- α	2/6	0 (0-1)*
0.5×10^7 / MyHC- α	0.5×10^7 / OVA	6/6	1.5 (1-3)*
0.5×10^7 / MyHC- α	none	5/5	2 (1-2)

*p < 0.05 for the comparison of severity grades in Th17 injected mice co-transferred with MyHC- α - vs. OVA-specific Th1 cells (Mann Whitney U).

5.7 CD8 α^+ DC treated with TNF- α or LPS- α CD40 have the same potential to stimulate T cells, but polarize different CD4 $^+$ T cell subsets

After showing that CD4 $^+$ Th1 cells conferred protection from EAM, we wondered whether an early polarization of IFN- γ -producing CD4 $^+$ Th1 cells negatively regulate CD4 $^+$ Th17 cell differentiation. For this purpose, we sought a specific subpopulation of APC that was able to especially differentiate CD4 $^+$ Th1 cells. We focused our efforts on splenic dendritic cells. In fact, among the five described splenic DC subpopulations (103), it has been reported that DC expressing CD8 α^+ are prone to induce IFN- γ -producing CD4 $^+$ Th1 cells *in vitro* and *in vivo* (104, 107). We hypothesized that a vaccination strategy with activated splenic CD8 α^+ DC pulsed with heart-peptide could induce an early

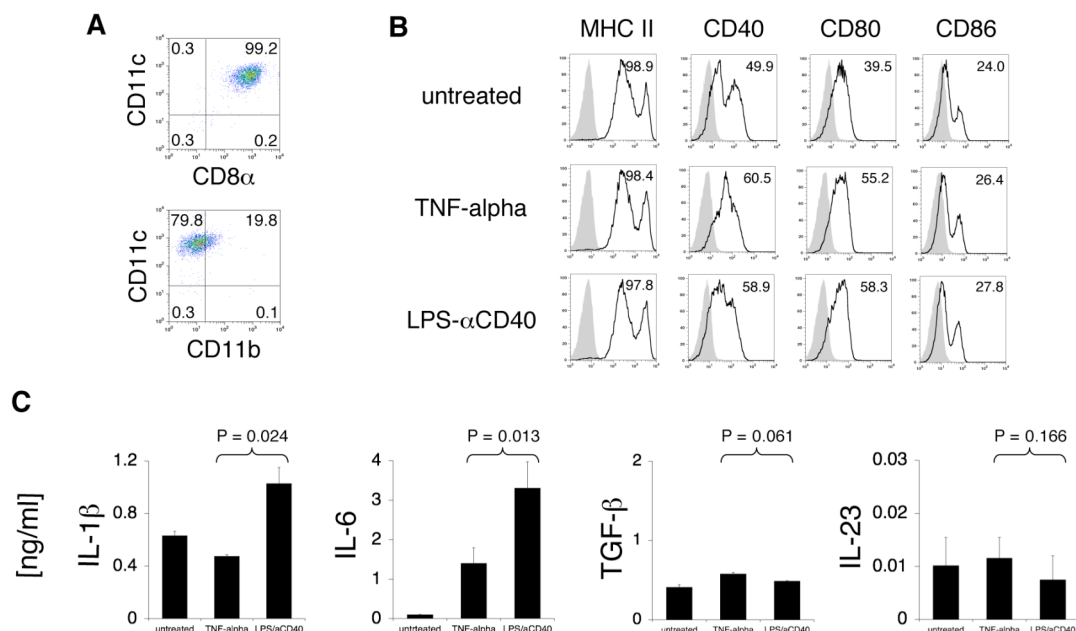


Figure 5.9. Surface marker phenotype and cytokine production of DC used to vaccinate mice. A, Characteristic surface markers of DC generated and sorted from FLT3L-treated mice. The expression of CD8 α^+ , CD11c, and CD11b was analyzed by FACS to check the purity of the sorted DC population.

B, Expression of surface costimulatory markers MHCII, CD40, CD80, and CD86 on CD8 α^+ DC stimulated for 4 h with different adjuvants, analyzed by FACS. The histograms were gated on CD8 α^+ /CD11c $^+$ DC. The grey area represents the isotype control.

C, CD8 α^+ DC sorted from FLT3L-treated mice were stimulated for 4 h with TNF- α or LPS- α CD40. Supernatants were tested for IL-1 β , IL-6, TGF- β , and IL-23 using commercially available ELISA kits.

polarization of IFN- γ -producing CD4⁺ Th1 cells, which, in turn, would inhibit the proliferation of pathogenic and auto-aggressive IL-17-producing CD4⁺ Th17 cells.

CD8 α ⁺ dendritic cells were sorted from FLT3L-treated spleens and then stimulated *in vitro* for 4 h with TNF- α or LPS plus α -CD40. Cell suspensions were immediately harvested and centrifuged to collect pure supernatants for cytokines measurement, while DC were analyzed by FACS for surface activation markers. As shown in Fig. 5.9A, splenic DC highly expressed CD8 α and CD11c, but low CD11b, indicating that these DC belong to the lymphoid lineage. The surface marker phenotype of TNF/CD8 α ⁺ and LPS- α CD40/CD8 α ⁺ displayed elevated levels of the costimulatory molecules CD40 and CD80, while CD86 and MHC II expression was similar in unstimulated and stimulated cells (Fig. 5.9B). Sandwich ELISA with DC culture supernatant

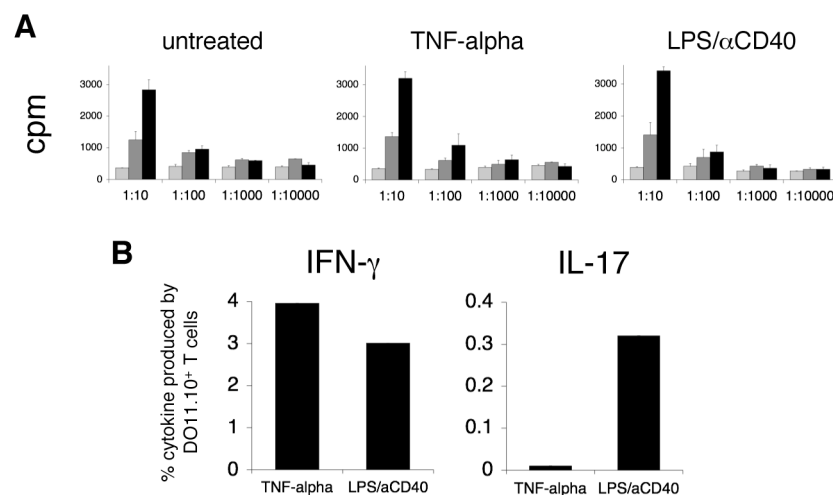


Figure 5.10. Potential of CD8 α ⁺ DC to stimulate and polarize CD4⁺ Th cells. A, CD8 α ⁺ DC were sorted from FLT3L-treated mice, pulsed with OVA³²³⁻³³⁹-peptides and untreated or stimulated with TNF- α or LPS/ α CD40 for 4 h, washed, and finally resuspended with titrating amounts of CD4⁺ T cells sorted from OVA-transgenic DO11.10 mice. Co-cultures were incubated for 24 h (□), 48 h (▣), and 72 h (■). Proliferation of CD4⁺ T cells was assessed by ³H-thymidine incorporation.

