The opposing roles of Interferon-γ and IL-17 in inflammation

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Summary

Inflammation is the normal immune response to infection or injury, helping the body to fight pathogens and regain the integrity of the organism. Inflammatory processes develop in an unwanted and harmful degree in autoimmunity and chronic inflammation. The aim of the thesis was to identify the role of IFNγ and T cell Th1 commitment in inflammation using two separate models; experimental autoimmune myocarditis (EAM) and graft versus host mediated idiopathic pneumonia syndrome (IPS).

Using knock out mice for Th1 (T-bet), we show that inflammatory $CD4^+$ T cell responses in EAM depend not on Th1, but rather on newly described Th17. Neither Th1 or Th2 but only Th17 induce myocarditis in EAM and *in vivo* IL-17 depletion markedly reduce disease severity in *T-bet*$^{-/-}$ mice. Mice deficient for both T-bet and a receptor for IL-23 (maintenance factor of the Th17 subset) were protected from disease. While heart-infiltrating *T-bet*$^{+/+}$ $CD8^+$ T cells secrete IFNγ, a negative regulator of EAM and inhibitor of IL-17 production, *T-bet*$^{-/-}$ $CD8^+$ lymphocytes lost their capacity to release IFNγ within the heart. These data show that severe IL-17-mediated EAM develops in the absence of T-bet, and that T-bet can regulate autoimmunity via the control of $CD8^+$ T cell bystander function in the inflamed target organ.

Idiopathic pneumonia syndrome (IPS) is a major complication after allogeneic bone marrow transplantation, found in 30-50% of patients. Transplantation of bone marrow together with IFNγ deficient $CD4^+$ T cells resulted in severe pulmonary inflammation that was lethal by day 13 in our animal model. Absence of IFNγ favoured the development of IL-17 producing $CD4^+$ T cells. Blocking of IL-17 could only reduce disease severity. Protection from pulmonary graft-versus-host disease was dependent on the presence of IFNγ in donor $CD4^+$ T cells or the IFNγ receptor on pulmonary parenchymal cells. The lung tissue seems to be a uniquely sensitive organ for $CD4^+$ T cell mediated immunopathology in absence of the protective IFNγ induced signalling cascade. In addition, pulmonary host antigen-presenting cells (APC) are rapidly replaced

by donor APC and do not seem to play a major role for the maintenance of the donor mediated allogeneic immune response against the host.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ag – antigen
APC – antigen presenting cells
BM-DCs – bone marrow-derived dendritic cells
CFA – complete Freund's adjuvant
DAMPs – danger associated molecular patterns
DCs – dendritic cells
DCM – dilated cardiomyopathy
DNA – deoxyribonucleic acid
EAE – experimental autoimmune encephalomyelitis
EAM – experimental autoimmune myocarditis
ELISA – enzyme-linked immunosorbent assay
HLA – human leukocyte antigen
IFN - interferon
GM-CSF – granulocyte and macrophage colony stimulating factor
GVHD – graft versus host disease
IL – interleukin
IPS – idiopathic pneumonia syndrome
LPS – lipopolysaccharide
LT - leukotrienes
MHC – major histocompatibility locus
mo - months
MyHC-α – peptide derived from the murine myosin alpha heavy chain
NO – nitric oxide
NOS – nitric oxide synthase
PAMPs – pattern associated molecular patterns
PCR – polymerase chain reaction
PG - prostaglandins
PRR – pattern recognition receptors

RNA – ribonucleic acid

RT-PCR reverse transcription – polymerase chain reaction

SCID – severe combined immunodeficiency

T-bet – T box expressed in T cells

TLR – Toll-like receptor

TcR – T cell receptor

Tg – transgene, transgenic

Introduction

Inflammation

As the first reaction to injury or infection the immune system starts a series of processes called inflammation to regain the integrity of the body. Already in the 1st century AD, the roman encyclopedist Celsus recorded the classical signs of inflammation with the words *calor, dolor, rubor* and *tumor* – which stand for heat, pain, redness and swelling. All of these observations translate into a series of events that take place in an inflammatory process. Small blood vessels adjacent to the area of tissue damage initially become dilated with increased blood flow, then flow along them slows down. Endothelial cells swell and partially retract so that they no longer form a completely intact internal lining. The vessels become leaky, permitting the passage of water, salts, and some small proteins from the plasma into the damaged area (exudation). One of the main proteins to leak out is fibrinogen. Circulating neutrophils initially adhere to the swollen endothelial cells (margination), then actively migrate through the vessel basement membrane (emigration), passing into the area of tissue damage. Later, small numbers of macrophages migrate in a similar way, as do lymphocytes. Several factors start an immediate response, eg. histamine acting on endothelium. A delayed response starts 2-3 hours after injury and lasts for up to 8 hours. This is mediated by factors synthesized by local cells, e.g. bradykinin; factors derived from complement; and factors released from dead neutrophils in the exudate. What contributes to pain and fever development seen during an inflammation? Two factors involved are leukotrienes (LT) and prostaglandins (PG), they are potent eicosanoid lipid mediators derived from phospholipase-released arachidonic acid that are involved in numerous homeostatic biological functions and inflammation. LT and PG are generated by cyclooxygenase isozymes and 5-lipoxygenase, respectively, and their biosynthesis and actions are blocked by clinically relevant nonsteroidal anti-inflammatory drugs, (e.g. Aspirin, Ibuprofen). Leukotrienes are made predominantly by inflammatory cells like polymorphonuclear leukocytes, macrophages, and mast cells. Prostaglandins are

formed by most cells in the body and act as autocrine and paracrine lipid mediators. They are synthesized de novo from membrane-released arachidonic acid when cells are activated by mechanical trauma or by specific cytokine, growth factor, and other stimuli (e.g. collagen and ADP in platelets, bradykinin and thrombin in endothelium). Prostaglandins elicit a hyperalgesic response, or increased sensitivity, to touch by sensitizing the free end of pain neurons in peripheral inflammation. Prostaglandins act both at peripheral sensory neurons and at central sites within the spinal cord and brain to evoke hyperalgesia. Bacterial lipopolysaccharides and other harmful factors induce cytokine networks that cause fever. These stimulate the neural pathways to raise body temperature [1]. As a subsequent hallmark of inflammation, macrophages and lymphocytes are entering the tissue, after they are attracted by various secreted chemokines, cytokines etc. to the target tissue. With this step, the specialized immune response is initiated.

Innate and adaptive immunity

Innate immunity is inborn in all metazoans to defend the organism against invading pathogens like bacteria, fungi and viruses. The innate immune response is characterized by unique features. Pathogens are recognized by receptors that are encoded in the germline. Those receptors have a broad specificity for pathogen associated molecular patterns (PAMPs) like polysaccharides and polynucleotides and differ little from one pathogen to the other, but are not found in the host. The innate response can be mounted immediately without any delay, but as a consequence there is no memory evolving after an encounter with a specific pathogen. Macrophages, dendritic cells and epithelial cells have a set of transmembrane receptors that recognize different types of PAMPs. These are called Toll-like receptors (TLRs) because of their homology to receptors first discovered and named in Drosophila. In macrophages and dendritic cells, the pathogen is exposed to the TLRs when it is inside the phagosome. Which TLR(s) it binds to will determine what the response will be. In this way, the TLRs identify the nature of the pathogen and turn on an effector response appropriate for

Table 1: TLR Recognition of microbial Components

Microbial components	***Species***	***TLR***
Bacteria		
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	Mycoplasma	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
LTA	Group B Streptococcus	TLR6/TLR2
PG	Gram-positive bacteria	TLR2
Porins	Neisseria	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
ND	Uropathogenic bacteria	TLR11
Fungus		
Zymosan	Saccharomyces cerevisiae	TLR6/TLR2
Phospholipomannan	Candida albicans	TLR2
Mannan	Candida albicans	TLR4
Glucuronoxylomannan	Cryptococcus neoformans	TLR2 and TLR4
Parasites		
tGPI-mutin	Trypanosoma	TLR2
Glycoinositolphospholipids	Trypanosoma	TLR4
Hemozoin	Plasmodium	TLR9
Profilin-like molecule	Toxoplasma gondii	TLR11
Viruses		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Envelope proteins	RSV, MMTV	TLR4
Hemagglutinin protein	Measles virus	TLR2
ND	HCMV, HSV1	TLR2
Host		
Heat-shock protein 60, 70		TLR4
Fibrinogen		TLR4
ND = not determined	adapted from [2]	

dealing with it. These signaling cascades lead to the expression of various cytokine genes. Mammals have 12 different TLRs each of which specializes in a subset of PAMPs (see Table 1).

The adaptive immune response is the more advanced and specialized arm of immunity and is found in vertebrates only. First of all, the receptors of adaptive immunity are generated randomly (gene rearrangement in B and T cell receptors, somatic hypermutation in B cells) and have a very narrow specificity for a small fraction of a molecule (epitope). As only a small fraction of cells will have a receptor for a certain antigen and those cells will need to undergo clonal expansion, the adaptive immune response develops slower (3-5 days) than the innate immune response. This specific antigen response comes with one major benefit, 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 / pathogen.

Immunological tolerance and autoimmunity

The immune system ensures the functional integrity and is the body`s protection against infection and disease. Several mechanisms are known to control the protection of reactivity against own tissues and proteins, including thymic deletion of autoreactive T cells by negative selection and the induction of anergy in the periphery [3]. This immunological paradigm is known as the *self versus non-self model*.

Central tolerance mechanisms that are designed to purge self-reactive T cells from the immune system are not totally effective. Autoreactive T cells are a normal component of the peripheral T cell repertoire. The immune system must use various control mechanisms to prevent generalized autoimmunity. This includes induction of T cell apoptosis or anergy, diversion of effector T cell phenotype and the influence of regulatory T cells [4]. The tolerance for “self” can be broken by mechanisms that are not all known till now. Many ways have been suggested for this phenomenon, such as sequence homologies between bacterial or viral proteins and self-proteins, shared antigens between foreign and

host that induce common T cell and and antibody reactivity or internal dysregulation of immunoregulatory processes without the participation of infectious agents; a process called *molecular mimicry*.

Autoimmune diseases can be roughly divided by their autoantigen targets into organ specific and systemic diseases. Organ specific diseases are for example type I diabetes mellitus, multiple sclerosis, goodpasture`s syndrome and systemic diseases include systemic lupus erythematosus, rheumatoid arthritis, antiphospholipid syndrome and scleroderma.

There are several obstacles with the view of self non-self discrimination by the immune system, as many observations can not be explained by it, like for instance transplant rejection, feto-maternal tolerance, development of autoimmunity or the need for adjuvants to elicit an immune response.

An alternative model has been suggested by Polly Matzinger, immunity is not designed to discriminate self from non-self but to recognize danger-associated molecular patterns (DAMPs), irrespective of their nature [5, 6]. By that view, immunity evolved as a system to recognize tissue damage rather than foreignness. Supporting this, endogenous ligands of PRRs have been found that have the ability to license APCs for T cell activation independent of infection [7]. DAMPs or danger signals can be DNA, RNA, heat shock proteins (HSPs), IFNα (mostly produced by virus infected cells), IL-1β and breakdown products of hyaluron (from damaged vessels), which are all recognized by Toll like receptors (TLR) expressed on the surface or inside the cell.

Animal models of human autoimmune diseases

In order to study and unravel disease mechanisms underlying human autoimmune diseases, scientists have created animal (especially mouse) models to find out more details of certain pathologies, which can then be translated into new therapies or treatments for humans. The mouse has a short reproduction time and since the arrival of gene knock out techniques, research with this animal has provided major insights into a vast array of diseases, developmental processes and physiological mechanisms. The best known and studied

autoimmune model of a human disease is EAE - experimental autoimmune encephalomyelitis, which is used to gain inside into human Multiple Sclerosis. Other animal models of autoimmunity are for instance EAU – experimental autoimmune uveitis, EAM – experimental autoimmune myocarditis, CIA – collagen induced arthritis or EAMG – experimental autoimmune myasthenia gravis. Some mouse strains show a spontaneous development of an autoimmune disease, like NZB/NZW F1 mice that get symptoms of Systemic lupus erythematosus when they reach a certain age, or the NOD mouse, an ideal model for insulin-dependent diabetes mellitus. Other models can only be induced by an active immunization with a strong adjuvant and the protein or peptide of interest, to which an autoimmune response is desired. Like in humans, different inbred mouse strains have defined major histocompatibility complex (MHC) - molecules on their cell surfaces, with the consequence that many autoimmune diseases can only be induced in one or a few strains (eg. EAE in the C57BL6 strain, EAM in the BALB/c strain, EAMG in the SJL strain). In the following paragraph some of the findings on cytokine responses in inflammatory (autoimmune) processes are described in detail. Many of the results in this field were obtained from experiments with the described mouse models of human pathologies.

A changing hypothesis – Th1 - Th2 - Th17

For over 35 years CD4 T cells (helper T cells) were divided into two functional classes; Th1 were thought to induce tissue damage and Th2 to drive antibody-mediated responses, especially in allergy. Each subset was assigned a distinct pattern of cytokines. The most important Th1 cytokine was Interferon γ, while Interleukin-4 was seen as the master Th2 cytokine. The transcription factors T-bet and GATA-3 were shown to regulate either Th1 or Th2 identity of CD4 T cells [8, 9]. In the last 4-5 years this idea had to be revised after experimental evidence was obtained that did not fit with the old Th1/Th2 concept (reviewed in [10]. The major flaw of the Th1/Th2 hypothesis became evident when experiments with IFNγ (or IFNγ receptor) deficient mice and blocking of IFNγ

showed the exact opposite effects than predicted. In two models of autoimmune diseases, EAE as well as EAM, the disease worsened dramatically in absence of IFNγ or its receptor [11-13].

A new T helper subset was found to be a major player in tissue damage and inflammation and is characterized by the production of IL-17 [14]. The transcription factor RORγT is the critical component for the identity of these T cells [15]. Transforming growth factor β and IL-6 were identified as the cytokines needed to initiate such an IL-17 producing CD4 T cell population [16]. The cytokine IL-23, consisting of a p40 subunit shared with IL-12 and a unique p19 subunit, was shown to be crucial for the maintenance of the IL-17 producing T cell subset [17-19]. IL-27, a member of the IL-12/IL-23 family was found to have a suppressive role on the population of IL-17 expressing T helper cells [20].

TGF β, a cytokine previously mainly known for its importance to downregulate immune responses, is now intriguingly found to have a dual role; acting immunosuppressive through Foxp3+ regulatory T cells and acting proinflammatory by advancing the generation of Th17 cells [21]. The current concept of the T helper subsets and their relations is summarized in the following figure.

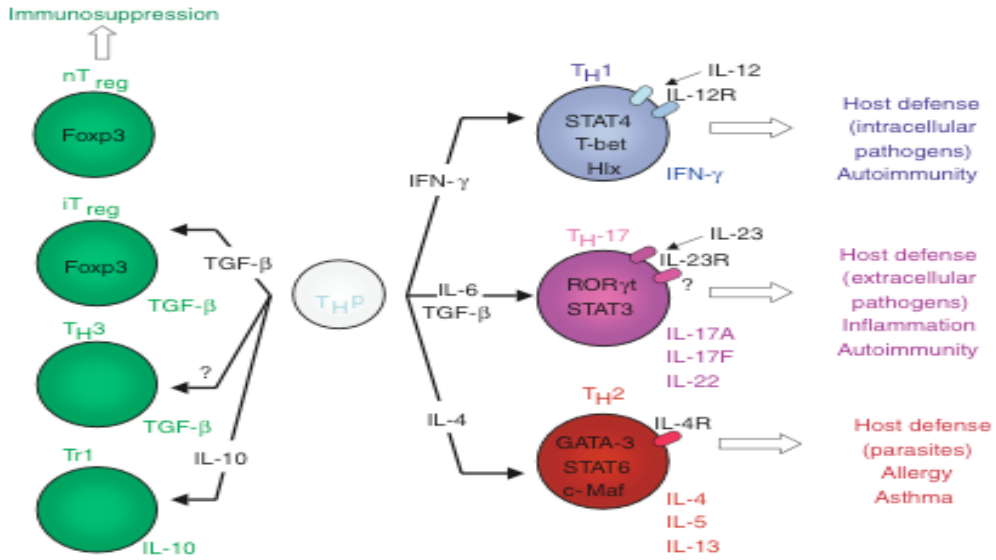


Figure 1 Differentiation of CD4+ T cell lineages. Peripheral naive CD4+ T cell precursor cells (T_Hp) can differentiate into three subsets of effector T cells (T_H1, T_H2 and T_H-17) and several subsets of T_{reg} cells, including induced T_{reg} cells (iT_{reg}), Tr1 cells and T_H3 cells. Naturally occurring T_{reg} cells (nT_{reg}) are generated from CD4+ thymic T cell precursors. The differentiation of these subsets is governed by selective cytokines and transcription factors, and each subset accomplishes specialized functions.

from: Bettelli E, Oukka M, Kuchroo VK. Th17 cells in the circle of immunity and autoimmunity, Nat Immunol 2007; 8:345-50 [21]

A major role for IL-17 has been described in various models of immune-mediated tissue damage, including organ-specific autoimmunity in the brain, heart, synovium and intestines, allergic disorders of the lung and skin, and microbial infections of the intestines and the nervous system [17, 19, 22-30].