B, OVA³²³⁻³³⁹-peptide-pulsed TNF/CD8 α ⁺ or LPS- α CD40/CD8 α ⁺ were intravenously injected into OVA-transgenic DO11.10 mice. Splenocytes were restimulated with OVA³²³⁻³³⁹-peptides for 24 h and with PMA/IO/BFA for the last 4 h to analyze intracellular cytokines production by FACS. The percentage of peptide-specific IFN- γ and IL-17 production refers to CD4⁺/KJ1-26⁺ cells.

was performed to measure cytokines production after 4 hours incubation with TNF- α or LPS/ α -CD40. The production of cytokines varied remarkably between the differentially stimulated DC. High levels of IL-1 β and IL-6 were secreted by LPS- α CD40/CD8 α^+ compared to TNF/CD8 α^+ , but high levels of TGF- β were measured by TNF/CD8 α^+ compared to LPS- α CD40/CD8 α^+ (Fig. 5.9C). The differences in IL-23 production were not significant ($p = 0.166$). The profile of cytokines produced by LPS- α CD40/CD8 α^+ suggests a high potential to differentiate naïve CD4 $^+$ T cells in pathogenic CD4 $^+$ Th17 cells, which are the most auto-aggressive mediators in EAM induction.

Dendritic cells are potent antigen presenting cells (83-85). They capture and process antigens displaying large amounts of MHC-peptide complexes at their surface, priming naïve T cells through the affinity between MHC-peptide complexes and TCR and the cooperation of other costimulatory molecules (83). To investigate the potential of CD8 α^+ DC to prime naïve T cells, we pulsed CD8 α^+ DC with OVA³²³⁻³³⁹-peptides in the presence or absence of TNF- α or LPS plus α -CD40 for 4 hours. DC were immediately washed to arrest the stimulation, and then resuspended in culture medium containing naïve DO11.10 transgenic CD4 $^+$ T cells. After 24, 48, and 72 hours, T cell proliferation was assessed by incorporation of ³H-thymidine. Of note, both TNF- α and LPS- α CD40 treatments did not affect the CD8 α^+ DC potential to prime and stimulate CD4 $^+$ T cells (Fig. 5.10A). Titrating amounts of untreated CD8 α^+ DC, as well as TNF/CD8 α^+ and LPS- α CD40/CD8 α^+ were equally able to induce proliferation after different incubation times. To verify which CD4 $^+$ Th cell subpopulations were primed by OVA³²³⁻³³⁹-pulsed TNF/CD8 α^+ or LPS- α CD40/CD8 α^+ , we injected CD8 α^+ DC intravenously into DO11.10 transgenic mice. After two days, splenocytes were collected from treated mice and restimulated with PMA, ionomycin, and Brefeldin A, to assess the intracellular cytokines production. Both CD8 α^+ DC stimulations induced an elevated antigen-specific production of IFN- γ , which suggested a predominant CD4 $^+$ Th1 cell development (Fig. 5.10B). On the contrary, only LPS- α CD40/CD8 α^+ induced a relevant antigen-specific production of IL-17 compared to TNF- α -stimulated CD8 α^+ , which did not produce any IL-17 at all (Fig. 5.10B). These data indicate that TNF/CD8 α^+ support the differentiation of IFN- γ -producing

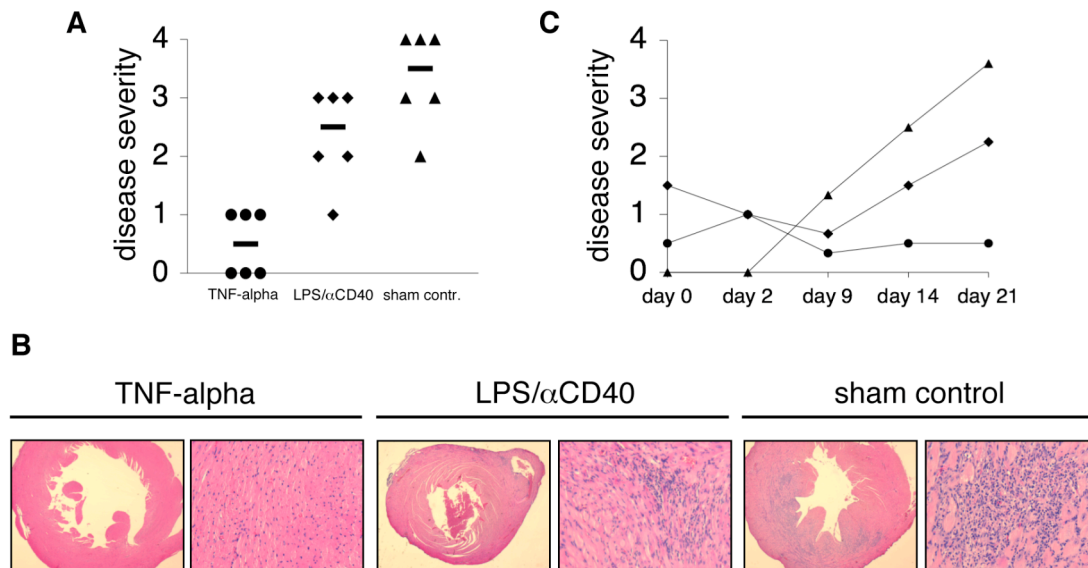


Figure 5.11. Vaccination with TNF- α -stimulated CD8 α^+ DC prevents EAM. A, Wild-type BALB/c mice were vaccinated 3 times at days -7, -5, -3, with 10^6 MyHC- α -loaded TNF/CD8 α^+ or LPS- α CD40/CD8 α^+ each time. Vaccinated mice were immunized with $150 \mu\text{g}$ of MyHC- α /CFA at day 0 and day 7, and sacrificed at day 21. Histological severity scores of individual mice are shown. $p = 0.0026$ for TNF- α - vs. sham-treatment, $p = 0.046$ for LPS- α CD40- vs. sham-treatment.

B, Hematoxylin/eosin-stained sections, 25X and 200X of the original magnification are shown. C, Histological severity scores after vaccination (day 0), and after both vaccination and immunization (day 2, day 9, day 14, and day 21). Median of at least 3 mice and SD values are shown.

CD4 $^+$ Th1 cells, which are favorable to protect from EAM, while LPS- α CD40/CD8 α^+ support the polarization of both CD4 $^+$ Th1 and pathogenic IL-17-producing CD4 $^+$ Th17 cells, which are auto-aggressive and favorable to induce EAM.

5.8 Pre-vaccination with TNF- α -stimulated CD8 α^+ DC prevents EAM

To analyze the potential of differentially stimulated CD8 α^+ DC to modulate EAM, TNF/CD8 α^+ or LPS- α CD40/CD8 α^+ were pulsed with MyHC- α -peptide for 4 h. Mice were pre-vaccinated three times, at days -7, -5, and -3. At day 0, we then immunized the pre-vaccinated animals with MyHC- α -peptides emulsified in CFA. The same mice were boosted at day 7. Sham-treated control mice were pre-vaccinated with PBS only and immunized at day 0 and

day 7 with MyHC- α -peptides emulsified in CFA. Twenty-one days after the first immunization, the hearts were removed and examined for heart-infiltrating cells. As expected, immunized sham-vaccinated control mice exhibited severe myocarditis at day 21, while mice that received LPS- α CD40/CD8 α^+ had only a slight decrease of EAM incidence (Fig. 5.11 A and B) that, however, was statistically significant ($p = 0.046$). In contrast, vaccination with TNF/CD8 α^+ led to a relevant prevention of the disease ($p = 0.0026$) (Fig. 5.11 A and B). To exclude that the vaccination altered the course of the disease, preceding the onset of myocarditis earlier than 21 days after the first immunization, we sacrificed vaccinated and vaccinated/immunized mice at different time points. We examined hearts for infiltrating cells at day 0, day 2, day 9, day 14, and, as mentioned before, at day 21. Examination of mice at day 0 allowed us to verify that the vaccination did not cause any side effect such heart inflammation or EAM. In fact, MyHC- α /CFA-immunized mice vaccinated with TNF/CD8 α^+ always showed very low or irrelevant heart score disease values (Fig. 5.11C), suggesting that this vaccination method, in addition to prevent EAM, was not pathogenic and did not cause EAM. Vaccination with LPS- α CD40/CD8 α^+ showed increased levels of EAM compared to TNF/CD8 α^+ at day 0, comparable levels after MyHC- α /CFA immunization from day 2 until day 9, but again constantly increased levels of heart-infiltrating cells from day 9 to day 21 (Fig. 5.11C). This suggested that LPS- α CD40/CD8 α^+ vaccination was not optimal to prevent and protect mice from EAM. Sham-vaccinated control mice immunized with MyHC- α /CFA displayed the usual development of inflammatory heart-infiltrating cells characteristic for EAM. Taken together, these data indicate that vaccination with serial injections of TNF- α -stimulated CD8 α^+ DC pulsed with MyHC- α -peptides can protect from EAM.