The role of IFN γ and IL-17 in two inflammatory diseases

In order to gain insight into this newly described Th17 subset and its implications in inflammation, I chose to study the roles of IFN γ and IL-17, their regulation and possible opposing roles in two disease models that were already established in our laboratory. To obtain a differential picture of inflammatory pathways, one part of the research was conducted in an autoimmune setting, whereas the second part dealt with a graft versus host transplantation model that affects the lung. This way I was hoping to identify common mechanisms that would be

comparable in two unrelated inflammation states. The first part of the work is performed in the mouse model for the human pathology of myocarditis and dilated cardiomyopathy; experimental autoimmune myocarditis. The second part was conducted in a bone marrow transplantation model (BMT), which shows the development of severe lung inflammation, translating to a pathology named idiopathic pneumonia syndrome, a major complication in humans with graft versus host disease after BMT.

Autoimmunity and heart diseases

In developed countries, ischemic heart disease represents the most common cause of heart failure. In young patients under the age of 40 and children, however, most cases of heart failure result from dilated cardiomyopathy or myocarditis [31]. Clinical and epidemiological data suggest that many cases of dilated cardiomyopathy also evolve from myocarditis [31-33]. 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" [34, 35].

Worldwide, infection with the parasitic protozoan Trypanosoma Cruzi (Chagas disease), which is endemic in Southern America, is the leading cause of myocarditis [36]. In Europe and North America, Enteroviruses, such as Coxsackievirus B3 (CVB3) and to a lesser extent Adenovirus, have been suggested as the most important microorganisms inducing inflammatory heart disease [31, 37, 38] [39]. Other common cardiotropic microorganisms include Cytomegalo- [40], Parvo- [41], Hepatitis C- [42], Human Immunodeficiency- [43] and Epstein-Barr virus [44]. In addition, recent findings suggest that bacteria such as Chlamydia pneumoniae and Borrelia burgdorferi might play a yet underestimated role in the development of heart failure following myocarditis [39].

Non-infectious myocarditis denotes cardiac inflammation with no evidence of infection, for example in the context of autoimmune diseases, drug-induced hypersensitivity, neoplasia and/or other systemic disorders [31].

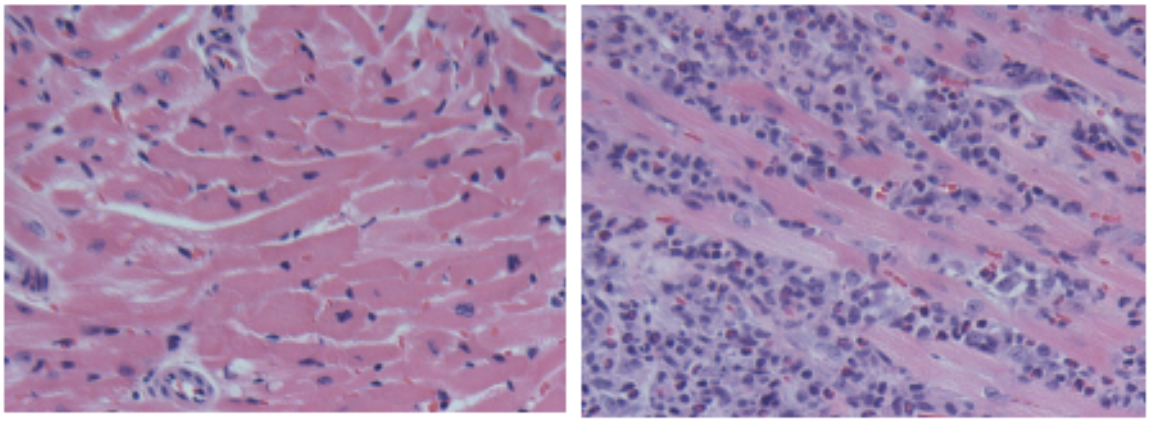
Virus infections directly contribute to the destruction of cardiac tissue by cleaving cytoskeletal dystrophin which leads to a disruption of the dystrophin-glycoprotein

complex [45]. It is hypothesized that this mechanism is responsible for enteroviral replication in the heart and development of virus associated chronic cardiomyopathy.

Interestingly, patients with dilated cardiomyopathy (DCM) frequently present autoantibodies against cardiac proteins such as myosin and the anti-adenine nucleotide translocator [46, 47]. Myocytes isolated from patients with diagnosed myocarditis and idiopathic DCM often display increased levels of MHC class I and class II proteins [47], and myocarditis commonly develops in patients with systemic autoimmune disease [31]. Peripheral blood lymphocytes from DCM patients can adoptively transfer disease to immunodeficient SCID mice [48]. 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 [49].

The mouse model experimental autoimmune myocarditis - EAM

Several animal models of myocarditis exist. Some models, such as Coxsackie virus B3 -induced myocarditis, attempt to replicate human myocarditis by examining the consequences of the host-pathogen interaction on disease progression [50]. Indeed, use of this model has uncovered an immunomodulatory role for the antiviral cytokine IFN γ in myocarditis [51]. Other models attempt to recreate the disease from the point of autoimmune induction. For example, the model of experimental autoimmune myocarditis (EAM) involves immunizing mice from susceptible strains with a peptide derived from the murine cardiac myosin alpha heavy chain (MyHCα) [52]. Mice develop severe myocarditis that reaches maximal severity at 21 days post-immunization. The immunological heart infiltrate consists predominantly of $CD11b^+$ macrophages, with lesser numbers of $CD4^+$ and $CD8^+$ T cells [53]. The induction of myocarditis has profound consequences on heart function; immunized mice display chronically impaired left-ventricular contractility and increased heart size [54].



OVA/CFA MyHC-α/CFA

While viral or bacterial infection can induce myocarditis, and while autoimmune mechanisms can promote chronic myocardial destruction, it remains to be determined how the two processes are linked. Studies using the EAM model have helped to address this question. One hypothesis postulates that heart-specific self-antigens may structurally mimic pathogenic ones, thereby leading the same T cells that cleared the initial infection to then attack the myocardium [53]. It is important to note that primed T cells do not require costimulation; antigen recognition alone is sufficient to induce effector responses. Further, heart-resident APCs from non-immunized mice present cardiac myosin-derived peptides in the context of MHC class II, and can induce the proliferation of myosin-specific T cell hybridomas *in vitro* [55]. It is therefore tempting to speculate that activated, pathogen-specific T cells that home to the heart are at the wrong place at the wrong time – that is, they are primed and able to respond to cardiac myosin.

The role of dendritic cells on deciding the fate of T helper cells

Whether a naïve T cell will become for instance a Th1 cell producing IFNγ, or a Th17 cell producing IL-17, depends largely on two factors, 1) the predominating cytokine milieu in the area of T cell priming and 2) the signalling events in and from the dendritic cell that will activate the T cell. IL-12, IL-23 and other factors that influence T cell effector fate are produced by DCs upon activation through

pattern-recognition receptors (PRRs), which bind components of microbes or viruses [56]. The importance of the dendritic cell in the induction of an immune response and its maturation status is nicely illustrated in animal models using antigen loaded DCs that are in vitro activated with innate stimuli like LPS and signalling through costimulatory molecules. DC immunization was shown to induce experimental autoimmune myocarditis, autoimmune diabetes, experimental autoimmune encephalomyelitis and recently autoimmune uveitis [54, 57-59].

Role of different cytokine subsets in EAM

The concept of $CD4^+$ T helper separation into Th1 and Th2 responses was a longstanding hypothesis in immunology. In this concept, Th1 responses were thought to be responsible for autoimmunity and Th2 bias was believed to protect from disease.

The prototypic Th1 cytokine IFN γ was long seen as the essential factor for the expansion and effector function of autoreactive $CD4^+$ T cells. Studies in human show mainly IFN γ producing Th1 cells in target organs of patients with organ-specific autoimmunity. Through cytokine knockout studies in mice this perception was proven to be wrong; IFN γ- and IFN γR- deficient mice developed severe myocarditis with increased mortality and signs of severe dilated cardiomyopathy compared to wildtype controls [13, 60].

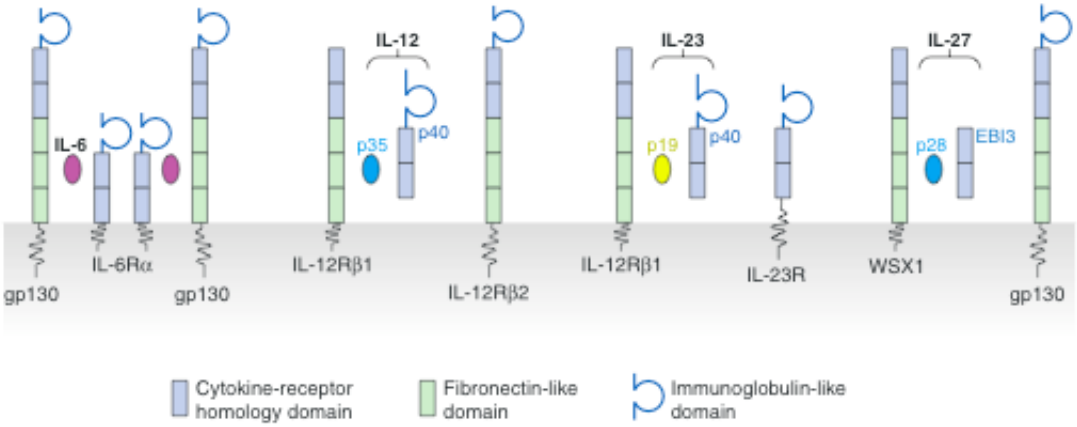
As for the Th2 master cytokine Interleukin-4, it was shown that IL-4 deficient and IL-4Rα deficient mice are susceptible to induction of EAM [22, 60, 61].

Interleukin-6 deficient mice were described to be protected from EAM [62], now knowing that IL-6 is essential for induction of an IL-17 response by its interplay with TGF-β [16].

IL-6 is part of a bigger cytokine family, consisting of IL-12, IL-23 and IL-27.

Two cytokines of this family, IL-12 and IL-23, share a common subunit IL-12p40 as well as a receptor molecule IL-12Rβ1. IL-12 as a link of innate to adaptive immunity [63], is crucial for induction of a Th1 response, while IL-23 is now known as the maintenance factor of an Th17 response [17]. In EAM, it was

previously shown that IL-12p40 deficient mice are protected from disease [60], while IL-12p35 deficient mice are susceptible to EAM and CVB3 induced myocarditis [22, 64].



from: Kastelein RB, Hunter CA, Cua DJ: Discovery and Biology of IL-23 and IL-27: Related But Functionally Distinct Regulators of Inflammation [65]

Graft versus host disease after bone marrow transplantation

For haematological malignancies, non-malignant chronic haematological diseases, cancer and autoimmune diseases, bone marrow transplantation is increasingly used as the most promising therapy in humans. Chances to find an exact HLA -match (an identical twin), are low, so often an allogeneic bone marrow transplantation is performed. Patients receiving a bone marrow transplantation undergo a strict preparation of chemotherapy and/or radiation to kill as many malignant cells as possible and to suppress the immune system of the patient to allow the donor cells to grow. This pre-treatment is called the "patient conditioning".

Graft versus host disease is a complication resulting from MHC (major histocompatibility complex) - fully- or partially- mismatched bone marrow transplantation. Three criteria have to be fulfilled for diagnosis of GVHD; 1) the graft must contain immunologically competent cells, 2) the host must possess important transplantation alloantigens that are lacking in the donor graft so that the host appears foreign to the graft and can therefore stimulate it antigenically, 3) the host itself must be incapable of mounting an effective immunologic

reaction against the graft, or it must at least allow for sufficient time for the latter to manifest its immunologic capabilities, ie, it must have the security of tenure [66]. Graft versus host disease can appear in an acute or chronic form, the acute form developing within 100 days following transplantation, whereas the chonic form can begin during or after three months post transplantation. Complications result in damage of skin, lung, gastrointestinal tract and liver, but other organs can be inflicted too.

The idiopathic pneumonia syndrome - IPS

The lung is a target of graft versus host disease in 30-50% of patients receiving allogeneic bone marrow transplantation including infections and pulmonary GVHD [67]. One pathological condition, namely idiopathic pneumonia syndrome (IPS), is characterized by the absence of an infectious agent and histological evidence for bronchiolitis, vaskulitis and interstitial pneumopathy. This noninfectious lung injury is associated with significant morbidity and mortality and responds poorly to standard therapeutic approaches. Possible etiological factors of IPS can include toxicity effects of chemoradiotherapy, occult pulmonary infections, but are mostly believed to appear through immunological dysregulation by i) enhanced proinflammatory cytokine and chemokine expression (mainly TNFα), ii) release of endogenous endotoxin (LPS), iii) donor-derived cellular effectors (alloreactive T cells, macrophages and monocytes as well as neutrophils) and iiii) through endothelial cell apoptosis or activation.

IPS can be observed early and late after stem cell transplantation. A recent study by Fukuda showed that patients receiving myeloablative conditioning before allogeneic BMT had significantly higher incidence of IPS than patients receiving nonmyeloablative conditioning, and that the severity of GvHD positively correlated with the frequency of IPS [68]. These findings suggest that inflammatory tissue injury associated with myeloablative conditioning and GVHD may be predisposing factors for the development of IPS.

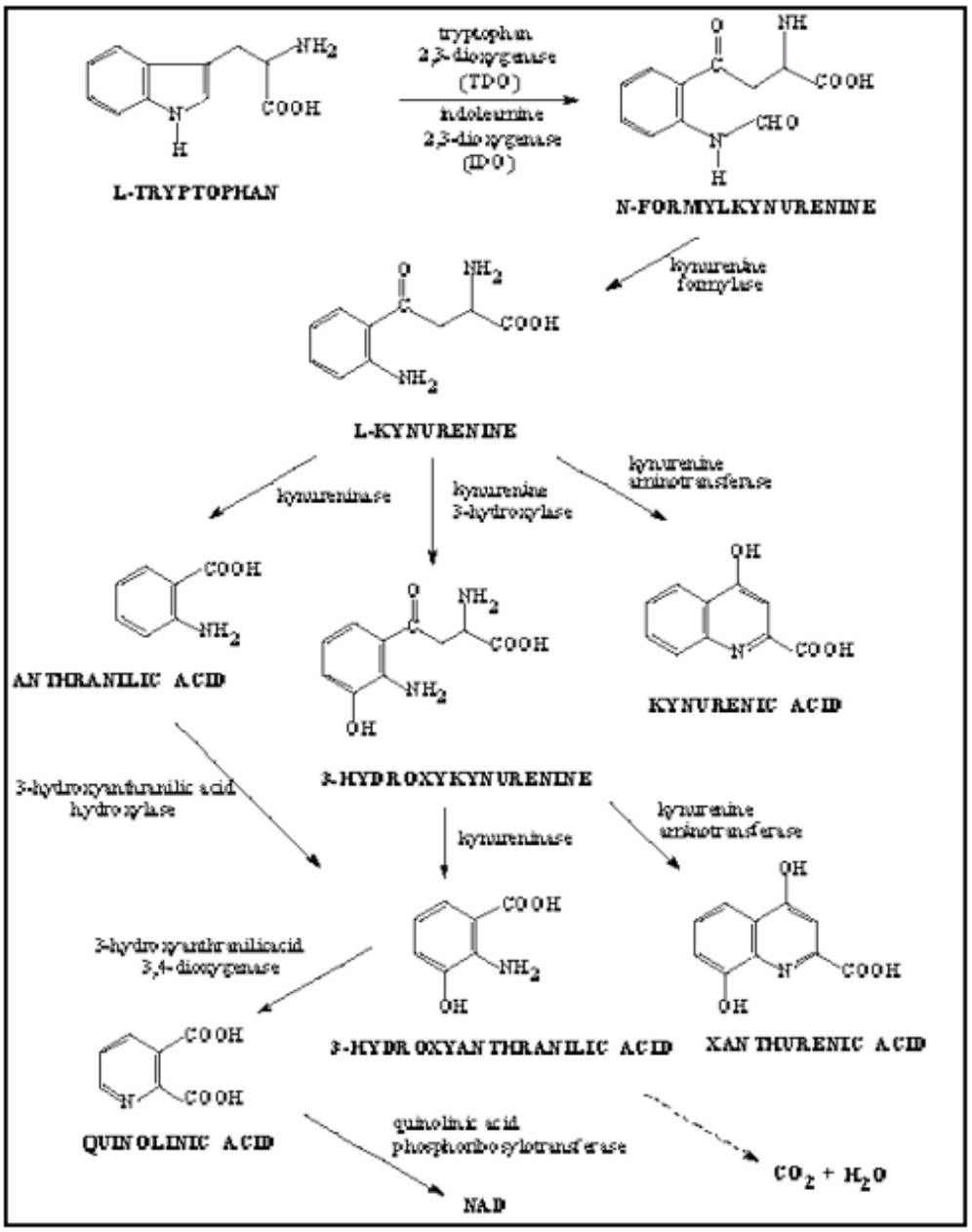
Several animal models for IPS have been established so far, and it has been shown that donor T cells are critical for disease development [69-71].

IFNγ dependent factors indoleamine 2,3-dioxygenase and nitric oxide as immunomodulators

Inducible indoleamine 2,3-dioxygenase (IDO) is a heme enzyme, which can degrade tryptophan, an essential amino acide. After food intake tryptophan is made available for protein synthesis through the liver and secreted to blood serum. Tryptophan is by 70% degraded in the liver by the kynurenine pathway through the enzyme tryptophan 2,3-dioxygenase (TDO). Outside the liver, IDO is the limiting enzyme of the kynurenine pathway [72]. During the degradation of tryptophan many metabolites are formed, which all have different effects and functions. IDO can be induced by IFNγ in fibroblasts, monocytes, macrophages and dendritic cells, this induction can be enhanced synergistically by TNFα and LPS [73-76]. IDO has immunomodulatory properties and was identified up until now in pregnancy, transplantation, asthma and tumor models [77-82]. In pregnancy, allogeneic fetuses were rejected by T cells, when IDO was systemically inhibited. In T cell deficient $Rag^{-/-}$ mice this rejection did not occur [78]. Allogeneic T cell responses can be inibited by IDO expressing dendritic cells [83]. The mechanism of the IDO mediated immune suppression can be explained by the tryptophan starvation model of Pfefferkorn [84]. T cells stay in the late G_1-phase of the cell cycle, in spite of antigen presentation and costimulation. Additionally, T cells cultured in low tryptophan medium show a higher sensitivity for CD95L induced apoptosis [85].

Nitric oxide is a gas and a free radical, there are three enzymes important for its synthesis; inducible nitric oxide synthase (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). In immunological processes NO plays a major role by killing bacteria and tumor cells and in apoptosis through cellular stress. Both oxidative and nitrosative stress are thought to play important roles in tissue injury related to GVHD and IPS [86-89]. Oxidative stress usually occurs as a byproduct of irradiation and some cytotoxic drugs used in conditioning and is generated during inflammatory responses. Nitrosative stress is more often associated with inflammation, which occurs after myeloablative conditioning as well as during

GVHD. An important role for NO in GVHD and IPS is certain, but it is not really clear, if that effect is detrimental or beneficial [90].



from Pawlak,D.,A.Tankiewicz, and W.Buczko, *Kynurenine and its metabolites in the rat with experimental renal insufficiency.* J Physiol Pharmacol, 2001. **52**(4 Pt 2): p. 755-66.[91]

Aim of the thesis

Experimental autoimmune myocarditis is the animal model for the human conditions of myocarditis and dilated cardiomyopathy. For many years autoimmune disease models in mice were categorized into Th1 or Th2 mediated diseases, based on their cytokine patterns. Conflicting data existed when EAM and other autoimmune models were induced in mice lacking critical cytokines of either Th1 or Th2 pathways. Mice deficient for IFNγ or its receptor were previously found to develop severe myocarditis. IL-4 ligand and IL-4 receptor deficient mice are also susceptible to disease. One aim of the thesis is to identify the main pathogenic cytokine involved in autoimmune myocarditis. To address this question we use mice deficient in the transcription factor T-bet (Tbx21, T box expressed in T cells), the most important transcription factor for the differentation of CD4 T helper cell subset 1. We speculate that the pathogenesis of autoimmune myocarditis requires function of the distinct, recently described IL-23/IL-17 effector axis. Further, we will study the role for T-bet in $CD8^+$ T cells.

In a second project the roles of IFNγ and IL-17 are analyzed in a newly established animal model of idiopathic pneumonia syndrome (IPS). IPS occurs as a major complication of graft versus host disease after bone marrow transplantation. Our hypothesis is that IPS is a CD4 T cell mediated disease that develops a severe phenotype when those CD4 T cells are deficient in IFNγ; mice receiving wt CD4 T cells develop only minor lung infiltration and inflammation. We want to find out if the severe phenotype is partly due to increased levels of IL-17 in the lung. Additionally, IFNγ -induced factors nitric oxide and indoleamine 2,3-dioxygenase (IDO) will be addressed, as they are known to have immunosuppressive properties that might be ineffective, when IFNγ deficient T cells are transplanted. It is known that IL-17 can further increase the severity of inflammation by attraction of other cell types to the site. We therefore want to study the role of neutrophils and antigen presenting cells in the lung during the induction of lung inflammation.

Part 1.

T-BET NEGATIVELY REGULATES AUTOIMMUNE MYOCARDITIS BY SUPPRESSING LOCAL PRODUCTION OF INTERLEUKIN-17

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Abstract

Experimental autoimmune myocarditis mirrors postinfectious heart disease, the most common cause of dilated cardiomyopathy in humans. Here we report the surprising finding that mice lacking T-bet, a T-box transcription factor required for Th1 cell differentiation and IFNγ production, develop severe autoimmune heart disease compared to *T-bet*$^{+/+}$ control mice. Genetic experiments in *T-bet*$^{-/-}$ *IL-4*$^{-/-}$ or *T-bet*$^{-/-}$ *IL4Rα*$^{-/-}$ double mutant mice and transfer of heart-specific Th1 and Th2 cell lines showed that autoimmune heart disease can develop independently of Th1 or Th2 polarisation. While Treg and dendritic cell functions appeared normal in *T-bet*$^{-/-}$ mice, loss of T-bet in the CD8^{+} compartment exacerbated the development of autoimmune heart disease. Intriguingly, whereas peripheral *T-bet*$^{-/-}$ CD8^{+} T cells retain their capacity to produce IFNγ, this function was completely lost in heart infiltrating CD8^{+} lymphocytes. These data identify a novel role of T-bet in the regulation in autoimmunity via the control of CD8^{+} T cell functions.

Introduction

Cardiovascular disease is a leading cause of morbidity and mortality in the Western world. Dilated cardiomyopathy (DCM), often resulting from Coxsackievirus B2 and Chlamydia-triggered myocarditis, may be present in up to one quarter of all cases of heart failure [39, 49]. Notably, many of the affected individuals develop heart-antigen-specific autoantibody responses [46], and immunosuppressive therapy can improve heart function in DCM patients, who display no evidence of viral or bacterial genomes in heart biopsies [44]. Interestingly, peripheral blood lymphocytes from patients with DCM could adoptively transfer disease to SCID mice lacking B and T cells [48].

Experimental autoimmmune myocarditis (EAM) is a mouse model of postinfectious myocarditis and cardiomyopathy [92]. EAM can be induced in susceptible mouse strains by immunization with a self-peptide derived from the myosin alpha heavy chain (MyHC-α). EAM is a $CD4^+$ T cell mediated disease [54, 92, 93],but the relative contributions of the $CD4^+$ Th1 and Th2 subsets are unclear [60, 94, 95]. The majority of immunological infiltrates in EAM are macrophages, suggesting that Th1 signals predominate. Signaling through the key Th2 cytokine IL-4 is dispensable for the development of EAM [60]. Furthermore, the p40 subunit of the Th1-driving cytokine IL-12 and the $\beta 1$ receptor subunit of the IL12R are essential for disease progression [60, 95]. However, in vivo IL-4 blockade has been shown to reduce disease severity [61]. At the same time, loss of either IFNγ or its receptor results in increased disease severity, implying that this key Th1 cytokine is a negative regulator of EAM [13, 60, 95].

As IFNγ signaling may not be absolutely required for the development of Th1 responses, we tested susceptibility of mice lacking T-bet, a T-box transcription factor essential for Th1 lineage differentiation [8, 96]. *T-bet*$^{-/-}$ $CD4^+$ T cells display a profound defect in IFN-γ production in vitro, and T-bet expression is critical for

the development of inflammatory autoimmune diseases such as experimental autoimmune encephalomyelitis, Crohn's disease, type 1 diabetes, and atherosclerosis [97-101].

Here we show that in marked contrast to other autoimmune models, mice lacking T-bet develop severe EAM. MyHC-α-specific $CD4^+$ T cells trigger autoimmune myocarditis regardless of Th1 or Th2 commitment. Analysis of *T-bet*$^{-/-}$ *IL12Rβ1*$^{-/-}$ and *T-bet*$^{-/-}$*IL12p35*$^{-/-}$ mice and antibody depletion experiments showed that IL-23 and IL-17 are critical for EAM pathogenesis. Loss of T-bet results in increased IL-17 production in the inflamed heart, and this tissue-specific repression depends on $CD8^+$ T cell-mediated bystander suppression. Our data indicate that T-bet expressed in the $CD8^+$ T cell compartment is a negative regulator of autoimmune heart disease.

Materials and methods

Mice

T-bet$^{-/-}$ mice have been described previously [96, 102] and were a gift from Dr. Laurie Glimcher (Harvard School of Public Health, Boston MA). *IFNγR*$^{-/-}$, *IL12Rβ1*$^{-/-}$, *IL12p35*$^{-/-}$ and CB17.SCID mice were purchased from the Jackson Laboratory. All mice were backcrossed to the BALB/c strain for >10 generations. Mice were used at 6-8 weeks of age for immunizations. All experiments were conducted in accordance with Swiss federal laws and institutional guidelines.

Bone marrow dendritic cell culture

Bone marrow cells were isolated from mice at 6-8 weeks of age. Cells were cultured in RPMI with 10% FCS (Gibco), as well as penicillin + streptomycin, L-glutamate and 2-mercaptoethanol (all from Sigma) (hereafter referred to as RPMI-complete). Media were supplemented with 200 U GM-CSF/mL (PeproTech). $2x10^6$ cells were cultured on non-tissue culture coated Petri dishes (Falcon, BD) for 8-12 days. Media was changed on d3, d6, d8 and d10 of culture.

Immunization protocols

Mice were immunized with 100 µg of the myosin-alpha heavy chain peptide (MyHC-$\alpha_{614-634}$) Ac-SLKLMATLFSTYASAD-OH emulsified 1:1 in PBS/CFA (1 mg/mL, H37Ra, Difco Laboratories) as described [62]. For some experiments, IFA (Difco Laboratories) substituted for CFA. For DC immunization, BM-DCs were pulsed for 1 hour with 10 µg/mL MyHC-α peptide, and stimulated for another 2 hours with 0.1 µg/mL LPS (O111:B4, E.coli LPS ultrapure, List Biological Laboratories) and 5 µg/mL anti-CD40 (BD Pharmingen) [54]. Recipient mice received $2.5x10^5$ pulsed and activated BM-DCs i.p. for three consecutive days and were sacrificed 10 days after the first injection. For some DC experiments, culture supernatant was harvested for ELISAs. In IL-17 depletion experiments, mice were immunized with 50 μg MyHC-α/CFA, injected with either anti-mouse-IL-17 (R&D Systems) or PBS on d9, 12 and 15, and sacrificed on d17.

Histopathology, immunohistochemistry and autoantibody detection.

Myocarditis was scored on hematoxylin/eosin stained sections using grades from 0 to 4: 0 - no inflammatory infiltrates; 1 - small foci of inflammatory cells between myocytes; 2 - larger foci of more than 100 inflammatory cells; 3 - more than 10% of a cross-section involved; 4 - more than 30% of a cross-section involved [54]. At least 3 levels of sectioning were scored per mouse, with each level being 100-200 μm apart. The severity score for each heart corresponded to the highest score observed for any level. For immunohistochemistry, OCT™ (Sakura Finetek) embedded frozen heart sections were fixed in acetone and then processed for antibody staining according to standard protocols. The following antibodies were used: anti-CD4 (YTS 191), anti-CD8 (YTS 169) (BD Pharmingen), and anti-CD68 (FA-11) (Serotec). Autoantibody-responses were determined by ELISA as described [54], using alkaline phosphatase-labeled goat anti-mouse IgM and IgG subclass antibodies (Southern Biotechnology Associates).

T cell functions.

$CD4^+$ and $CD8^+$ cells were purified using magnetic beads ($CD4^+$ and $CD8^+$ T cell isolation kits; Miltenyi Biotech). 5-10x10^4 $CD4^+$ T cells were restimulated for 72 hours on 2 µg/ml MyHC-α peptide pulsed, irradiated (20 Gy) syngeneic splenocytes (2x$10^{5)}$. In some experiments, $CD4^+$ or $CD8^+$ T cells were stimulated with either 1 µg/mL each of plate-bound anti-CD3 and anti-CD28 (BD Pharmingen), or with 1 µg/mL soluble anti-CD3 on 2x10^5 irradiated syngeneic splenocytes. For regulatory T cell assays, $CD4^+$ T cells were sorted into $CD4^+CD25^-$ (Teff) and $CD4^+CD25^+$(Treg) populations using a high-speed cell sorter (FACSAria, BD Pharmingen). 5x10^4 of Teff cells were co-cultured with Treg cells on 2x10^5 irradiated syngeneic splenocytes, in the presence or absence of 1 µg/mL soluble anti-CD3 (BD Pharmingen) for 72 hrs. ^{3}H-thymidine incorporation was measured as a read-out for proliferation responses. For some analyses of heart-infiltrating T cell cytokine production, cells were either stimulated in the presence of soluble anti-CD3/ anti-CD28, or cultured for 72 hours in supernatants from mature, LPS stimulated BMDC cultures (conditioned medium). IFNγ levels in conditioned medium were always below the detection limits of our ELISA kits (<50 pg/ml). Cytokine levels were measured using commercially available Quantikine ELISA kits for detection of IFNγ, TGF-β1, TNFα, IL-4, IL-6, IL-10, IL-12p40, IL-12p70 and IL-17 (R&D Systems). For analysis of *in vivo* expansion, $CD8^+$ T cells were incubated for 10 minutes with 10 µM CFSE (Molecular Probes) at 37°C prior to injection. All T cells were cultured in RPMI-complete media.

MyHC-α-specific Th1 or Th2 $CD4^+$ lines and adoptive transfer.

$CD4^+$ T cells were purified from diseased *T-bet*$^{+/+}$ or *T-bet*$^{-/-}$ mice and cultured with irradiated (20 cGy) splenocytes at a 1:2 ratio in the presence of 2 µg/mL MyHC-α for 7 days. Cells were then washed and rested in the presence of 10 U/mL of recombinant IL-2 for additional 7 days. This pulse/rest cycle was repeated at least three times. Finally, $CD4^+$ T cells were restimulated for 4 days

before i.p. injection of $1x10^7$ $CD4^+$ T cells per syngeneic recipient. For reconstitution of SCID mice, naïve $CD4^+$ and $CD8^+$ were isolated and $5x10^6$ $CD4^+$ and $2.5x10^6$ $CD8^+$ were injected i.p. into same-day-immunized recipients.

FACS analysis.

Cell suspensions were stained using fluorochrome-conjugated mouse-specific antibodies against CD3, CD4, CD8α, CD8β, Qa-1^b, CD11c, CD80, MHC class II (I-A^d), CD25 (all BD Pharmingen). Prior to intracellular staining, cells were restimulated for 4 hours with 20 ng/ml phorbol myristyl acetate (PMA) and 1µM ionomycin in the presence of 10 µg/ml Brefeldin A (Sigma). Intracellular staining was conducted on PMA-Ionomycin activated cells that were fixed with 2% paraformaldehyde for 20min at RT, after fixation cells were permeabilized with 0.1% Saponin in FACS-Buffer (PBS, 1% FCS, 2mM EDTA). Appropriate antibodies for intracellular cytokines (IFNγ, IL4, IL6, IL17, TNFα) were added and incubated for 20min at RT in the dark, cells were washed once in permeabilization buffer and finally resuspended in FACS-Buffer. Samples were analyzed on a FACSCalibur (BD Pharmingen) or CyAn (DAKO) using FlowJo (TreeStar) software.

Cytotoxicity assays. *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ (H-2^d) BALB/c mice were primed by i.p. injection of allogeneic (H-2^b) spleen-derived DCs. $CD8^+$ T cells were isolated from primed mice using a biotinylated anti-CD8β antibody (BD Pharmingen) and anti-biotin microbeads (Miltenyi). After *in vitro* restimulation on irradiated H-2^b splenocytes, cytotoxic $CD8^+$ T cells were used as effector cells in a Calcein release assay using calcein loaded EL-4 (H-2^b) target cells. Spontaneous calcein release (SR) was determined by addition of medium; maximal release (MR) by addition of lysis buffer to target cells. Calcein release was measured after 2 hours of incubation in a fluorescence multiwell plate reader (SpectraMax Gemini XS; Molecular Devices). Percent specific lysis was calculated as (Sample release-SR)/(MR-SR)x100%.

Heart digestion buffer: The buffer consists of the following chemicals;
120 mM NaCl, 5mM $CaCl_2$, 5.4 mM KCl, 1.2 mM NaH_2PO_4, 20 mM $NaHCO_3$, 5.6 mM glucose, 5 mM taurine, 1.6 mM $MgCl_2$, 10 mM 2,3-butanedione monoxime (all Sigma). The buffer is sterile filtrated through a 0.22μm filter. Collagenase D (Worthington Biochemicals Corporation) was used at a concentration of 0.895 mg/ml and Protease IV (Sigma) at a concentration of 0.5 mg/ml.