5.9 Antigen-specific IFN γ -producing CD4 $^+$ Th1 confer protection from EAM

To assess whether the mechanism of EAM prevention in mice vaccinated with TNF- α -stimulated CD8 α^+ DC was dependent on antigen-specific T cells, we adoptively transferred DO11.10 transgenic CD45.2 CD4 $^+$ T cells into CD45.1 wild-type mice. One day later, TNF/CD8 α^+ or LPS- α CD40/CD8 α^+ -sorted from CD45.2 mice were injected into the same CD45.1 recipient mice. Two days

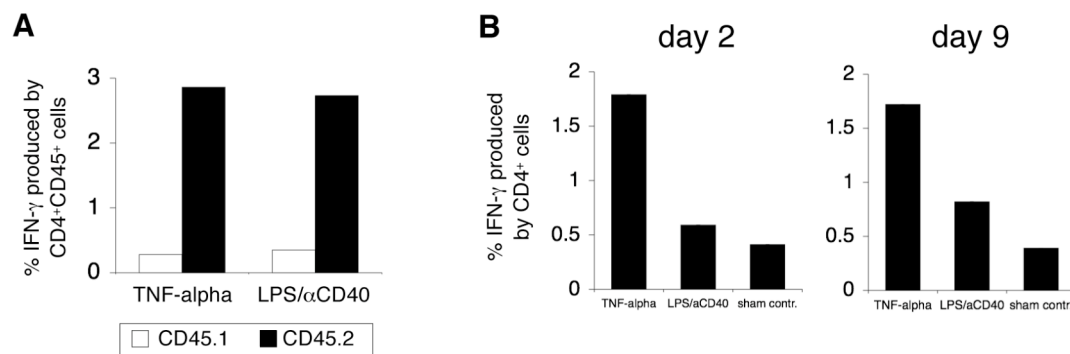


Figure 5.12. TNF/CD8 α^+ prevent EAM supporting antigen-specific IFN- γ -producing CD4 $^+$ Th1 cells.

A, 10^6 CD4 $^+$ T cells sorted from OVA transgenic DO11.10 mice were intravenously injected into each CD45.1 wild-type mouse. One day later, 10^5 OVA³²³⁻³³⁹-pulsed CD8 α^+ DC stimulated with TNF- α or LPS- α CD40 were intravenously injected into the same CD45.1 recipient mice. After two days, spleens were extracted and splenocytes were restimulated with PMA/IO/BFA for 4 h. FACS staining for intracellular IFN- γ measurement is shown for both CD4 $^+$ CD45.1 and CD4 $^+$ CD45.2 $^+$ cells.

B, Mice vaccinated and immunized as described in Fig. 6.3 were sacrificed at day 2 and day 9. After 48h restimulation with 2 μ g/ml MyHC- α -peptides, CD4 $^+$ Th cells were intracellularly analyzed for IFN- γ production by FACS.

after CD8 α^+ DC injection, spleens were removed and cell suspensions were analyzed for intracellular cytokine production. As shown in Fig. 5.12A, restimulated CD4 $^+$ T cells from CD45.1 recipient mice produced low levels of IFN- γ after injection of TNF/CD8 α^+ or LPS- α CD40/CD8 α^+ , suggesting that CD8 α^+ DC did not induce peptide-unspecific T cells responses. In contrast, adoptively transferred DO11.10 transgenic CD45.2 CD4 $^+$ T cells released high amounts of IFN- γ , indicating that CD8 α^+ DC triggered antigen-specific CD4 $^+$ Th1 cells to produce IFN- γ (Fig. 5.12A). As mentioned before, CD4 $^+$ T cells from mice vaccinated with peptide-loaded CD8 α^+ DC showed an increased antigen-specific production of IFN- γ . To understand whether the self-peptide-specific CD4 $^+$ Th1 cell response was restricted to the vaccination period, mice vaccinated with MyHC- α -pulsed CD8 α^+ DC and then immunized with MyHC- α /CFA were examined 2 days and 9 days after the first immunization. Splenocytes suspensions were restimulated with MyHC- α -peptide for 48

hours for intracellular cytokine analysis. As expected, IFN- γ was highly produced by CD4⁺ Th1 cells primed by TNF/CD8 α ⁺ at day 2 and day 9, reflecting the protective role of IFN- γ in EAM (Fig. 5.12B). On the contrary, the vaccination effect of LPS- α CD40/CD8 α ⁺ was already reduced 2 days after the first immunization, as demonstrated by low IFN- γ production compared to CD4⁺ Th1 cells from TNF/CD8 α ⁺ vaccinated mice (Fig. 5.12B). These data indicate that vaccination with TNF/CD8 α ⁺ supports and induces CD4⁺ Th1 polarization, allowing the suppression of pathogenic and auto-aggressive IL-17-producing CD4⁺ Th17 cells. In addition, the success of this vaccination strategy emphasizes the protective role of IFN- γ -producing CD4⁺ Th1 cells in EAM.

5.10 GITR⁺ Treg play no role in TNF- α -DC-mediated protection from EAM

Several CD4⁺ T cell subpopulations produce IFN- γ . Given the efficacy of the DC vaccination strategy, the question arises whether protection is indeed conferred by self-antigen-specific CD4⁺ Th1 cells, or whether the pre-vaccination strategy also acts on regulatory T cells. For this reason, we specifically addressed the role of regulatory T cells (Treg) in our vaccination strategy. To this end, we analyzed the number of CD4⁺FoxP3⁺GITR⁺ Treg and their potential to produce IFN- γ in the spleen of vaccinated mice (day 0). Surface marker analysis revealed that both TNF/CD8 α ⁺- or LPS- α CD40/CD8 α ⁺-vaccinated mice did not display relevant differences compared to sham-vaccinated control mice among the CD4⁺FoxP3⁺GITR⁺ Treg population (Fig. 5.13A). Importantly, levels of IFN- γ expressed by Treg were very low and comparable in both CD8 α ⁺ DC-vaccinated mice and sham-vaccinated control mice (Fig. 5.13A).

As $CD4^+FoxP3^+GITR^+$ Treg are known to modulate and protect from EAM (115), we investigated whether $CD8\alpha^+$ DC vaccination altered the inhibitory potential of Treg. For this purpose, we isolated splenocytes from a mouse with myocarditis and co-cultivated them with titrating amounts of $GITR^+$ Treg sorted from $CD8\alpha^+$ DC-vaccinated or sham-vaccinated mice at day 0. As shown in Fig. 5.13B, all $GITR^+$ Treg were equally efficient in inhibiting proliferating MyHC- α -specific splenocytes. Thus, we did not observe any functional difference between Treg sorted from TNF/ $CD8\alpha^+$ -, LPS- $\alpha CD40/CD8\alpha^+$ -, or sham-vaccinated mice. These findings suggest that the immunomodulatory potential of Treg was not affected by our pre-vaccination strategy. Accordingly, $CD4^+FoxP3^+GITR^+$ Treg are not involved in the protective mechanism induced by $CD8\alpha^+$ DC vaccination.

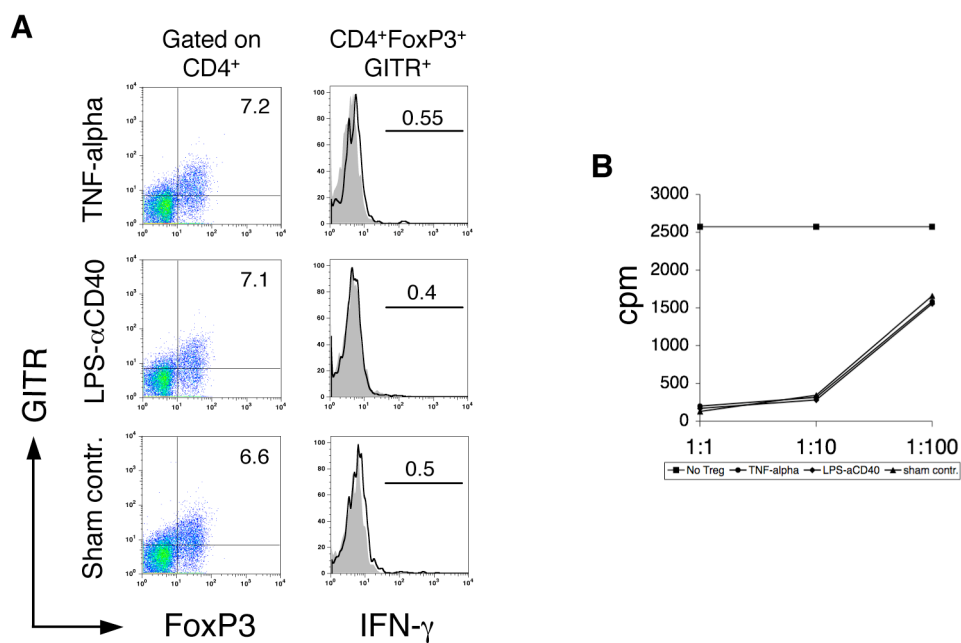


Figure 5.13. $CD4^+FoxP3^+GITR^+$ Treg did not show any difference in number and inhibitory potential. A, Mice vaccinated at days -7, -5, and -3, with TNF/ $CD8\alpha^+$ or LPS- $\alpha CD40/CD8\alpha^+$ were sacrificed at day 0. Splenocytes were stained for surface markers with CD4, GITR, and for intracellular markers with FoxP3. For intracellular IFN- γ detection by FACS, spleen cell suspensions were restimulated for 4 h with PMA/IO/BFA. B, 10^5 splenocytes from a mouse with myocarditis were co-cultivated for 48 h with titrating amount of $GITR^+$ Treg (10^4 , 10^3 , 10^2) sorted from differently vaccinated mice (day 0) in the presence of 2 $\mu g/ml$ MyHC- α -peptides. Splenocytes proliferation was measured by 3H -thymidine incorporation.

6 Discussion

In the first part of my thesis, I provided evidence for a dual role of CD11b⁺ monocytes, the major heart-infiltrating cells during the disease course of EAM. In the short term, CD11b⁺ monocytes represent the major cellular substrate of IL-17-induced acute inflammation. In the long term, however, CD11b⁺ monocytes mediate a disease limiting IFN- γ -triggered negative feedback loop, which suppresses heart-specific T cell responses.

In the second part of my thesis, I developed the basics for a novel vaccination strategy to prevent EAM. In addition, I provided for the first time *in vivo* evidence that an established Th1 memory might prevent Th17-mediated autoimmunity *in vivo*. Pretreatment with serial injections of self-peptide-pulsed and TNF- α -stimulated splenic CD8 α ⁺ DC triggered a Th1 biased MyHC- α -specific CD4⁺ T cell response and protected mice from EAM by suppressing CD4⁺ Th17 cell lineage expansion.

6.1 The dual role of CD11b⁺ monocyte-like cells in EAM

In the EAM model, the extent of the cardiac infiltrates peaks around day 21 after the first immunization. According to our data, IL-17 released by autoreactive CD4⁺ Th17 cells directly promotes the recruitment of bone marrow-derived CD11b⁺ monocytes to the heart. CD11b⁺ monocytes represent the major population of heart-infiltrating cells. Later on, during the disease course, mononuclear infiltrates and intra-cardiac IL-17 release spontaneously resolve in wild-type mice. This, however, is not the case in IFN- γ R^{-/-} or IFN- γ ^{-/-} mice, which show ongoing severe inflammation and a persistent autoreactive T cell response. Our data indicate that heart-infiltrating CD11b⁺ monocytes are key players in a negative feedback loop suppressing autoreactive heart-specific T cells. This negative feedback loop is IFN- γ -dependent and nitric oxide-mediated. In fact, IFN- γ signaling is required on CD11b⁺ monocytes but not on T cells, because IFN γ -R^{+/+} CD11b⁺ monocytes were sufficient to abrogate EAM in IFN- γ R^{-/-} mice.

So far, EAM has been recognized as an autoimmune disease developing independently of Th1/Th2 polarization (47). It has been shown recently that

IL-6, which is essential for the induction of Th17 T cells (46), and IL-23-promoting Th17 expansion, are both critical for EAM development (47, 57). In extension of these findings, our data prove the direct pathogenic role of IL-17 in EAM development, because IL-17 depletion prevents EAM development. Furthermore, we show that IL-17 is responsible for the recruitment of myeloid-derived monocytes representing the major heart infiltrating cells in EAM. Although the exact mechanism could be speculative, it might include direct chemotactic effects, as suggested *in vitro* for pulmonary macrophages (116). Indeed, we found markedly suppressed MCP-1 and MIP-1 α mRNA levels in hearts of anti-IL-17-treated immunized mice compared to controls. Furthermore, mice lacking the CCR2 receptor for MCP-1 and the CCR5 receptor for MIP-1 α , respectively, had been reported to be resistant to EAM (114). In addition, we found reduced mRNA expression of the chemotactic ligand SDF-1 acting through the CXCR4 receptor on injected monocytes. Nevertheless, we cannot exclude that other IL-17-dependent mediators or receptors might also be involved in the recruitment of inflammatory cells. IL-17 could also act on stromal endothelial cells in inflamed tissue to induce the secretion of neutrophil-attracting factors such as IL-8, CXCL1 or GM-CSF (117-119).

Phenotypically, the CD11b⁺ heart-infiltrating cells represent a heterogeneous population of largely immature CD45, CD14, F4/80 and CXCR4 positive monocytes expressing low levels of MHC class II and Gr-1. Culturing bone marrow in the presence of M-CSF yields a population of cells with largely identical surface marker expression pattern. Interestingly, recent data demonstrated that polymicrobial sepsis results in the expansion of an immature Gr-1⁺CD11b⁺ cell population with T cell suppressing properties (120). In contrast to our observations in the EAM model, these cells were Gr-1⁺ and most expressed CD31, a marker of immature myeloid cells. Obviously, the severe systemic inflammatory response associated with sepsis results in the mobilization of a more immature myeloid lineage committed cell population from bone marrow. Nevertheless, the heart-infiltrating CD11b⁺ cells still show some maturation capacity, because they up-regulate NOS2 and MHC class II in the presence of combined IFN- γ exposure and TLR4

stimulation (see Fig. 5.6). This final maturation step appears critical for the induction of the above mentioned negative feedback loop.