Isolation of heart-infiltrating cells. Mice were injected i.p. with 1000 units Heparin at least 10min before euthanasia with CO_2. The heart was quickly removed from the chest with great care to keep all vessels intact and placed on ice in heart digest buffer. The remaining lung and thymus tissue was removed with fine scissors and the aorta was identified. The pump was started with a flow rate of ca. 0.6ml/min (only heart digest buffer) and the aorta of the heart pulled over the canule, fixated first with a clip and later with suture thread on the canule that is connected with the pump (Langendorff machine from Mouse Specifics Inc., Boston, MA). When the heart was visibly drained from blood, the buffer was exchanged for enzyme containing heart digest buffer (6ml) and the flow rate subsequently increased to 2.0-2.5 ml/min. The flow through was collected in a new tube, later the heart was placed in a petridish together with the flow through solution. The heart was cut with a razor blade into small pieces and tissue fragments were digested at 37°C for 30-45 min. The enzymes were quenched with PBS containing 10% FCS and tissue suspensions were passed sequentially through 70µm, 40µm cell strainers (BD Falcon) and finally through 15µm self-assembled strainers (Sefar AG). For some assays, isolated infiltrating cells were purified on the basis of surface marker expression on MACS (Miltenyi) columns.

Statistics. The Mann-Whitney *U* test was used for the evaluation of severity scores. Dichotomous data were analyzed using Fisher's exact test. Proliferation responses and cytokine levels were compared using ANOVA and Student's *t*-

test. Statistical analysis was conducted using Prism 4 software (GraphPad Software).

Results

T-bet$^{-/-}$ mice develop severe myocarditis

IFNγ production characterizes Th1 cells, and IFNγ secreted from Th1 cells strongly activates macrophages and actively represses transcription of IL-4 [103]. Absence of IFNγ receptor signaling, however, does not exclude Th1 commitment in EAM because CD4$^+$ T cells from MyHC-α immunized *IFNγR*$^{-/-}$ mice were highly competent to produce IFNγ upon in vitro restimulation (Figure 1.).

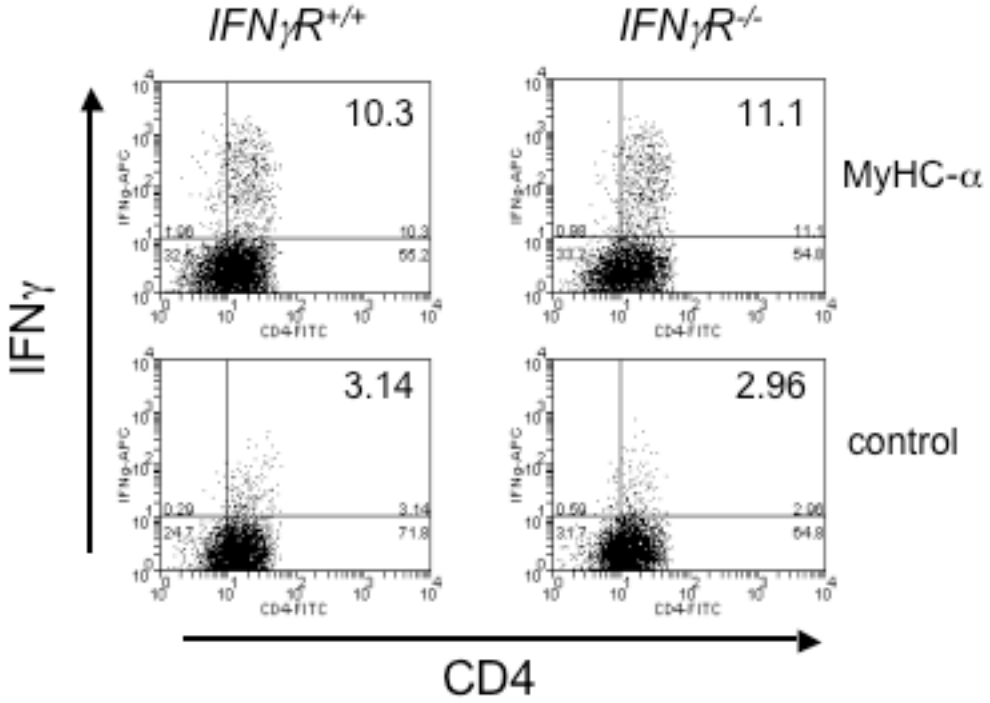


Figure 1. CD4+ from MyHC-α-immunized IFN-γR-/- mice produce IFNγ. IFNγR+/+ and IFNγR-/- mice (n = 3 each) were immunized, and CD4+ T cells were isolated 21 d later. IFNγ production was measured in CD4$^+$ splenocytes after 48 h of MyHC-α restimulation using intracellular staining and FACS analysis. Representative data is shown.

We therefore clarified the role of Th1 responses in EAM using mice genetically deficient for T-bet, a T-box transcription factor driving helper T cell differentiation into the Th1 cell lineage commitment [8]. Surprisingly, *T-bet*$^{-/-}$ mice

developed severe EAM mice after immunization with MyHC-α emulsified in CFA (Figure 2A and B, and Table 2), and also upon injection of activated MyHC-α loaded *T-bet*$^{+/+}$ bone marrow-derived DCs (BMDCs)(Table 2).

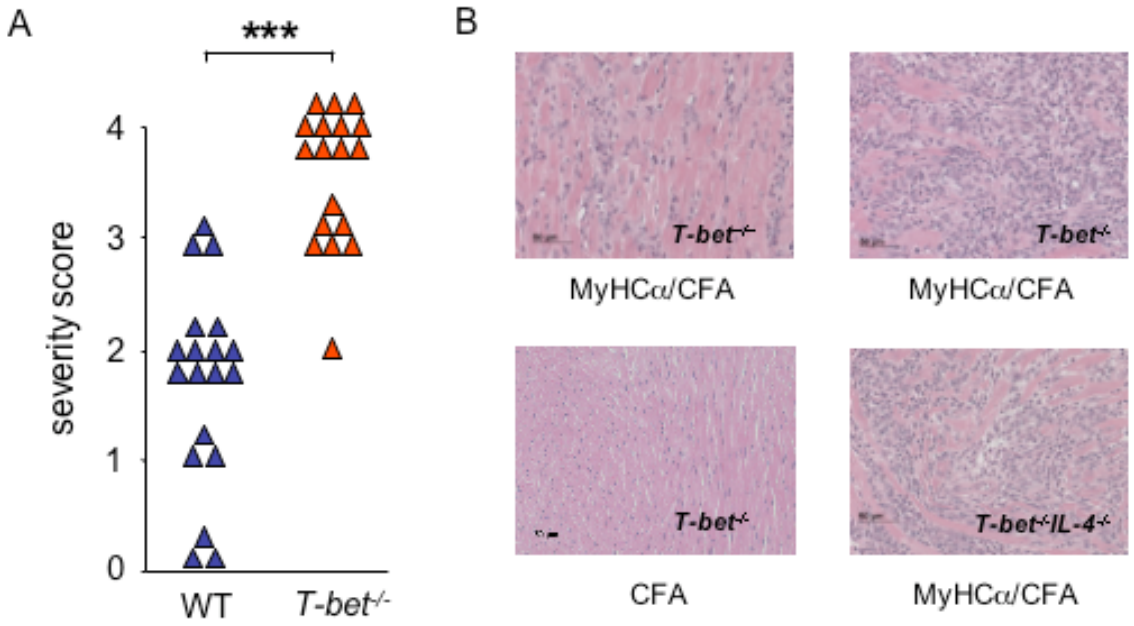


Figure 2. *T-bet*$^{-/-}$ mice develop EAM of increased severity: *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ mice were immunized with MyHC-α peptide in CFA and sacrificed 21 days later. (A) Severity scores of individual diseased *T-bet*$^{+/+}$ vs *T-bet*$^{-/-}$ mice, ***p<0.000001 (B) Hematoxylin/eosin stained sections from hearts of immunized mice. MyHC-α-immunized *T-bet*$^{+/+}$ (upper left), MyHC-α-immunized *T-bet*$^{-/-}$ (upper right), *T-bet*$^{-/-}$ immunized with CFA alone (lower left), and MyHC-α-immunized *T-bet*$^{-/-}$*IL-4*$^{-/-}$. 40X original magnification.

Inflammatory infiltrates in the hearts of immunized *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ mice consisted of granulocytes and mononuclear cells, including macrophages and lymphocytes. *T-bet*$^{-/-}$ hearts showed slightly greater eosinophilia and less fibrosis as compared with *T-bet*$^{+/+}$ hearts, suggesting a Th2 - biased response (not depicted). Immunohistochemical analysis, however, showed no apparent differences in relative numbers of CD68^{+} macrophages, CD4^{+} T cells, and CD8^{+} T cells between diseased *T-bet*$^{-/-}$ and *T-bet*$^{+/+}$ hearts (Figure 3). FACS analysis indicated that proportions of CD4^{+} versus CD8^{+} heart-infiltrating cells were also comparable in *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ mice (not depicted).

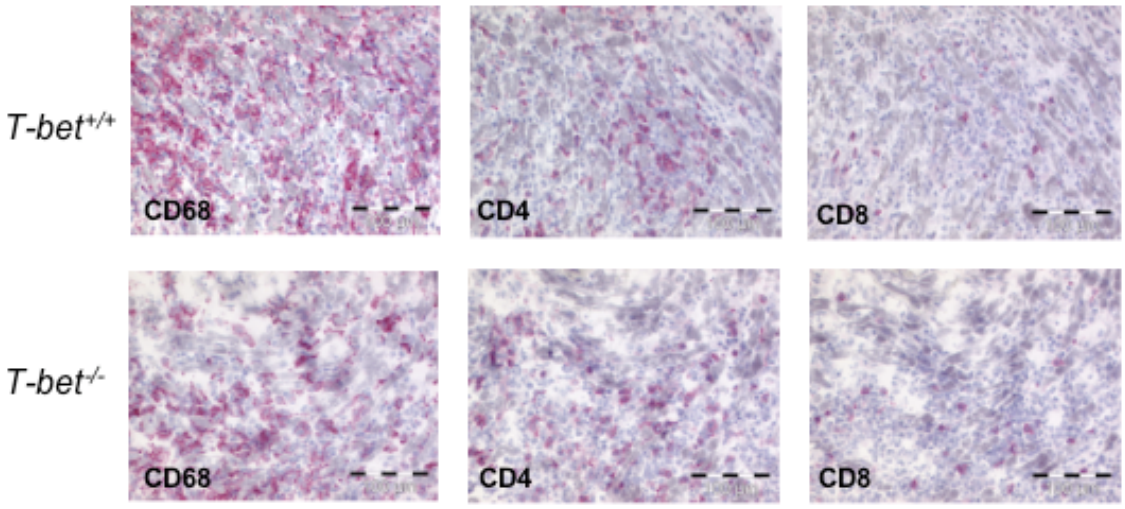


Figure 3. Characterization of heart infiltrating cells in *T-bet*$^{-/-}$ mice: Frozen heart sections from MyHC-α immunized *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ mice were stained to determine proportions of infiltrating macrophages (CD68, left panel) and lymphocytes (CD4, middle panel; CD8, right panel).

The increase in EAM severity observed in *IFNγ*$^{-/-}$ mice has been explained by impaired apoptosis of activated CD4^{+} T cells as well as by unrestricted expansion of CD4^{+}CD44^{high} T cells [104]. However, we saw no evidence of impaired cell death in CD4^{+} T cells isolated from MyHCα-immunized *T-bet*$^{-/-}$ mice, nor did we observe any increase in the numbers of peripheral CD4^{+}CD44^{high} cells in these mice (not depicted). Furthermore, most *T-bet*$^{-/-}$ mice, but none of the *IFN-γR*$^{-/-}$ animals, slowly recovered from disease within 2 mo after immunization (not depicted), suggesting that loss of T-bet does not mimic IFNγ deficiency in EAM.

T-bet$^{-/-}$ *mice develop an autoreactive Th2 response*

Consistent with the idea of CD4^{+} T cell-mediated disease and enhanced disease severity in *T-bet*$^{-/-}$ mice, in vitro recall responses of *T-bet*$^{-/-}$ CD4^{+} T cells to MyHC-α were increased in comparison with *T-bet*$^{+/1}$ controls (Fig. 4 A). To exclude the possibility that T-bet does not act as a Th1 transcription factor in BALB/c mice, we measured IFNγ and IL-4 production in *T-bet*$^{-/-}$ and *T-bet*$^{+/+}$ CD4^{+} T cells. In fact, CD4^{+} T cells from immunized *T-bet*$^{-/-}$ mice produced no IFNγ but significant

amounts of IL-4 relative to *T-bet*$^{+/+}$ CD4^{+} T cells upon in vitro restimulation (Fig. 4 B). Furthermore, diseased *T-bet*$^{-/-}$ mice displayed a complete lack of myosin-specific IgG2a, a Th1-dependent antibody isotype (Fig. 4 C). Collectively, these data indicate that Th1 differentiation is not required for the development of autoimmune myocarditis and confirm a Th2 phenotype of MyHC-α-specific *T-bet*$^{-/-}$ CD4^{+} T cells.

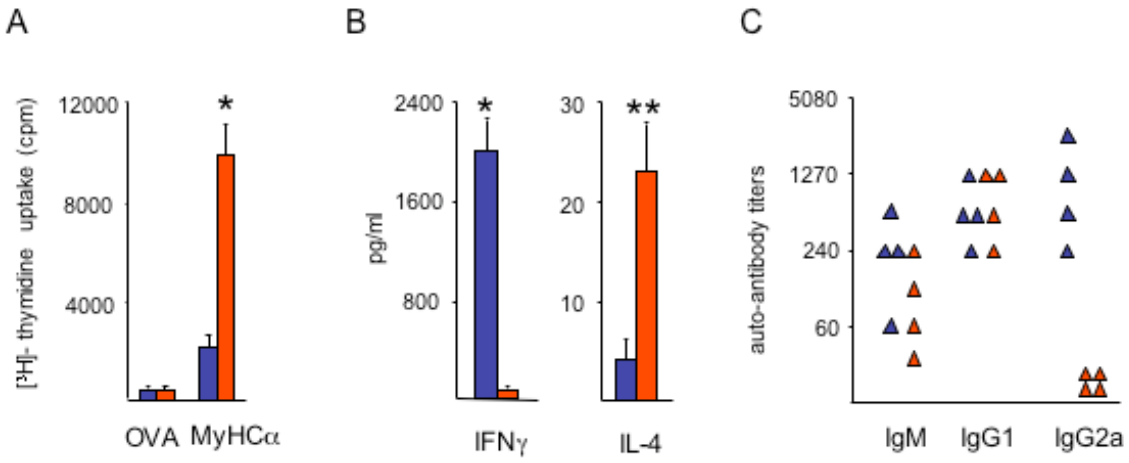


Figure 4. CD4^{+} T-cell responses and cytokine production patterns of immunized *T-bet*$^{-/-}$ vs. *T-bet*$^{+/+}$ mice. CD4^{+} T cells from *T-bet*$^{+/+}$ (blue bars) and *T-bet*$^{-/-}$ mice (red bars) were restimulated with either MyHC-α or control OVA peptide. (A) Proliferative responses after 48 hours. (B) IFNγ and IL-4 production in supernatants after 40 hours of restimulation. (C) Serum MyHC-α autoantibody responses from immunized *T-bet*$^{+/+}$ (blue) and *T-bet*$^{-/-}$ (red) mice at day 21. *p<0.05, **p<0.01 (Student`s t-test).

EAM develops regardless of Th1 or Th2 differentiation

IL-4 is a key determinant of Th2 differentiation [105]. To test the possibility that the exaggerated production of IL-4 seen from *T-bet*$^{-/-}$ CD4^{+} T cells could increase disease severity, we crossed T-bet mutant mice onto IL-4- and IL-4Rα– deficient strains. Intriguingly, both *T-bet*$^{-/-}$*IL4*$^{-/-}$ and *T-bet*$^{-/-}$*IL4Rα*$^{-/-}$ mice developed severe EAM relative to *T-bet*$^{+/+}$ and *IL-4*$^{-/-}$ or *IL4Rα*$^{-/-}$ controls.

Table 2. Myocarditis prevalence and severity in MyHC-α-immunized mice.

Mice [genotype]	*Immunization*	*Disease prevalence [#diseased/#treated]*	*Mean score of diseased mice*
T-bet$^{-/-}$	*MyHC-α/CFA*	*18/18*[q]	*3.56*[a,e,h,l,r]
T-bet$^{+/+}$	*MyHC-α/CFA*	*16/19*[j,m]	*2*[a,b,c,d,g,k,p]
IL-4$^{-/-}$	*MyHC-α/CFA*	*3/4*	*1.67*[b,f]
IL-4Rα$^{-/-}$	*MyHC-α/CFA*	*3/4*	*2*[c,i]
T-bet$^{-/-}$ IL-4$^{-/-}$	*MyHC-α/CFA*	*4/4*	*3.75*[d,e,f]
T-bet$^{-/-}$ IL-4Rα$^{-/-}$	*MyHC-α/CFA*	*5/5*	*3.8*[g,h,i]
IL-12Rβ1$^{-/-}$	*MyHC-α/CFA*	*0/4*[j,n]	*n.a.*
IL-12p35$^{-/-}$	*MyHC-α/CFA*	*5/5*	*1.6*[k,l,o]
T-bet$^{-/-}$ IL-12Rβ1$^{-/-}$	*MyHC-α/CFA*	*0/6*[m,n,o]	*n.a.*
T-bet$^{-/-}$ IL-12p35$^{-/-}$	*MyHC-α/CFA*	*6/6*	*3.67*[p,q,r]
T-bet$^{+/+}$	*MyHC-α pulsed* T-bet$^{+/+}$ *dendritic cells*	*4/5*	*1.5*[s,t]
T-bet$^{-/-}$	*MyHC-α pulsed* T-bet$^{+/+}$ *dendritic cells*	*5/5*	*2.6*[s]
T-bet$^{+/+}$	*MyHC-α pulsed* T-bet$^{-/-}$ *dendritic cells*	*3/3*	*1.67*[t]
IL-12p35$^{+/+}$	*MyHC-α pulsed* IL-12p35$^{+/+}$ *dendritic cells*	*4/4*	*1.87*[o]
IL-12p35$^{+/+}$	*MyHC-α pulsed* IL-12p35$^{-/-}$ *dendritic cells*	*4/4*	*2.25*[u]

H&E sections were scored as described in "Methods". CFA/MyHC-α immunized mice were sacrificed on d21, DC-immunized mice were sacrificed on d10. Statistical significance was assessed using the Mann-Whitney U test unless otherwise indicated. [a] *$p < 0.000001$,* T-bet$^{-/-}$ *vs* T-bet$^{+/+}$; [b] *$p = n.s.$,* T-bet$^{+/+}$ *vs* IL-4$^{-/-}$; [c] *$p = n.s.$,* T-bet$^{+/+}$ *vs* IL-4R_$^{-/-}$; [d] *$p < 0.0008$,* T-bet$^{+/+}$ *vs* T-bet$^{-/-}$IL-4$^{-/-}$; [e] *$p = n.s.$,* T-bet$^{-/-}$ *vs* T-bet$^{-/-}$IL-4$^{-/-}$; [f] *$p < 0.029$,* IL-4$^{-/-}$ *vs* T-bet$^{-/-}$IL-4$^{-/-}$; [g] *$p < 0.0002$,* T-bet$^{+/+}$ *vs* T-bet$^{-/-}$IL-4R_$^{-/-}$; [h] *$p = n.s.$,* T-bet$^{+/+}$ *vs* T-bet$^{-/-}$IL-4R_$^{-/-}$; [i] *$p < 0.036$,* IL-4R_$^{-/-}$ *vs* T-bet$^{-/-}$IL-4R_$^{-/-}$; [j] *$p < 0.004$,* T-bet$^{+/+}$ *vs* IL-12R_1$^{-/-}$ *(Fisher's exact test);* [k] *$p = n.s.$,* T-bet$^{+/+}$ *vs* IL-12p35$^{-/-}$; [l] *$p < 0.0001$,* T-bet$^{-/-}$ *vs* IL-12p35$^{-/-}$; [m] *$p < 0.0005$,* T-bet$^{+/+}$ *vs* T-bet$^{-/-}$IL-12R_1$^{-/-}$ *(Fisher's exact test);* [n] *$p = n.s.$,* IL-12R_1$^{-/-}$ *vs* T-bet$^{-/-}$IL-12R_1$^{-/-}$ *(Fisher's exact test);* [o] *$p < 0.000007$,* T-bet$^{-/-}$ *vs* T-bet$^{-/-}$IL-12R_1$^{-/-}$ *(Fisher's exact test);* [p] *$p < 0.002$,* T-bet$^{+/+}$ *vs* T-bet$^{-/-}$IL-12p35$^{-/-}$; [q] *$p < 0.009$,* IL-12p35$^{-/-}$ *vs* T-bet$^{-/-}$IL-12p35$^{-/-}$; [r] *$p = n.s.$,* T-bet$^{-/-}$ *vs* T-bet$^{-/-}$IL-12p35$^{-/-}$; [s] *$p < 0.0317$,* T-bet$^{+/+}$ *(*T-bet$^{+/+}$ *DC-immunized) vs* T-bet$^{-/-}$ *(*T-bet$^{+/+}$ *DC-immunized);* [t] *$p < n.s.$,* T-bet$^{+/+}$ *(*T-bet$^{+/+}$ *DC-immunized) vs* T-bet$^{+/+}$ *(*T-bet$^{-/-}$ *DC-immunized);* [u] *$p < n.s.$, IL-12p35$^{+/+}$ (*IL-12p35$^{+/+}$ *DC-immunized) vs IL-12p35$^{+/+}$ (*IL-12p35$^{-/-}$ *DC-immunized). n.a., not applicable*

However, disease in *T-bet$^{-/-}$IL4$^{-/-}$* and *T-bet$^{-/-}$IL4Rα$^{-/-}$* mice was comparable in severity to that seen in *T-bet$^{-/-}$* mice (Figure 2B and Table 2). This demonstrates that the exacerbated disease seen in *T-bet$^{-/-}$* mice is not due to increased IL-4 signaling.

To directly examine the pathogenic role of MyHC-α specific CD4^{+} T cells, we derived MyHC-α-specific CD4^{+} lines from immunized *T-bet$^{-/-}$* and *T-bet$^{+/+}$* mice. T-bet$^{+/+}$ CD4^{+} T cell lines were skewed toward a Th1 phenotype, as they produced large amounts of IFN-γ, but no IL-4, upon restimulation. In contrast, *T-bet$^{-/-}$* lines were Th2 skewed, as they produced no IFN-γ yet generated high levels of IL-4 (Fig. 5 A). Adoptive transfer of both *T-bet$^{+/+}$* and *T-bet$^{-/-}$* lines induced myocarditis of comparable disease severity in *T-bet$^{+/+}$* recipient mice (Fig. 5 B). These data suggest that EAM can develop regardless of whether Th1 or Th2 signals predominate in MyHC-α-specific CD4^{+} T cells.

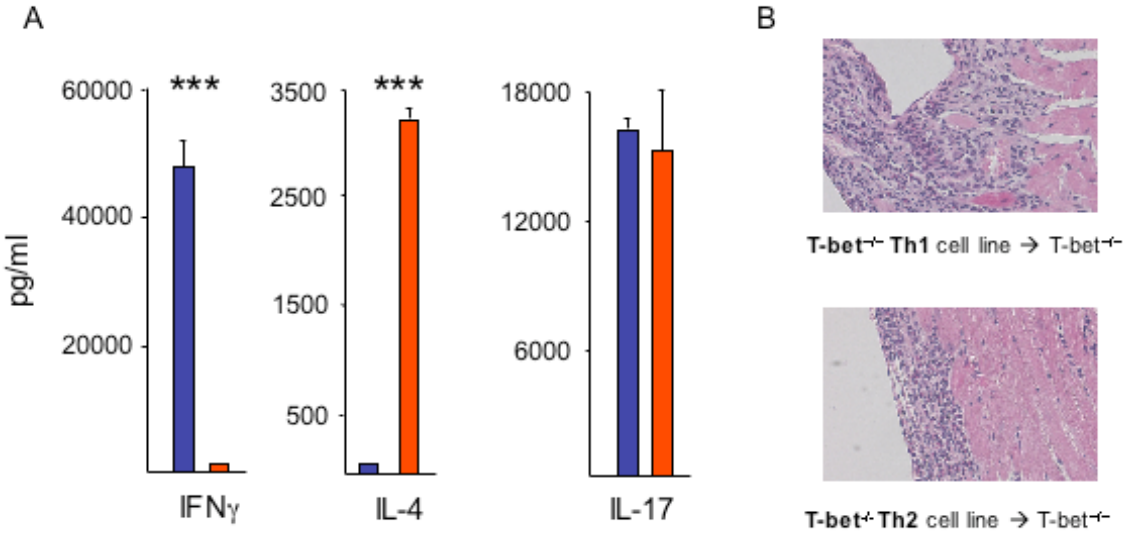


Figure 5. Th1 as well as Th2 CD4$^+$ T cell lines trigger autoimmune myocarditis. (A) MyHC-α specific lines were generated by subjecting CD4$^+$ splenocytes from immunized *T-bet*$^{+/+}$ (blue) or *T-bet*$^{-/-}$ (red) mice to multiple rounds of antigen re-stimulation followed by rest in minimal IL-2. Supernatant was collected for ELISA 48 hrs after the 3rd antigen re-stimulation. ***p<0.0001 (Student's t-test). One representative experiment is shown. (B) Both MyHC-α specific Th1 cell lines (upper panel) and Th2 cell lines (lower panel) are pathogenic in wild type recipient mice. Hematoxylin/eosin stained sections, 40X original magnifications.

Myocarditis induction in T-bet$^{-/-}$ mice is IL-23 dependent

IL-12 is produced in large amounts by activated DCs and macrophages, and it strongly induces Th1 differentiation [106]. By inducing the up-regulation of IL-12Rβ2, T-bet can increase Th1 cell sensitivity to IL-12 and thereby stabilize Th1 lineage commitment [107, 108]. In addition, *IL12p40*$^{-/-}$ and *IL12Rβ1*$^{-/-}$ mice are resistant to EAM [60, 95]. However, IL-12 shares the p40 subunit with the related cytokine IL-23, and the IL-23 receptor heterodimer contains IL-12Rβ1. Indeed, many of the immunological and pathogenic functions initially attributed to IL-12 have now been described as IL-23-dependent [63]. We therefore sought to examine whether the severe EAM that develops in the absence of T-bet could be regulated by either IL-12 or IL-23. We crossed *T-bet*$^{-/-}$ mice onto an IL-12p35-deficient strain, which specifically lacks IL-12. *IL-12p35-/-* mice developed disease of a severity comparable to wild-type mice, whereas *T-bet*$^{-/-}$*IL-12p35*$^{-/-}$ mice developed severe EAM at a level similar to *T-bet*$^{-/-}$ mice (Table 1). DCs are a major source of IL-12 production [63]; however, activated and MyHC-α pulsed

IL-12p35$^{-/-}$ BMDCs were as capable as *IL-12p35*$^{+/+}$ counterparts of inducing myocarditis in wild-type recipients (Table 1). We then examined the effect of loss of T-bet on an *IL-12Rβ1*$^{-/-}$ strain, which lacks both the IL-12 and the IL-23 receptors. Strikingly, *IL12Rβ1*$^{-/-}$ and *T-bet*$^{-/-}$*IL12Rβ1*$^{-/-}$ were resistant to EAM (Table 2). These data show that IL-23, rather than IL-12, is a key pathogenic cytokine in EAM. Moreover, they indicate that loss of IL-23 signaling can abrogate the development of exacerbated EAM seen in *T-bet*$^{-/-}$ mice.

Increased IL-17 in the heart, but not peripheral lymphoid tissue, of immunized T-bet$^{-/-}$ mice

Recent findings suggest that IL-23 plays an important role in the expansion of an IL-17-producing subset of CD4$^+$ T cells (Th$_{IL-17}$) in vitro [109, 110]. These cells play an essential role in the infectious response [111, 112] as well as in autoimmunity [14, 18, 19]. Further data have shown that the Th$_{IL-17}$ lineage is distinct from Th1, and that IFN-γ and T-bet can repress CD4$^+$ T cell production of IL-17 [14, 110]. In light of our finding that deficiency in IL-23 signaling could rescue *T-bet*$^{-/-}$ mice from severe EAM, we examined whether increased generation of Th$_{IL-17}$ cells could explain the exacerbated disease seen in *T-bet*$^{-/-}$ mice. Restimulation of CD4$^+$ T cells from both *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ mice resulted in small numbers of IL-17$^+$CD4$^+$ T cells that were IFNγ$^-$ (Fig. 6). Despite the identification of Th$_{IL-17}$ as a distinct CD4$^+$ effector T cell lineage, a proportion of IL-17$^+$ T cells can still produce IFN-γ [110]. Indeed, our MyHC-α-specific, pathogenic *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ CD4$^+$ T cell lines, which produced elevated levels of IFN-γ and IL-4, respectively, both secreted significant but comparable amounts of IL-17 (Fig. 5 A). These findings suggest that although IL-17 production from MyHC-α-specific CD4$^+$ T-cells may play a role in EAM in the absence of classical Th1 and Th2 signals, T-bet does not repress the generation of such cells.

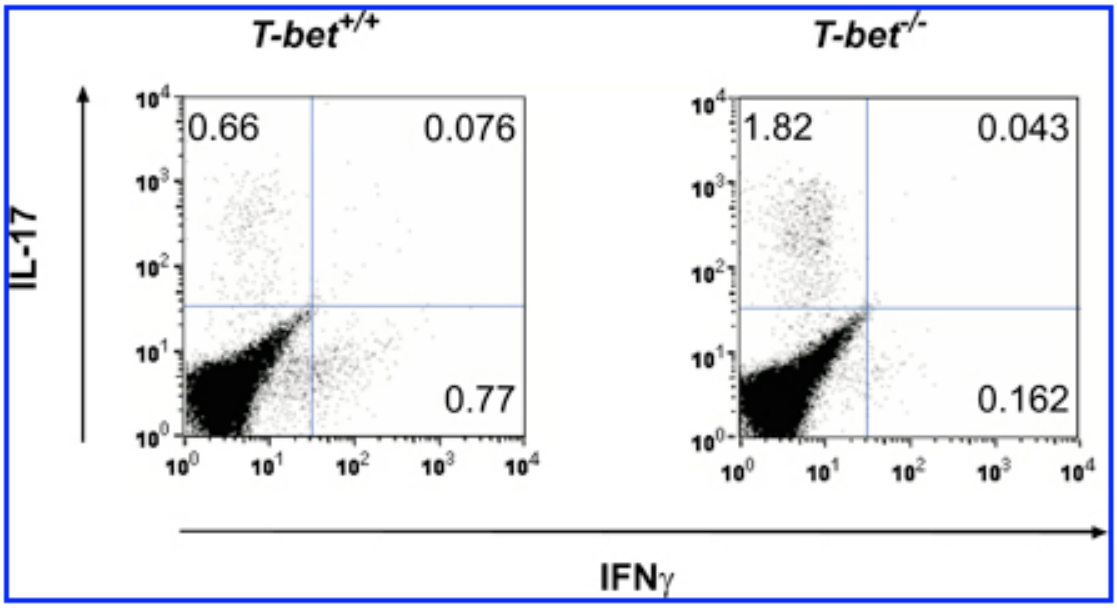


Figure 6. CD4$^+$ from MyHC-α-immunized T-bet$^{+/+}$ and T-bet$^{-/-}$ mice produce IL-17. Splenocytes from immunized T-bet$^{+/+}$ and T-bet$^{-/-}$ mice were restimulated for 48 h with MyHC-α and analyzed for intracellular production of IFN-γ and IL-17. Cells were gated on the CD4+ population. A representative of three individual experiments is shown.

Given that IL-17 promotes the infiltration of neutrophils to local sites of inflammation [113], we asked whether heart-infiltrating T cells are competent to release IL-17 in immunized mice. Surprisingly, heart-infiltrating CD3$^+$ T cells from immunized *T-bet*$^{-/-}$ mice made significantly more IL-17 than those taken from *T-bet*$^{+/+}$ counterparts (Fig. 7 A). In vivo depletion of IL-17 in immunized *T-bet*$^{-/-}$ mice markedly reduced EAM severity relative to non-depleted *T-bet*$^{-/-}$ controls (Fig. 7 B). These data suggest that the heightened disease severity seen in *T-bet*$^{-/-}$ mice may be explained by an increase in T cell-mediated IL-17 production in the heart.