Mice lacking T-bet^{-/-}, a T-box transcription factor required for Th1 cell differentiation and IFN- γ production, show a heart-localized up-regulation of IL-17 production and higher disease scores (47). In the same report, it was demonstrated that the IL-23 subunit IL-12p40, but not the IL-12p70 specific subunit IL-12p35, are critical for myocarditis development. The idea that IL-23 is critical for Th17 T cell expansion and EAM development fits to the observation that STAT4^{-/-} and STAT4^{-/-}IFN- γ ^{-/-} mice, but not IFN- γ ^{-/-} mice, are protected from EAM (50). In fact, STAT4 signaling is required for IL-17 production in response to IL-23 (54). Furthermore, our findings fit earlier observations of enhanced myocarditis susceptibility in IFN- γ ^{-/-} and IFN- γ R^{-/-} mice (48-50). Indeed, we found markedly increased IL-17 levels after *in vitro* re-stimulation of heart infiltrating T cells from IFN- γ ^{-/-} and IFN- γ R^{-/-} mice (Fig. 5.7B). Whereas the capacity of heart infiltrating T cells to release IL-17 successively decreases in wild-type mice after day 21, IL-17 release and infiltrations with mononuclear inflammatory cells persist in the hearts of IFN- γ ^{-/-} and IFN- γ R^{-/-} mice up to two months after immunization. Anti-IL-17 treatment reduced monocyte infiltrations and myocarditis scores in IFN- γ ^{-/-} mice, underscoring the relevance of persistent intra-cardiac IL-17 release for the persistence of cardiac inflammation in the absence of IFN- γ . Together with the enhanced disease scores of IFN- γ ^{-/-} mice (48, 50) and the increased intra-cardiac IL-17 levels, the latter observations suggest a direct anti-inflammatory role for IFN- γ in Th17-mediated autoimmune myocarditis *in vivo* and fit the perception that IFN- γ negatively regulates Th17 T cell expansion (53, 121).

Importantly, my data argue against an IFN- γ -dependent role for regulatory T cells in EAM suppression (122-124), because wild-type monocytes alone were sufficient to suppress disease in IFN- γ R^{-/-} mice lacking IFN- γ signaling on T cells. Mechanistically, the *in vitro* data rather suggest that upon IFN- γ exposure, monocytes take a final maturation step and mediate T cell suppressive effects by release of nitric oxide (Fig 6.1). Another IFN- γ -dependent mechanism might include the induction of indoleamine-2,3-dioxygenase (IDO) on antigen-presenting cells (125). However, differences in

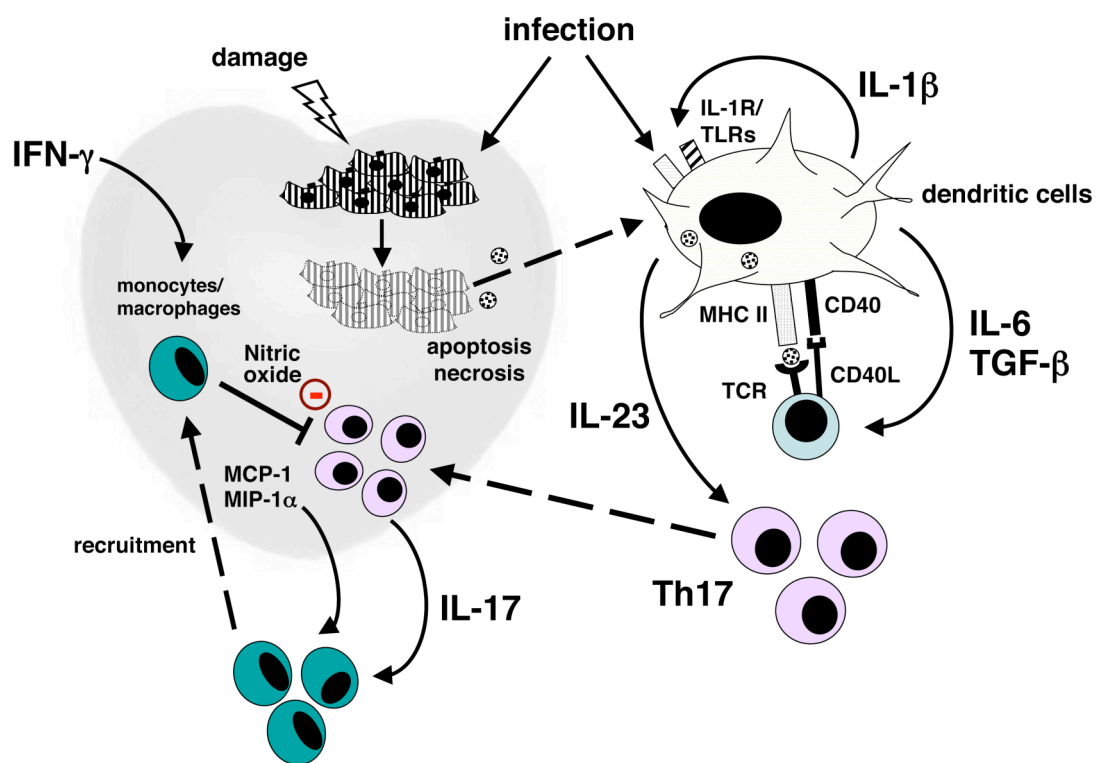


Figure 6.1. The negative feedback mechanism which confines EAM. Cardiac damage results in release of heart-specific self-antigens. In the presence of non-specific adjuvants, APC-internalizing cardiac self-antigens get activated beyond a threshold level and promote heart-specific autoimmunity. Pro-inflammatory cytokines, such as IL-1, IL-6, and TGF- β , support the differentiation of auto-aggressive CD4⁺ Th17 cell, while IL-23 is necessary for survival and expansion of the CD4⁺ Th17 lineage. Heart-specific CD4⁺ Th17 cells infiltrate into the heart inducing inflammatory signals. IL-17 and other macrophage-attracting chemokines support the recruitment of inflammatory cells into the heart. IFN- γ stimulates heart-infiltrating monocytes/macrophages to produce nitric oxide. Nitric oxide reversibly inhibits T cell proliferation, resolving the disease.

systemic serum levels of IDO metabolites between IFN- $\gamma^{+/+}$ and IFN- $\gamma^{-/-}$ mice were never found. According to our data, the IFN- γ -triggered NOS2 upregulation and nitric oxide release is synergistically enhanced by the stimulation of Toll like receptor 4, but not by TNF- α (Fig. 5.6).

The role of nitric oxide in immune-mediated diseases is still controversial (126, 127). Nitric oxide has been shown to act as a potent cytotoxic effector molecule in immune defence and to induce tissue damage in autoimmune diseases (128, 129). In fact, increased levels of human NOS2 mRNA have

been measured in the brain of multiple sclerosis patients and have been correlated to chronic active demyelinating lesions (130), while EAE in susceptible mice was reduced by administration of a NO-inhibitor (131). On the other hand, many studies reported that NO exerts anti-inflammatory effects. In fact, nitric oxide represents a short living mediator of reversible T cell growth arrest. Importantly, physiological levels of nitric oxide inhibit T cell proliferation *in vitro* reversibly, without induction of apoptosis or affecting the capacity of the cells to produce cytokines (80, 132, 133). In fact, several lines of evidence suggest that NO-dependent feedback mechanisms confining T cell expansion might play an important modulatory role in various diseases. In autoimmune disease models, for example, NO limits autoreactive T cell expansion in myocarditis (134), myasthenia gravis (135), experimental allergic encephalomyelitis (136), and in a mouse model of pulmonary graft versus host-disease (137). In the context of atopic asthma, IFN- γ -releasing Th1 cells function in concert with human bronchial epithelial cells, which serve as a major source of NO to inhibit T cell proliferation at the potential lesional sites in asthma. *In vitro*, the NO-induced T cell growth arrest was reversible and STAT5-dependent (133).

In the EAM model, MyHC- α /CFA immunization results in the generation of heart-specific Th17 as well as Th1 T cells. In wild-type mice, however, heart-infiltrating CD8⁺ T cells, but not the CD3⁺CD8⁻ T cells, represent the major source of IFN- γ within the inflamed myocardium at day 21 after immunization (47). Nevertheless, co-injection of MyHC- α specific Th1 T cells prevented disease development in RAG2^{-/-} mice 12 days after adoptive transfer of MyHC- α specific Th17 T cells. These findings indicate a negative regulatory role for Th1 T cells in autoimmune myocarditis. Given the fact that heart-infiltrating CD4⁺ T cells do not release IFN- γ at day 21, Th1 cells must either exert their inhibitory effects early during disease development or outside the heart. Nevertheless, IFN- γ released from heart-infiltrating CD8⁺ T cells or NK cells might still activate heart-infiltrating CD11b⁺ monocytes triggering local nitric oxide release and suppression of activated, heart-infiltrating Th17 cells. This negative feedback mechanism allows the immune system to promote the quick IL-17-dependent recruitment of monocytes to a potentially infected site.

IFN- γ , on the other hand, is required for the optimal activation of monocytes and macrophages dealing with an infective agent. At the same time, IFN- γ limits the expansion of activated T cells and protects from exaggerated or even auto-aggressive T cell responses. This negative feedback mechanism might regulate the expansion of CD4⁺ Th17 cells in the peripheral lymphatic organs. In fact, a biased expansion of heart-specific IFN- γ -producing CD4⁺ Th1 cells would negatively act on the polarization and expansion of pathogenic CD4⁺ Th17 cells. This could completely prevent the development of heart-specific CD4⁺ Th17 cells in the periphery, protecting from EAM.

Our data suggest that the view on the role of heart-infiltrating monocytes in autoimmune heart disease needs to be revised. So far, histological evaluation of cardiac infiltrates represented the diagnostic gold standard. Accordingly, severity grading of myocarditis strongly depends on the extent of mononuclear infiltrates, including monocytes and macrophages (26, 138). Our experiments suggest that these heart-infiltrating monocytes also reflect the first step of a negative feedback loop suppressing heart-specific T cells and initiating the healing process. This idea nicely fits the observation of a favorable disease prognosis of patients with fulminant myocarditis characterized by massive infiltrations of mononuclear cells (139). Regarding histology as readout for the disease progression into chronic myocarditis, we postulate that not the extent of infiltrates per se but rather the presence of IL-17-producing autoreactive CD4⁺ Th17 cells would correlate with potential adverse outcome.

In conclusion, we provide for the first time evidence for a protective role of bone marrow-derived CD11b⁺ monocytes in autoimmune myocarditis. In fact, heart-infiltrating monocytes represent a key element in an IFN- γ -dependent negative feedback loop limiting cardiac inflammation.

6.2 A CD8 α ⁺ DC-based vaccination strategy against EAM

Serial intravenous injections of MyHC- α -pulsed and TNF- α -stimulated CD8 α ⁺ dendritic cells (TNF/CD8 α ⁺) were used to develop a vaccination strategy to prevent EAM. In fact, providing evidence that a heart-specific CD4⁺ Th1-induced IFN- γ response supported protection from EAM, we hypothesized

that a vaccination strategy to mediate the polarization of IFN- γ -producing CD4⁺ Th1 cells in the peripheral lymphnodes negatively regulated the expansion of autoreactive CD4⁺ Th17 cells. For the differentiation of CD4⁺ Th1 cells, we used splenic CD8 α ⁺ DC, which have been described to prime IFN- γ -producing T cells. Other DC subsets were not helpful for this purpose. Indeed, splenic CD8 α ⁻ DC polarized IL-4-producing T cells, while bmDC cultured in GM-CSF induced a CD4⁺ Th17-biased response.

We showed a tolerogenic potential of TNF/CD8 α ⁺, which can be explained by the low proinflammatory cytokine secretion upon stimulation. In fact, TNF/CD8 α ⁺ produced low levels of IL-1 β and IL-6, and not significant increased levels of IL-23, when compared to LPS- α CD40/CD8 α ⁺. Phenotypically, unlike the untreated CD8 α ⁺ DC, TNF/CD8 α ⁺ expressed higher levels of the costimulatory surface molecules CD40, and CD80. However, TNF/CD8 α ⁺ and LPS- α CD40/CD8 α ⁺ essentially did not differ in their expression of costimulatory molecules and their priming capacity for naïve T cells. They differed, however, in their potential to polarize the CD4⁺ Th response. In fact, TNF/CD8 α ⁺ supported the differentiation of IFN- γ -producing CD4⁺ Th1, while LPS- α CD40/CD8 α ⁺ supported both, IFN- γ -producing CD4⁺ Th1 and IL-17-producing CD4⁺ Th17. These findings are in line with *in vitro* and *in vivo* data, suggesting that CD8 α ⁺ DC promote peripheral tolerance (140) and polarize IFN- γ -producing CD4⁺ Th1 cells (104-106, 141). We demonstrated, as well, the ability of TNF/CD8 α ⁺ to promote a CD4⁺ Th1 cell-biased response, but the potential of LPS- α CD40/CD8 α ⁺ to promote both CD4⁺ Th17 and Th1 cells was not yet described. This observation can be explained by TLR stimulation, increasing the production of various inflammatory cytokines (142). This was described in an autoimmune arthritis mouse model with mice lacking TLR4, which were protected against severe disease, demonstrating that TLR4 modulates the CD4⁺ Th17 cell population and IL-17 production (142). Studies in EAM reported, as well, that TLR4 activation supports the development of CD4⁺ Th17 cells (58). Taken together, CD4⁺ Th1 polarization is a prerogative of TNF- α /CD8 α ⁺, while differentiation of CD4⁺ Th17 is supported by the TLR-activated CD8 α ⁺ DC cells.

Compared to LPS- α CD40/CD8 α^+ -treated mice, TNF/CD8 α^+ -pretreated mice mount a Th1 response and are protected from subsequent MyHC- α /CFA immunization. Indeed, two and nine days after the first immunization, MyHC- α -specific IFN- γ production showed similar levels in LPS- α CD40/CD8 α^+ -vaccinated mice and sham-prevaccinated mice. In contrast, Elevated IFN- γ levels were increased in TNF/CD8 α^+ -prevaccinated mice at the same time points. Thus, it is then conceivable that the *in vivo* expansion of antigen-specific CD4 $^+$ Th1 cells, i.e. generation of a Th1 memory, allows a rapid response of primed IFN- γ -producing T cells at the draining lymphnodes after MyHC- α /CFA immunization. This rapid CD4 $^+$ Th1 response abrogates/suppresses the pathogenic CD4 $^+$ Th17 responses immediately. This mechanism has already been shown and demonstrated *in vitro* (51, 53, 143, 144). In fact, neutralization of IFN- γ *in vitro* increased the number of IL-17-producing Th17 cells. The number of CD4 $^+$ Th17 cells was then further increased by the addition of IL-4-neutralizing antibodies, indicating that both IFN- γ and IL-4 can inhibit the expansion of CD4 $^+$ Th17 cells *in vitro* (51, 53). We, on the other hand, provide for the first time *in vivo* evidence that CD4 $^+$ Th1 responses negatively regulate CD4 $^+$ Th17-mediated autoimmunity.