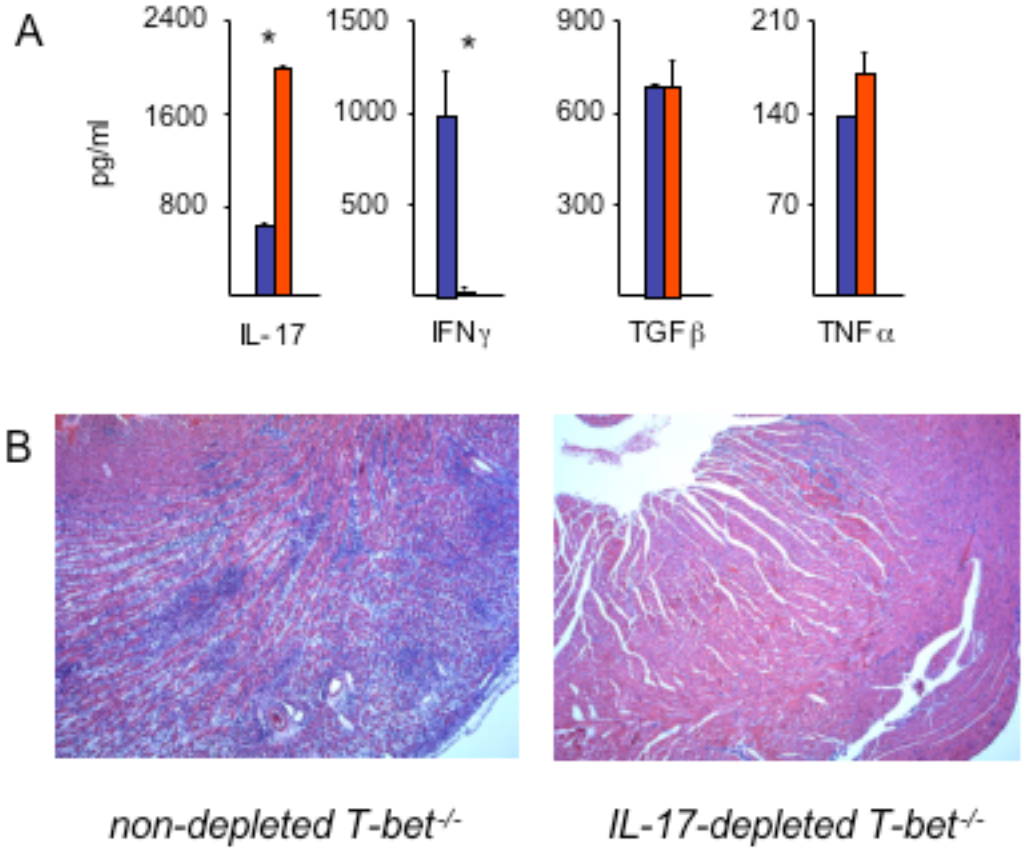


Figure 7. Heart infiltrating T-bet$^{-/-}$ CD3^{+} T cells display an altered cytokine profile and IL-17 depletion improves EAM severity in T-bet$^{-/-}$ mice. Cytokine production profile of T-bet$^{-/-}$ heart-infiltrating T cells. Infiltrating CD3^{+} cells were isolated from hearts of immunized T-bet$^{+/+}$ (blue) or T-bet$^{-/-}$ (red) mice at d21, and re-stimulated with anti-CD3/anti-CD28 for 24 hours. Production of indicated cytokines was assessed by supernatant ELISA. *p<0.005. Representative of two individual experiments. (B) T-bet$^{-/-}$ mice were immunized with 50 μg MyHC–α and injected with either 100 μg anti-mouse IL-17 or with 100 μL PBS on d9, 12, 15. Mice were sacrificed on d17. Hematoxylin/eosin stained sections, 5X original magnification.

Normal function of *T-bet*$^{-/-}$ BMDCs and regulatory T (T reg) cells

Peripheral CD4^{+} cells from *T-bet*$^{+/+}$ -and *T-bet*$^{-/-}$ -immunized mice produced comparable levels of IL-17 (Fig. 6 and 7 A). We therefore examined whether loss of T-bet in a different compartment might be responsible for the increase in EAM severity observed in *T-bet*$^{-/-}$ mice. Because T-bet expression is up-regulated in stimulated DCs [114, 115], we first evaluated the pathogenicity of activated MyHC-α loaded *T-bet*$^{-/-}$ DCs using a model of DC-induced autoimmune

myocarditis [54, 116]. Stimulated and MyHC-α-pulsed *T-bet*$^{-/-}$ BMDCs were as pathogenic as *T-bet*$^{+/+}$ BMDCs (Fig. 8 A and Table 2). *T-bet*$^{-/-}$ BMDCs produced similar amounts of IL-6, TNFα, and IL-12p40, and up-regulated MHC class II and CD80 to comparable levels, as wildtype controls upon in vitro stimulation (not depicted). Thus, in our model system of DC-induced EAM, loss of T-bet in DCs does not enhance autoimmune heart disease.

We next examined the potential role of T-bet in the maintenance of peripheral tolerance. CD4$^+$CD25$^+$ T reg cells suppress proliferation of CD4$^+$CD25$^-$ effector T cells in vitro and in vivo [117]. Furthermore, Afanasyeva et al. [104] recently described impaired apoptosis of CD4$^+$CD25$^+$ T cells in IFN-γ$^{-/-}$ mice using the EAM model. *T-bet*$^{-/-}$ CD4$^+$CD25$^+$ T reg cells were as capable as their T-bet$^{+/+}$ counterparts of suppressing the proliferation of CD4$^+$CD25$^-$ effector cells (Fig. 8 B). This data, as well as that of others [97], indicates that loss of T-bet does not affect the suppressive function of CD4$^+$CD25$^+$ T reg cells in vitro.

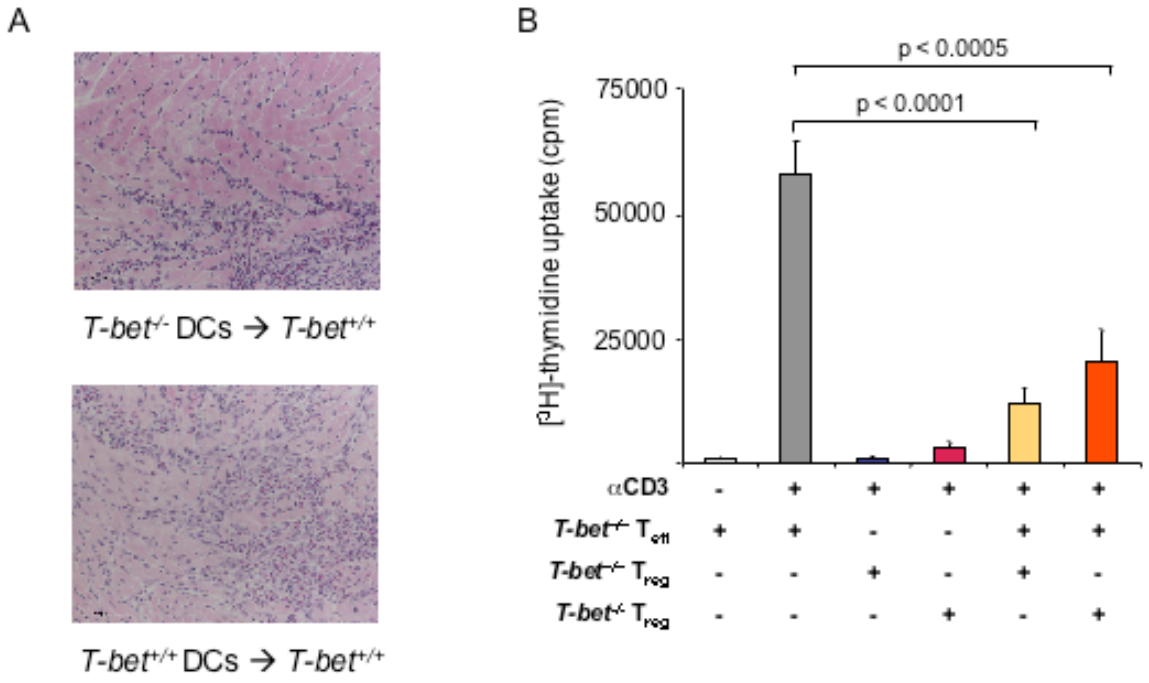


Figure 8. *T-bet*$^{-/-}$ DCs and CD4$^+$CD25$^+$ Treg cells function normally. (A) *T-bet*$^{+/+}$ mice were immunized with activated, MyHC-α-pulsed, *T-bet*$^{+/+}$ or *T-bet*$^{-/-}$ BMDCs. (B) *T-bet*$^{+/+}$ CD4$^+$CD25$^-$ T-cells (T_{eff}) were stimulated with anti-CD3 with or without *T-bet*$^{+/+}$ or *T-bet*$^{-/-}$ CD4$^+$CD25$^+$ cells (T_{reg}). Proliferation was measured at 72 h by [45]-thymidine incorporation. Data are representative of three independent experiments.

Loss of T-bet in the CD8$^+$ compartment exacerbates EAM

Besides CD4$^+$ T cells, CD8$^+$ cells are also found in the heart infiltrates of diseased mice (Fig. 3), and T-bet is expressed in CD8$^+$ T cells [96, 100, 118]. It is unclear whether CD8$^+$ cells promote or modulate EAM pathogenesis [119, 120]. A direct pathogenic role for CD8$^+$ T cells has been shown in transgenic mice expressing a class I-restricted OVA-derived peptide specifically in cardiomyocytes [120]. In nontransgenic mice, however, EAM is CD4$^+$ T cell dependent and CD8$^+$ T cells do not mediate disease [54]. In contrast, it appears that the loss of CD8$^+$ T cells exacerbates rather than protects against EAM as CD8-deficient mice develop exacerbated EAM [119].

We first examined the functional capabilities of *T-bet*$^{-/-}$ CD8$^+$ T cells by several methods. CD8$^+$ T cells from immunized *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ mice did not respond in vitro to irradiated splenocytes pulsed with whole myosin (not depicted), excluding antigenic spreading to class I-restricted myosin epitopes as a pathogenic mechanism. Further, *T-bet*$^{-/-}$ CD8$^+$ T cells mediated cytotoxic lysis of allogeneic targets to a similar degree as *T-bet*$^{+/+}$ counterparts (Fig. 9 A).

Table 3. Myocarditis prevalence and disease severity in SCID mice reconstituted with *T-bet*$^{+/+}$ CD4$^+$ T cells and *T-bet*$^{+/+}$ versus *T-bet*$^{-/-}$ CD8$^+$ T cells

Table II. Myocarditis prevalence and disease severity in SCID mice reconstituted with *T-bet*$^{+/+}$ CD4$^+$ T cells and *T-bet*$^{+/+}$ versus *T-bet*$^{-/-}$ CD8 T cells

Recipient	Reconstitution	Immunization	Disease prevalence (no. diseased/no. treated)	Mean score of diseased mice
SCID	*T-bet*$^{+/+}$ CD4$^+$ T cells *T-bet*$^{+/+}$ CD8$^+$ T cells	MyHC-α/CFA	4/15	1[a]
SCID	*T-bet*$^{+/+}$ CD4$^+$ T cells *T-bet*$^{-/-}$ CD8$^+$ T cells	MyHC-α/CFA	6/13	2.3[a]

SCID mice were reconstituted with *T-bet*$^{+/+}$ CD4$^+$ cells and either *T-bet*$^{+/+}$ or *T-bet*$^{-/-}$ CD8$^+$ cells (2:1 ratio of CD4$^+$/CD8$^+$). Recipients were immunized with MyHC-α at the time of reconstitution and killed 21 d later.
[a]$P < 0.02$ (Mann Whitney *U*).

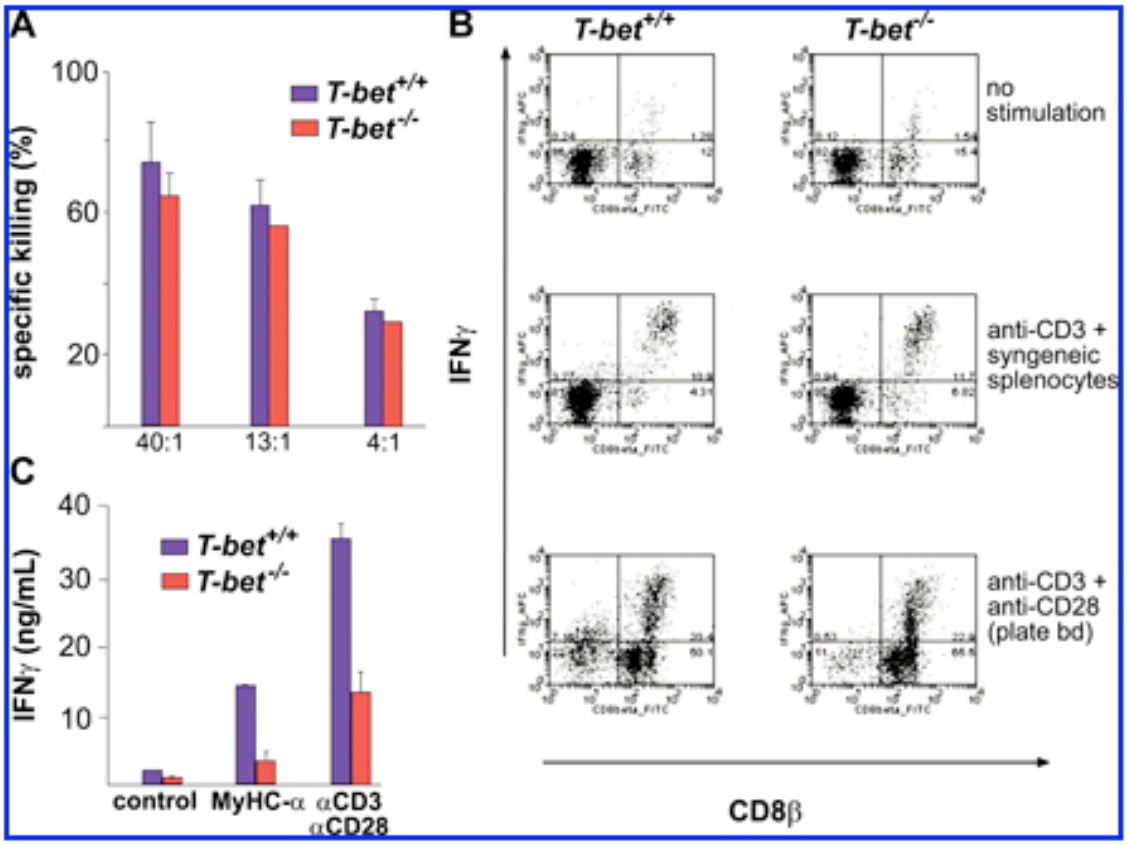


Figure 9. Peripheral T-bet$^{-/-}$ CD8+ T cells function normally. (A) Cytotoxicity assay of T-bet$^{+/+}$ versus T-bet$^{-/-}$ CD8^{+} T cells. Allo-primed and restimulated CD8^{+} T cells from either T-bet$^{+/+}$ or T-bet$^{-/-}$ mice were used at 40:1, 13:1, or 4:1 ratios on calcein-labeled EL-4 target cells. Results are expressed as mean of percent-specific lysis from quadruplicates ± SD. (B) T-bet$^{+/+}$ and T-bet$^{-/-}$ peripheral CD8β^{+} cells were stimulated with soluble anti-CD3 plus γ-irradiated syngeneic splenocytes (APCs), or with plate-bound anti-CD3/anti-CD28. IFN-γ production was assessed using intracellular cytokine staining. Representative data from three mice each is shown. (C) Whole T-bet$^{+/+}$ or T-bet$^{-/-}$ splenocytes from immunized mice were restimulated with MyHC-α or anti-CD3/anti-CD28. IFNγ production was measured from supernatants at 48 h.

IFNγ has been shown to inhibit the generation of IL-17^{+} CD4^{+} T cells in vitro [14, 110]. We therefore assessed the ability of *T-bet*$^{-/-}$ CD8^{+} cells to produce IFNγ. In agreement with previous findings [96], we found that peripheral *T-bet*$^{-/-}$ CD8^{+} display apparently normal IFNγ production upon polyclonal stimulation, regardless of whether they were taken from naive mice (Fig. 9B) or from immunized ones (not depicted).

As *T-bet*$^{-/-}$ heart-infiltrating lymphocytes produced exacerbated IL-17, we examined IFNγ production from *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ T cells isolated from the hearts of immunized mice. Intriguingly, we found that heart-infiltrating CD3^{+} T

cells from immunized *T-bet*$^{+/+}$ mice generated substantial amounts of IFNγ when stimulated with anti-CD3/anti-CD28, whereas heart infiltrating CD3^{+} cells from their *T-bet*$^{-/-}$ counterparts did not (Fig. 7A). This finding differed from the situation in the periphery: anti-CD3/anti-CD28-stimulated splenocytes from immunized *T-bet*$^{-/-}$ mice could be induced to make IFNγ (Fig. 9C). Furthermore, heart-infiltrating *T-bet*$^{+/+}$, but not *T-bet*$^{-/-}$, CD8^{+} T cells also released IFNγ in a TCR-independent manner when cultured in conditioned supernatants from LPS-activated BM-DCs (not depicted). Importantly, CD3^{+}CD8^{+}, and not CD3^{+}CD8^{-}, T cells were the only heart-infiltrating cells capable of releasing IFNγ in an antigen-independent manner in *T-bet*$^{+/+}$ mice (Fig. 10 A). This finding suggests that T cell-specific production of IFNγ in the inflamed heart is restricted to the CD8^{+} compartment; furthermore, this function is T-bet dependent. Of note, we found no differences in the production of the immunosuppressive cytokines TGFβ (Fig. 7 A) and IL-10 (not depicted) between heart-infiltrating *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ CD3^{+} T cells.

A role for tissue-resident CD8^{+} T cells in mediating non-cognate bystander suppression has recently been described in a model of allergic airway inflammation [121]. In this model, bystander suppression depended on IFNγ production by lung resident memory CD8^{+} T cells. Together with the fact that IFNγ negatively regulates autoimmune myocarditis [13, 60, 95], our findings suggest that loss of the IFNγ producing capacity of *T-bet*$^{-/-}$ CD8^{+} T cells might play an important role in the enhanced disease severity in *T-bet*$^{-/-}$ mice. To resolve this question in vivo, we simultaneously immunized SCID mice with the

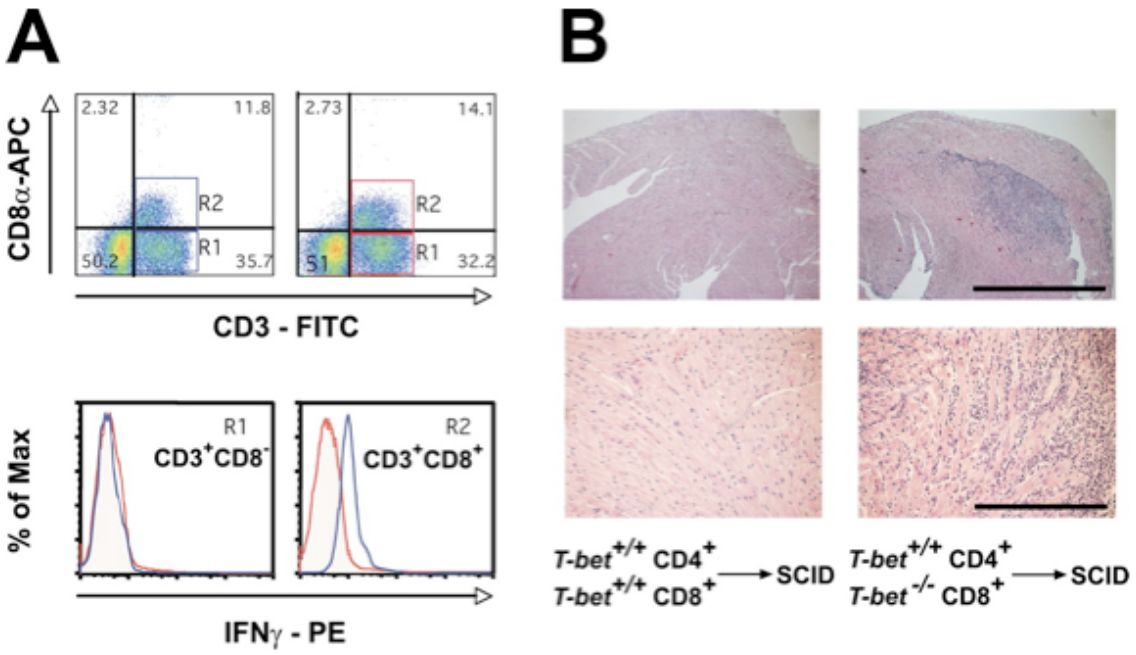


Figure 10. Heart-infiltrating *T-bet*$^{-/-}$ CD8$^+$ T cells display impaired IFNγ production, and loss of T-bet in CD8$^+$ T cells exacerbates myocarditis. A) Heart infiltrating mononuclear cells from immunized mice were restimulated with anti-CD3/anti-CD28, stained for CD3, CD8, and intracellular IFNγ, and analyzed by flow cytometry. Blue boxes and histograms, *T-bet*$^{+/+}$, red boxes and histograms, *T-bet*$^{-/-}$. A representative of two individual experiments is shown. B) SCID mice were reconstituted with either *T-bet*$^{+/+}$ CD4$^+$ plus *T-bet*$^{+/+}$ CD8$^+$ or *T-bet*$^{+/+}$ CD4$^+$ plus *T-bet*$^{-/-}$ CD8$^+$ and immunized. Mice were killed 21d later. Hematoxylin and eosin-stained sections of hearts from mice receiving *T-bet*$^{-/-}$ CD8$^+$ T cells (right) versus mice receiving *T-bet*$^{+/+}$ CD8$^+$ cells (left) are shown. Bars, (top) 1mm; (bottom) 100μm.

autoimmune MyHC-α peptide and reconstituted them with naïve *T-bet*$^{+/+}$ CD4$^+$ cells plus either *T-bet*$^{+/+}$ or *T-bet*$^{-/-}$ naïve CD8$^+$ cells. Intriguingly, mice that received *T-bet*$^{-/-}$ CD8$^+$ cells developed significantly more severe disease than those receiving *T-bet*$^{+/+}$ CD8$^+$ (Fig. 10B and Table 3). One explanation for these findings could be that *T-bet*$^{-/-}$ CD8$^+$ T cells have a proliferative defect in vivo, thereby leading to excessive expansion of co-transferred *T-bet*$^{+/+}$ CD4$^+$ and subsequently to exacerbated disease. To exclude this possibility, we CFSE-labeled *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ CD8$^+$ and transferred them to SCID mice. *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ CD8$^+$ cells diluted CFSE to comparable levels upon transfer (not depicted), demonstrating that *T-bet*$^{-/-}$ CD8$^+$ cells proliferate normally in lymphopenic hosts. Taken with the knowledge that genetic ablation of the CD8$^+$ T cell compartment exacerbates autoimmune myocarditis [119], our results

demonstrate that $CD8^+$ T cells display down-regulatory properties rather than disease-promoting roles in EAM, and that these suppressive functions are impaired in the absence of T-bet.

In conclusion, expression of T-bet in heart-infiltrating $CD8^+$ T cells appears critical for their ability to mediate antigen-nonspecific bystander suppression of local inflammation in the EAM model. This observation parallels the impaired release of IFNγ and the increased production of IL-17 from heart-infiltrating *T-bet*$^{-/-}$ T cells.

Discussion

To date it has been unclear whether EAM is a Th1- or Th2- mediated disease. Although mice lacking IL-4 signaling could develop EAM, the same was also true for mice deficient in IFNγ or IFNγR [13, 60, 95]. However, our data and that of others [122] indicate that Th1 responses can progress in the absense of IFNγ signaling. We therefore examined the development of EAM in mice lacking the T-box transcription factor T-bet, which is critical for Th1 differentiation [8] and essential for $CD4^+$ cell tissue infiltration in Th1-mediated models of inflammation [123]. Our findings showed that in sharp contrast to other autoimmune disease models [97-101, 124], *T-bet*$^{-/-}$ mice developed EAM of exacerbated severity relative to *T-bet*$^{+/+}$ controls. Although *T-bet*$^{-/-}$ mice were previously described to develop transiently increased disease severity relative to wild-type controls in a model of *Staphylococcus aureus*-induced arthritis [125], these mice also suffered from greatly increased bacterial load, making it likely that increased arthritis severity was due to impaired infectious clearance rather than to autoimmune mechanisms. We therefore describe the first model in which T-bet negatively regulates autoimmune disease.

As expected, immunized *T-bet*$^{-/-}$ mice developed defective Th1 responses, as measured by the presence of MyHC-α-specific, IgG2a isotype autoantibodies and by IFNγ production by restimulated CD4+ T cells. We then found that *T-bet*$^{-/-}$ *IL12p35*$^{-/-}$ mice were also highly susceptible to EAM, excluding the possibility that

IL-12 can promote severe Th1-driven autoimmunity in the absense of T-bet. At the same time,Th1- and Th2- polarized, MyHC-α-specific $CD4^+$ T cell lines were equally effective in transferring disease to healthy recipients, and both *Tbet*$^{-/-}$*IL-4*$^{-/-}$ and *T-bet*$^{-/-}$*IL4Rα*$^{-/-}$ mice still developed severy myocarditis. Therefore, our results describe EAM as an autoimmune disease that can develop independently of Th1/Th2 dysregulation. However, we cannot exclude that Th1 or Th2 polarization might contribute to the broad pattern of different morphologic phenotypes and clinical courses in patients with inflammatory heart disease [49, 126].

Interestingly, IL-23 signaling was absolutely required for EAM development, as *IL-12Rβ1*$^{-/-}$ and *T-bet*$^{-/-}$*IL12Rβ1*$^{-/-}$ mice, but not *IL-12p35*$^{-/-}$ and *T-bet*$^{-/-}$*IL-12p35*$^{-/-}$ mice, were protected from disease. IL-23 is essential for the generation of IL-17-producing $CD4^+$ T cells in vivo, and IL-17 is a pathogenic cytokine critical for the progression of experimental autoimmune encephalomyelitis [18]. IL-17-producing $CD4^+$ T cells (Th_{IL-17}) have been described as representing an effector cell type independent from Th1 or Th2 cells [14, 16, 110, 127]. Although recent evidence argues against a role for IL-23 in the initiation of Th17 differentiation [16], it is clear that IL-23 is at least essential for the expansion or survival of Th17 cells in vivo. We therefore examined whether loss of T-bet could increase IL-17 production, and by extension disease severity, in our EAM model. However, both $CD4^+$ T cells from immunized *T-bet*$^{+/+}$ *and T-bet*$^{-/-}$ mice, as well as in vitro-expanded and pathogenic MyHC-α-specific Th1 *T-bet*$^{+/+}$ and Th2 *T-bet*$^{-/-}$ $CD4^+$ T cell lines, produced comparable levels of IL-17. This suggested that although IL-17 production might characterize pathogenic $CD4^+$ T cells in EAM, T-bet did not repress IL-17 production in the peripheral $CD4^+$ compartment.

However, in contrast to peripheral T cells, we observed that IL-17 production from heart-infiltrating T cells was markedly greater in immunized *T-bet*$^{-/-}$ mice as compared with *T-bet*$^{+/+}$ controls. IL-17 can act on stromal endothelial cells in inflamed tissue to induce secretion of neutrophil-attracting factors, such as IL-8, CXCL1 and GM-CSF [113]. This suggests that the heart-

localized up-regulation of IL-17 production in *T-bet*$^{-/-}$ mice could be pathologically relevant. Indeed, depletion of IL-17 in vivo markedly reduced EAM severity in *T-bet*$^{-/-}$ mice. Recent reports have suggested that loss of T-bet results in increased IL-17 production from CD4$^+$ T cells in vitro [110], and from restimulated draining LN cells taken from MOG peptide-immunized mice [14]. Our data strongly suggest a direct pathogenic role for IL-17$^+$CD4$^+$ T cells in EAM. Furthermore, they establish for the first time that T-bet-mediated repression of IL-17 can modulate the severity of an autoimmune disease.

IFNγ can inhibit the production of IL-17 from CD4$^+$ T cells. We therefore analyzed the capacity of heart-infiltrating T cells to produce IFNγ. In *T-bet*$^{+/+}$ mice, heart-infiltrating CD3$^+$CD8$^+$, but not CD3$^+$CD8$^-$, cells made IFNγ upon polyclonal stimulation. Intriguingly, CD8$^+$ T cells appear to be responsible for almost all of the IFNγ released within the diseased heart of wild-type mice in autoimmune myocarditis. This secretion might act to suppress local production of IL-17. Furtermore, CD8$^+$ T cells from both immunized *T-bet*$^{+/+}$ and *T-bet*$^{-/-}$ mice could not proliferate in response to whole myosin, suggesting that heart-infiltrating CD8$^+$ T cells in EAM secrete IFNγ in an antigen-nonspecific manner. Importantly, *T-bet*$^{-/-}$ heart-infiltrating T cells were wholly deficient in their capacity to produce IFNγ yet were still able to produce other cytokines, such as TGFβ and TNFα. This absence of infiltrating lymphocyte-specific production of IFNγ parallels the observed increase in IL-17 production in *T-bet*$^{-/-}$ hearts.

Although IFNγ has been well described as the prototypical Th1 effector cytokine, it is becoming clear that is also possesses immunomodulatory properties. IFNγ can induce APC expression of immunoregulatory molecules, such as inducible nitric oxide synthase and indoleamine-2,3-dioxygenase, and IFNγ is essential for the generation of T reg cells with in vivo suppressive function [128]. IFNγ production is suppressed in arthritic joints despite significant T cell infiltration, suggesting that local down-regulation of IFNγ production contributes to autoimmune inflammation in rheumatoid arthritis [129], and *IFNγ*$^{-/-}$ and *IFNγR*$^{-/-}$ mice are highly susceptible to severe EAM [13, 60, 95]. Bystander, noncognate

$CD8^+$ -mediated production of IFNγ has also been implicated in the suppression of inflammation; $CD8^+$ T cells directed against an influenza-derived epitope were able to protect against OVA-mediated allergic airway inflammation [121]. We found that although cotransfer of *T-bet*$^{+/+}$ $CD8^+$ T cells with *T-bet*$^{+/+}$ $CD4^+$ T cells resulted in minimal disease in immunized SCID recipients, transfer of *T-bet*$^{-/-}$ $CD8^+$ T cells and *T-bet*$^{+/+}$ $CD4^+$ T cells caused significantly more severe EAM. These findings point toward a critical T-bet-dependent role of $CD8^+$ T cells in modulating autoimmune myocarditis severity.

Our findings that organ-infiltrating but not peripheral *T-bet*$^{-/-}$ $CD8^+$ T cells lack the capacity to release IFNγ complement recent findings that antigen-specific, pancreatic LN-localized *T-bet*$^{-/-}$ $CD8^+$ T cells loose the capacity to release IFNγ and to mediate disease in the rat insulin promoter-lymphocyte choriomeningitis virus transgenic model of autoimmune diabetes [100]. In contrast to autoimmune myocarditis, however, the transgenic autoimmune diabetes model depends on a T-bet- and IFNγ-dependent T cell response against an MHC class I-restricted antigen. Nevertheless, it might be that the common defect in IFNγ production by organ-infiltrating *T-bet-/-* $CD8^+$ T cells results in impaired cytotoxicity in an MHC class I-restricted immune response, yet leads to impaired bystander suppression in an MHC class II-mediated disease model. We propose that the defect of heart-infiltrating *T-bet*$^{-/-}$ $CD8^+$ T cells to release IFNγ impairs their capacity to suppress local inflammation in EAM.

Although we and others have found that peripheral *T-bet*$^{-/-}$ $CD8^+$ T cells can produce IFNγ, the question arises as to why heart-infiltrating $CD8^+$ cells require T-bet expression to produce IFNγ. Peripheral TCR-transgenic *T-bet*$^{-/-}$ $CD8^+$ cells display defective IFNγ production upon stimulation with specific peptide, suggesting that upon strong TCR stimulation, other factors can compensate for the loss of T-bet in driving IFNγ [130]. Recently, the transcription factor Eomes was identified as an inducer of IFNγ in $CD8^+$ T cells [130, 131]. Although Eomes and other factors may have functions that overlap with T-bet in peripheral $CD8^+$ cells, our data show that T-bet is clearly essential for IFNγ production from heart-infiltrating $CD8^+$ cells. It is possible that upon migration to

the heart, $CD8^+$ T cells undergo a process of epigenetic reprogramming that renders T-bet essential for IFNγ generation. Alternately, heart-infiltrating $CD8^+$ cells may arise from a subset of peripheral $CD8^+$ T cells that absolutely require T-bet for the production of IFNγ. Further research is required to distinguish between these and other scenarios.

Clinical studies suggest that inflammation is a major factor contributing to the pathogenesis of cardiovascular diseases. Here we have shown that mice lacking T-bet are highly susceptible to autoimmune myocarditis. Polymorphisms in the gene encoding T-bet as well as its promoter have been associated with differential susceptibility to asthma in human subjects [132-134]. Epidemiological studies have found a significant association between idiopathic DCM and a history of asthma [135, 136], making a tempting to speculate that loss of T-bet may provide a common molecular mechanism underlying these pathologies.

Intriguingly, induction of EAM appears to be independent of classical Th1 or Th2 effector responses and is rather mediated by a subset of $CD4^+$ T cells characterized by IL-17 production. Indeed, the ablation of Th1 signaling in *T-bet*$^{-/-}$ mice results in severe EAM, in part by abolition of a T-bet-dependent, $CD8^+$ T cell-mediated mechanism of inflammatory suppression. T-bet plays an essential role in the pathogenesis of multiple autoimmune diseases [97-101, 124] and has thus been proposed as a potential target of immunomodulatory therapy [137]. However, the findings presented here imply that care must be taken in manipulating Th1 and Th2 responses in the treatment of autoimmune diseases so as to avoid potentially devastating side effects.

Part 2.

Interferon gamma negatively regulates idiopathic pneumonia syndrome, an IL-17^{+}CD4^{+} T cell-mediated graft versus host disease

A version of this part is currently submitted for publication.

Abstract

Hematopoietic stem cell transplantation is an effective therapy for many hematological diseases. However, toxicity, infections and graft-versus-host are major limiting factors. Here we show massive enhanced CD4 T cells mediated pulmonary graft-versus-host disease in absence of Interferon-γ and T-bet signalling, whereas the other organs typical involved in graft-versus-host disease did not showed increased severity. Absence of Interferon-γ favoured the development of IL-17 producing CD4 T cells. Blocking IL-17 by neutralizing antibodies could reduce disease severity. However, protection from pulmonary graft-versus-host disease was dependent on the presence of Interferon-γ in CD4 T cells or the Interferon-γ receptor on pulmonary parenchymal cells. Therefore the lung tissue seems to be a uniquely sensitive organ for CD4 T cell mediated immunopathology in absence of the protective Interferon-γ induced signalling cascade. In addition, pulmonary host antigen-presenting cells (APC) are rapidly replaced (by day 10) by donor APC and do not seem to play a major role for the maintenance of the donor mediated allogeneic immune response against the host.