The factors regulating CD4 $^+$ Th1 cell differentiation are essential for the expansion, the stability, and the survival of the lineage. The expansion of effector and memory Th1 cells is defined by several factors, like the duration of antigenic stimulation (145), the type of antigen presenting dendritic cells, and the cytokine milieu (146). In fact, the duration of antigen stimulation determines whether naïve or effector Th cell will be activated or deleted. Under prolonged stimulation, for instance, effector Th cells present a decreased capacity to proliferate and die earlier (145). Thus, it was not surprising that vaccination with high amounts of peptide-pulsed and stimulated CD8 α^+ DC did not protect from EAM after induction of the disease. This can be explained by a too strong induction of CD4 $^+$ Th1 cells by CD8 α^+ DC, which led effector and memory CD4 $^+$ Th1 cells to death after the second and the third injection. Moreover, we used MyHC- α -loaded and TNF- α -stimulated or LPS- α CD40-stimulated bmDC (see "Material and methods") as control, to evaluate the differences between bmDC and CD8 α^+ DC. The

potential of bmDC to induce proliferation of naïve CD4⁺ T cells *in vitro* was considerably increased compared with CD8α⁺ DC. Furthermore, we already knew that bmDC were high pathogenic and induced strong autoimmune myocarditis between 10 and 14 days after the first injection (44-46). As expected, mice vaccinated three times with bmDC (stimulated with TNF-α or with LPS-αCD40) showed severe myocarditis with huge amounts of heart-infiltrating cells at day 10, while vaccination with CD8α⁺ DC was not pathogenic and induced only irrelevant myocarditis. On the level of the CD4⁺ Th cells, bmDC-pre-vaccinated diseased mice displayed high antigen-specific proliferation and IL-17 production, while CD8α⁺ DC-prevaccinated mice showed no relevant antigen-specific proliferation and no IL-17 production. In summary, TNF/CD8α⁺ DC were tolerogenic by promoting a non-pathogenic, protective peptide-specific IFN-γ-producing CD4⁺ Th1 cell response, while bmDC were immunogenic, supporting peptide-specific IL-17-producing CD4⁺ Th17 cells.

The cytokine milieu is very important for the polarization of CD4⁺ Th1 and Th17 cell lines. IL-12 has been and is still considered the most important cytokine to differentiate CD4⁺ Th1 cells (146-148). However, as mentioned in the "Introduction", IL-12 is a heterodimer composed of two subunits, the p40 and the p35. The p40 subunit is shared with IL-23, which is composed of the p40 and the p19 subunits. It has been shown that IL-23 is necessary for the survival and the expansion of the CD4⁺ Th17 lineage. For this reason, we did not take in consideration a measurement of IL-12 in the supernatants of stimulated CD8α⁺ DC, as IL-12p40 can support both CD4⁺ Th1 and Th17 lineages (149). Other crucial cytokines involved in the polarization of the CD4⁺ Th17 lineage were detected in LPS-αCD40/CD8α⁺ supernatants and reflected the cytokine pattern necessary for CD4⁺ Th17 polarization (51-53). In fact, LPS-αCD40/CD8α⁺ were able to support the *in vivo* expansion of CD4⁺ Th17 cells, while the development of CD4⁺ Th1 cells was supported by both TNF/CD8α⁺ and LPS-αCD40/CD8α⁺.

IFN-γ is produced by several cells. Besides T cells, also DC, NK cells, and Treg produce relevant amounts of IFN-γ (123, 150-153). For this reason, we attempted to measure the production of IFN-γ in the supernatants of

stimulated CD8 α^+ DC. In general, we observed that IFN- γ production was not high, and comparing differentially stimulated CD8 α^+ DC with unstimulated CD8 α^+ DC, only LPS- α CD40/CD8 α^+ cells produced increased levels of IFN- γ . However, this was not sufficient to explain any CD4 $^+$ Th1 or Th17 differentiation potential by CD8 α^+ DC. Afterwards, we investigated the role of GITR $^+$ Treg to produce IFN- γ and to protect vaccinated mice from EAM. In fact, as Treg, under particular stimulatory signals, produce IFN- γ , creating a regulatory mechanism to inhibit Th cells (123), we sought for a possible involvement of Treg in the protecting mechanism following vaccination. We decided to sort GITR $^+$ Treg cells because they have been described as the only CD4 $^+$ T cell population expressing the typical Treg transcription factor FoxP3, which protect mice from EAM (115). GITR $^+$ Treg sorted from vaccinated mice (day 0) did not present increased amounts of IFN- γ , suggesting that Treg were not a determinant source of IFN- γ .

The last cell population we analyzed for IFN- γ production was the natural killer population. NK cells belong to the innate immune system and act unspecifically in the early phase of inflammatory processes. The role of NK cells is still under investigation, but first results suggest that splenic NK cells from LPS- α CD40/CD8 α^+ -treated mice present a reduced production of IFN- γ compared to TNF/CD8 α^+ or sham-treated mice. However, it appears that this difference is not big enough to be considered significant. All together, the non-relevant production of IFN- γ by CD8 α^+ DC, Treg, and NK cells, supports our hypothesis that CD4 $^+$ Th1 cells are the crucial IFN- γ -producing cells responsible for EAM protection.

Taken together, my thesis provides a broader insight in the specific role of IFN- γ - and IL-17-producing CD4 $^+$ T cells in inflammatory heart disease. I found that IFN- γ can act as a negative regulator of CD4 $^+$ Th17-mediated inflammatory heart disease. Mechanistically, IFN- γ inhibited the proliferation of autoreactive CD4 $^+$ Th17 cells promoting nitric oxide from heart-infiltrating CD11b $^+$ monocytes. Nitric oxide, on the other hand, induced reversible growth arrest in activated CD4 $^+$ Th cells. Based on these findings, I used TNF- α -activated, self-antigen-loaded splenic CD8 α^+ DC to selectively promote the

development of a heart-specific CD4⁺ Th1 memory response. Enhancing the heart-specific CD4⁺ Th1 response suppressed pathogenic CD4⁺ Th17 responses and autoimmune myocarditis development after subsequent immunization. Our idea about the complementary role of IFN- γ and IL-17 in inflammatory heart diseases represents a first step towards a novel vaccination strategy against heart-specific autoimmunity.

7 List of abbreviations

APC	Antigen-presenting cells
BALB/c	Mouse strain (IA ^d haplotype)
BFA	Brefeldin A
bmDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	complementary DNA
CFA	Complete Freund's adjuvant
CFSE	5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
cpm	Counts per minute
CTLA	Cytotoxic T lymphocyte antigen
CVB3	Coxsackievirus B3
DC	Dendritic cells
DCM	Dilated cardiomyopathy
EAE	Experimental autoimmune encephalomyelitis
EAM	Experimental autoimmune myocarditis
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell-sorting
FBS	Fetal bovine serum
FLT3	Fms-like tyrosine kinase 3
FoxP3	Forkhead box P3
GITR	Glucocorticoid-induced TNFR family-related protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
H&E	Hematoxilin & eosin
IFN- γ	Interferon gamma
IL	Interleukin
Ig	Immunoglobulin
IO	Ionomycin
i.p.	Intraperitoneal
i.v.	Intravenous
KO	Knock-out

L-NAME	N-nitro-L-arginine-methyl-ester
LN	Lymph node
LPS	Lipopolysaccharide
MACS	Magnetic affinity cell sorting
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MyHC- α	Myosin heavy-chain-alpha
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
n.s.	Not significant
OVA ³²³⁻³³⁹	Ovalbumin 323-339 peptide
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PMA	Phorbol myristate acetate
RAG	Recombination activating gene
RT-PCR	Reverse transcriptase polymerase chain reaction
s.c.	Subcutaneous
SD	Standard deviation
SDF-1	Stromal derived factor 1
STAT	Signal transducers and activators of transcription
TCR	T cell receptor
TGF- β	Transforming growth factor beta
Th	T helper cells
TLR	Toll-like receptors
mTECs	Medullary thymic epithelial cells
TNF- α	Tumor necrosis factor alpha
Treg	Regulatory T cells
TSA	Tissue-specific antigens
Wt	Wild-type

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10 Curriculum vitae

Personal data

Name: Valaperti
First Name: Alan
Date of Birth: 30 June 1980
Birth place: Lugano (TI)
Civil status: Married
Nationality: Swiss
Addresses: Johanniterstrasse 15 Via Monte Boglia 16
4056 Basel 6900 Lugano
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Education

2005 - 2008 PhD, Biomedical Research, University of Basel
2000 - 2005 MSc, Molecular Biology, Immunology
University of Basel

Working experience

2005 - 2008 PhD thesis, University Hospital, Basel,
Switzerland, Prof. Urs Eriksson, Prof. Ed
Palmer
Title: "Mechanisms regulating autoreactive T
cell responses in inflammatory heart disease"
2004 - 2005 Diploma thesis, Departement für Klinisch-
Biologische Wissenschaften (DKBW),
University of Basel, Switzerland, Prof. Daniela
Finke
Title: "The role of thymic stromal lymphopietin
in Payer's patch development"

Course Certificates

- "Radiological Safety Protection Course" organized by the "Department of Biomedicine" of the University Hospital Basel, September 2006, Basel, Switzerland

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- LTK1 Module 1E: Introductory Course in Laboratory Animal Science (Swiss Ordinance on the Education and Training of Persons Conducting Animal Experiments), 2006, Zürich, Switzerland

Laboratory skills

- **Animal handling**

Maintenance of mouse breedings; genotyping; generation of chimeric mice; blood isolation; mouse dissection and isolation of lymphnodes, heart, lung, liver, gut, and bones; isoflurane gas anesthesia; bronchoalveolar lavage (BAL); intraperitoneal, intravenous, and subcutaneous injection; induction of experimental autoimmune myocarditis.

- **Cell cultures**

Cultivation of bone marrow-derived macrophages and dendritic cells; cultivation and expansion of T cell lines; proliferation assays of co-cultures with T cells plus antigen presenting cells; digestion of heart, spleen, and lymphnodes for cell isolation; cultivation and expansion of stem cell-like colonies; sorting with Magnetic Activated Cell Sorting (MACS) and AutoMacs (Miltenyi Biotec) machine; ELISA; nitric oxide measurement with Griess reaction.

- **Flow Cytometry**

CyanTM ADP Analyzer (Dako) and FACSCaliburTM (BD Biosciences); data analysis using FlowJo Software (Tree Star Inc.).

- **Immunohistochemistry and Microscopy**

Cut and stain of frozen organs; cytospin and stain of cultivated cells; fluorescence microscopy; evaluation of myocarditis score severity with light microscopy.

- **Molecular Biology and Biochemistry**

DNA and RNA extraction; PCR; quantitative RT-PCR; cloning by enzymatic digestion; gel electrophoresis; Western Blot.

Languages

Italian (mother tongue), German, English, French.

Oral presentations

- Macrophages suppress autoimmune myocarditis and heart-specific T cell responses. Immunomeeting, Department of Biomedicine (ZLF), 12 March 2007.
- A vaccination strategy to prevent experimental autoimmune myocarditis. Immunomeeting, Department of Biomedicine (ZLF), 10 October 2008.

Publications

- **Original articles**

- Blyszczuk P, Berger CT, Kania G, Marty RR, **Valaperti A**, Germano D, Dirnhofer S, Lüscher TF, Bogdan C, Hunziker L, Eriksson U. Type I IFN signaling promotes heart-specific autoimmunity. (submitted)
- Blyszczuk P, Kania G, Dieterle T, Marty RR, **Valaperti A**, Berger CT, Matter CM, Dirnhofer S, Lüscher TF, and Eriksson U. IL-1/MyD88 mediates post-inflammatory fibrosis and heart failure after experimental autoimmune myocarditis. (submitted)
- Germano D, Blyszczuk P, **Valaperti A**, Kania G, Dirnhofer S, Landmesser U, Lüscher TF, Hunziker L, Zulewski H, and Eriksson U. Prominin-1/CD133⁺ lung

epithelial progenitors protect from bleomycin-induced pulmonary fibrosis. (submitted)

- Kania G, Blyszczuk P, **Valaperti A**, Dieterle T, Leimenstoll B, Dirnhofer S, Zulewski H, and Eriksson U. Prominin-1⁺/CD113⁺ bone marrow-derived heart-resident cells suppress experimental autoimmune myocarditis. *Cardiovasc. Res.* 2008. 80: 236-245
- **Valaperti A**, Marty RR, Kania G, Germano D, Mauermann N, Dirnhofer S, Leimenstoll B, Blyszczuk P, Dong C, Mueller C, Hunziker L, Eriksson U. CD11b⁺ monocytes abrogate Th17 CD4⁺ T cell-mediated experimental autoimmune myocarditis. *J. Immunol.* 2008. 180: 2686-2695

■ Reviews

- Blyszczuk P, **Valaperti A**, and Eriksson U. Future therapeutic strategies in inflammatory cardiomyopathy: insights from the experimental autoimmune myocarditis model. *Cardiovasc Hematol Disord Drug Targets.* 2008. 80: 229-237

■ Book chapters

- **Valaperti A** and Eriksson U. The role of IL-17 in experimental autoimmune myocarditis. In: *Progression in Inflammatory Research, Volume: Th 17 Cells: Role in Inflammation and Autoimmune disease*, Quesniaux V, Ryffel B, and Di Padova F. Birkhäuser Publishing Ltd., Basel, Switzerland. 2009. (in press, due: December 2008)

■ Abstracts

- **Valaperti A**, Germano D, Kania G, Blyszczuk P, and Eriksson U. CD8 α ⁺ dendritic cell vaccination prevent CD4⁺ Th17-mediated experimental autoimmune myocarditis. Poster presentation at the "14th Cardiovascular Biology and Clinical Implications Meeting", October 2008, Muntelier, Switzerland.
- **Valaperti A**, Marty RR, Kania G, Germano D, Blyszczuk P, Dirnhofer S, and Eriksson U. CD11b⁺ monocytes abrogate Th17 CD4⁺ T cell-mediated experimental autoimmune myocarditis. Poster presentation at the "Jahrestagung der Schweizerischen Gesellschaft für Kardiologie (SGK)", Mai 2008, Bern, Switzerland. Poster presentation at the Annual Meeting SUK Project "Heart Remodeling in Health and Disease", April 2008, Bern Switzerland. Poster presentation at the "BioValley Science Day", October 2007, Basel, Switzerland.
- **Valaperti A**, Kania G, Blyszczuk P, and Eriksson U. Immunomodulatory potential of heart stem/progenitor cells. Poster presentation at the "3rd Swiss Stem Cell Network (SSCN) Annual Meeting", December 2006, Lausanne, Switzerland.