Introduction

Allogeneic stem cell transplantation is increasingly used to treat hematological malignancies, non-malignant chronic haematological diseases, cancer and autoimmune diseases. Pulmonary complications including infections and pulmonary graft-versus-host disease (GvHD) affect 30-50% of allogeneic stem cell recipients [67]. Whereas obliterative bronchiolitis represents a late

manifestation of graft-versus-host (GVH) disease, the idiopathic pneumonia syndrome (IPS) can be observed early and late after stem cell transplantation.
To address the mechanisms responsible for IPS development, several mouse models have been established and it has been recognised that T cells are critical for disease development [69, 71, 138]. So far little is known about the T cell subsets mediating the IPS phenotype. In particular, we have no idea about the role of $CD4^+$ T cells in general and, more specifically, about the relevance of the different $CD4^+$ T cell subsets involved in disease development. Regarding the role of the Th1 specific cytokines, however, indirect evidence points to a protective role of the Th1 key cytokine IFNγ, whereas another Th1 cytokine, TNFα aggravates pulmonary graft-versus host disease [138-140][141]. This picture is further clouded by the observation that apart from "classical" Th1 and Th2 helper cells, another subset of $CD4^+$ T cells characterised by IL-17 production might mediate organ-specific inflammation and recruitment of macrophages.
Here we show markedly enhanced pulmonary inflammation after transfer of semi-allogeneic bone marrow and donor CD4 T cells, lacking IFNγ. Importantly, $CD8^+$ T cells played a minor role in IPS. Disease severity strongly correlated with the expansion of IL-17^+CD4^+ T cells in the absence of IFNγ. Moreover, in vivo depletion of IL-17 reduced disease severity suggesting a direct pathogeneic role in IPS. Mechanistically, IFNγ signalling is critically required on radioresistant pulmonary parenchymal cells of the host to maintain a largely nitric-oxide independent negative feedback loop preventing the expansion of semi-allogeneic $CD4^+$ T cells.

Material and Methods

Mice: Sex-matched BALB/c wild-type (H-2^d), C57BL/6 (H-2^b) were bred and maintained at the animal facility of the Department of Research (University of Basel, Switzerland). CB6F1 (H-$2^{b,d}$) were obtained from Charles River (Sulzfeld, Germany) or bred in house. $Rag2^{-/-}$ ($H2^d$) were purchased from Taconic (Lille

Skensved, Denmark), IFNγ$^{-/-}$ (H-2^{d}), IFNγR$^{-/-}$ (H-2^{d}), T-bet$^{-/-}$ (H-2^{d}), IL12Rβ1$^{-/-}$ (H2^{d}) were supplied from The Jackson laboratories (Bar Harbor, MA). CB6F1 IFNγR$^{-/-}$, IL12Rβ1$^{-/-}$ IFNγR$^{-/-}$ (H2^{d}) , T-bet$^{-/-}$ IL12Rβ1$^{-/-}$ (H2^{d}) and T-bet$^{-/-}$ IL12p35$^{-/-}$ (H2^{d}) mice were bred and maintained at the animal facility of the Department of Research (University of Basel, Switzerland). CD45.1 (H2^{d}) congenic mice (BALB/c) were a kind gift of Antonius Rolink, Basel, Switzerland. All mice were bred mice under SPF conditions, the age of mice used as donors and recipients was 5-10 weeks. Mice were housed in sterile isolators after irradiation. All animal experiments were in accordance with institutional and Swiss national regulations and were approved by the local authorities.

Induction of graft versus host disease: For induction of graft versus host disease, mice were lethally irradiated (1300cGy, split in 2 doses) by a ^{60}Co source. CB6F1 (H-2^{b,d}) mice received intravenously either 1 x 10^{7} T cell depleted BALB/c (H-2^{d}) bone marrow or RAG2$^{-/-}$ BALB/c (H-2^{d}) bone marrow together with, depending on the experimental setting, either 5 x 10^{6} wild-type or IFN-γ$^{-/-}$ splenocytes or 2 x 10^{6} MACS sorted wild-type or IFN-γ$^{-/-}$ donor T cells from BALB/c, corresponding to a semi-allogeneic or haploidentical transplantation model. Control mice received 1 x 10^{7} bone marrow cells of Rag2$^{-/-}$ BALB/c or T cell depleted bone marrow of wild-type BALB/c (both H-2^{d}) with or without syngeneic T cells. Mice were kept on acidified drinking water supplemented with trimethoprim/sulfamethoxazole (Bactrim, Roche Pharmaceuticals, trimethoprim 32 mg/L, sulfamethoxazole 160 mg/L) for the duration of the experiments.

Broncho-alveolar lavage: Mice were euthanized by intraperitoneal administration of 100μg Penthothal® (Abbott), the inferior vein was cut and lungs were flushed with 5ml cold PBS through the right ventricle until they became completely white. Tracheotomy was performed with a tracheal canule and lungs were washed with 1ml cold PBS/0.1%BSA. The recovered BAL fluid was kept on ice until centrifugation. Cells were pelleted by centrifugation at 1000rpm at 4°C and the supernatants were immediately used for nitric oxide determination

(Griess-Reagent System, Promega) and TNFα quantification by ELISA (BD Opteia). Cells were resuspended in PBS/0.1%BSA and the total cell number was determined by exclusion of dead cells by trypanblue. Cell differential counts were performed on cytospin slides (Histocom AG, Zug, Switzerland) prepared from BAL fluid and stained with DiffQuick™ (Medion Diagnostics GmbH, Düdingen, Switzerland). All slides were evaluated in a light microscope (Laborlux K, Leitz, Germany) and standard morphological criteria were used. Four hundred to 600 cells per sample were counted.

Lung digestion: The inferior vein was cut and lungs were flushed with PBS through the right ventricle until they became completely white. Lungs were then removed, cut into small pieces and transferred in GKN digestion buffer (11mM D-glucose, 5.5mM KCl, 137mM NaCl, 25mM Na_2HPO_4, and 5.5mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4) supplemented with 10% FCS, containing 1.8 mg/ml collagenase type 4 (Worthington Biochemical Corporation, Lakewood, NJ) and 0.1 mg/ml Deoxyribonucleic acid I (DNAse I; Sigma Chemicals) and incubated for 90 min at 37 ^{0} C in a shaking water bath. After 60 min an additional 0.1 mg/ml DNAse I was added. Tissue was further disrupted by gently pipetting and was then passed through a 70 μm cell filter to remove tissue debris. After washing with GKN-10% FCS , erythrocytes were lysed with ACK and washed once with GKN-10% FCS, then cells were suspended in PBS.

Reverse Transcription PCR: RNA was isolated using Trizol™ (Invitrogen) from collagen digested lung cell suspensions. Prior to reverse transcription, RNA was digested with DNAse I for preparation of DNA-free RNA, using 1μg RNA, 10x Reaction Buffer with $MgCl_2$, DEPC-treated water and Deoxyribonuclease I (RNase-free) for 30min at 37°C, then 1μl of 25mM EDTA was added and incubated at 65°C for 10min. First strand cDNA synthesis was peformed as follows: RNA (1μg) was incubated with oligo$(dT)_{18}$ for 5min at 70°C and chilled on ice. To the mix was added; 5x reaction buffer, 10mM 4dNTP mix, RNase inhibitor and DEPC treated water, the mix was incubated for 5min at 37°C, finally

RevertAID™ M-MuLV Reverse Transcriptase was added and the reaction mixture incubated for 60min at 42°C. The reaction was stopped by heating for 10min at 70°C and chilled on ice (all Fermentas). The synthesized cDNA was used for PCR for following genes: IDO (indoleamine 2,3-dioxygenase) with primer forward 5`>gaaggatccttgaagaccac<3` and primer reverse 5`>gaagctgcgatttccaccaa<3` with annealing temperature 55°C for 30sec with 30 cycles of amplification for a product of 499bp; iNOS(inducible nitric oxid synthetase) with primer forward 5`>atggaccagtataaggcaagc<3` and primer reverse 5`>tgttgcattggaagtgaagc<3` with annealing temperature of 60°C for 30sec with 40 cycles of amplification for a product of 396bp; as a house keeping gene β-tubulin was used with primer forward 5`>ggaacatagccgtaaactgc<3` and primer reverse 5`>tcactgtgcctgaacttacc<3` with annealing temperature 60°C for 30sec for a product of 319bp. PCR was performed using TaqPCR Master Mix Kit (Quiagen). cDNA of cell lines MC22 (negative) and MC24 (positive) were used as IDO controls as described before [76]. cDNA of non- or IFNγ- stimulated M-CSF derived BM-macrophages served as iNOS controls, respectively.

Flow cytometry of lung cells: Single-cell lung suspensions from diseased and control mice were Fc-blocked with anti-mouse CD16/32 (eBioscience) and stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) direct labeled, or secondary antibodies to biotin labeled antibodies using Streptavidin-allophycocyanin-Cy7 (SAv-APC-Cy7), Streptavidin PerCP-Cy5.5 (SAv-PerCP-Cy5.5) labeled antibodies against CD3e (145-2C11), CD4 (L3T4), CD8α (53-6.7), MHC-II (I-A/I-E, clone 2G9)CD11b (M1/70), CD11c (HL3), CD45.1 (A20), CD45.2 (104) and BrdU (all from BD Pharmingen). Intracellular stains were performed after stimulation with 50 ng/ml PMA and 500 ng/ml ionomycin, together with Brefeldin A (10 ng/ml) for 6h. Cells were fixed with 2% paraformaldehyde and incubated with 0.1% Saponin (all chemicals from Sigma) and PE labeled antibody to IL-17 (Pharmingen). Propidiumiodide (PI) exclusion was routinely used to differentiate living from dead cells, when applicable. BrdU staining procedures followed available protocols (BrdU flow kit,

BD Pharmingen). Samples were analyzed on FACSCalibur (BD Biosciences) or Cyan (DAKO), using FlowJo (Treestar) Software. Depending on the experiment, cell sorting was performed with FACSAria (BD Biosciences) to isolate > 99% pure subpopulations $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells.

Magnetic cell sorting: Antibodies and isolation kits were purchased from Miltenyi Biotec GmbH (Cologne, Germany). Magnetic labelled CD90 (Thy 1.2) antibodies were used to deplete T cells from bone marrow. T cell subpopulations were positively selected from whole splenocyte suspensions using CD90 (Thy 1.2), CD4 (L3T4) or CD8α (Ly-2) antibodies coupled to magnetic beads following manufacturers instructions.

In vivo treatment with TREM-1 / anti-IL17 / anti-TNFα: Mice were injected on days 0, 3, 6, 9 and 12 with 100μg anti-mouse IL-17 (rat anti-mouse mAb, clone 50104; R&D Systems) or control Rat IgG (kindly provided by B. Becher, University Zürich) in 200μl PBS intraperitoneally. For experiments blocking TREM-1, a synthetic peptide mimicking a short highly conserved domain of soluble TREM-1 was injected i.p. every 12h with 100μg in 200μl 0.9% NaCl (sequence LQVTDSGLYRCVIYHPP, synthesized by ANAWA, Wangen, Switzerland as described before in ref. Gibot, et al.).
For depletion of granulocytes, mice were treated every 72h with a rat anti-mouse Gr1 (RB6-8C5) antibody by i.p. injection of 0.25 mg (kindly provided by R.Zinkernagel, Zürich).
For neutralization of TNFα, mice were treated with 40μg anti-TNFα(Enbrel®, Wyeth Pharmaceuticals AG, Zug) or vehicle (PBS) alone by intravenous injection on days 4, 8 and 11.

Bone marrow dendritic cell culture
Bone marrow cells were isolated from mice at 6-8 weeks of age. Cells were cultured in RPMI with 10% FCS (Gibco), as well as penicillin + streptomycin, L-glutamate and 2-mercaptoethanol (all from Sigma). Media were supplemented

with 200 U GM-CSF/mL (PeproTech). $2x10^6$ cells were cultured on non-tissue culture coated Petri dishes for 8-12 days. Media was changed on d3, d6, d8 and d10 of culture.

Semiquantitative evaluation of lung histopathology: Pulmonary toxicity after bone marrow transplantation was determined by examination of lung histopathology. H&E-stained lung sections from individual mice were coded without reference to mouse type or prior treatment regimen and independently examined by a pathologist to establish an index of injury. Grade 0: normal lung tissue, Grade 1: beginning periluminal infiltrates (around airways and vessels 1-3 cell diameters thick)) with 5-25% of lung tissue involved, Grade 2: 4-10 cell diameter thick periluminal infiltrates with 25-50% of lung tissue involvement, Grade 3: severe generalised (>50% lung tissue involvement) destructive inflammation with giant cells.

Results

Lethal IPS after co-transfer of bone marrow and IFN-$\gamma^{-/-}$ T cells

In order to address the role of Interferon gamma in the pathogenesis of IPS we adoptively transferred either $5x10^6$ wild-type BALB/c or IFN$\gamma^{-/-}$ BALB/c splenocytes together with $1x10^7$ RAG2$^{-/-}$ bone marrow cells (B and T cell deficient) into BALB/c X C57BL/6 (CB6F1) mice. Both groups of mice showed clinical signs of graft versus host disease with 20-25% weight lost and reduced physical activity 4 to 6 days after transplantation and no relevant differences in the weight curve between wild-type and IFN$\gamma^{-/-}$ transplanted mice could be observed so far (Fig. 11a). Whereas wild-type donor T cell reconstituted mice regained weight and survived up to 3-4 months after transplantation, mice transferred with IFN$\gamma^{-/-}$ donor T cells deteriorated rapidly developing signs of respiratory failure and had to be euthanized on day 13 (Fig. 11a).

The same lethal IPS phenotype developed when CB6F1 mice deficient for the IFNγR were reconstituted with $1x10^7$ RAG2$^{-/-}$ bone marrow cells and T cells,

irrespective of IFNγ deficiency in the T cell compartment (wild-type - or IFNγ$^{-/-}$ splenocytes) (Fig. 11a).

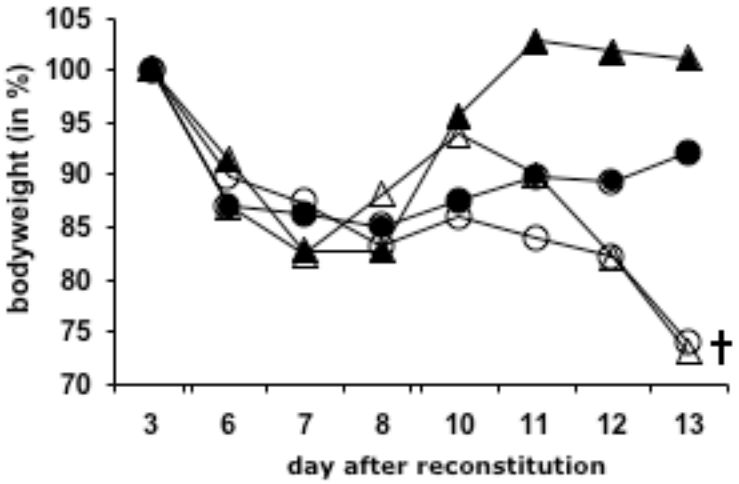


Figure 11a.) Lethal IPS induced by donor T cell IFNγ deficiency or host IFNγ Receptor deficiency. *CB6F1* and *CB6F1 IFNγR*$^{-/-}$ mice were irradiated and reconstituted with bm of BALB/c Rag2$^{-/-}$ and received either 5x10^6 BALB/c wt splenocytes (▲) or BALB/c IFNγ$^{-/-}$ splenocytes (Δ), or 2x10^6 BALB/c wt CD4$^+$ (●) or BALB/c IFNγ$^{-/-}$ CD4$^+$ (❍). Bodyweight was determined until day 13 after transplantation before IFNγ$^{-/-}$ CD4$^+$ recipients show mortality due to IPS.

Histological analysis of IFNγ$^{-/-}$ donor T cell treated and the IFNγR$^{-/-}$ recipients in semi-allogeneic bone marrow transplanted mice revealed almost complete destruction of the lung parenchyma, including massive infiltrates around airways and vessels and parenchymal pneumonitis. Infiltrates consisted of mononuclear cells, granulocytes, eosinophils, and several giant cells (Fig. 11b). In contrast, we observed only minimal mononuclear infiltrates mainly around blood vessels in the semi-allogeneic transplanted control group receiving wild-type donor T cells. No or only minimal inflammation was present in the lung of syngeneic transplanted mice (Fig. 11b). Of note, all semi-allogeneic transplanted mice developed intermediate hepatic graft versus host disease and intestinal involvement independent of the donor T cell phenotype.

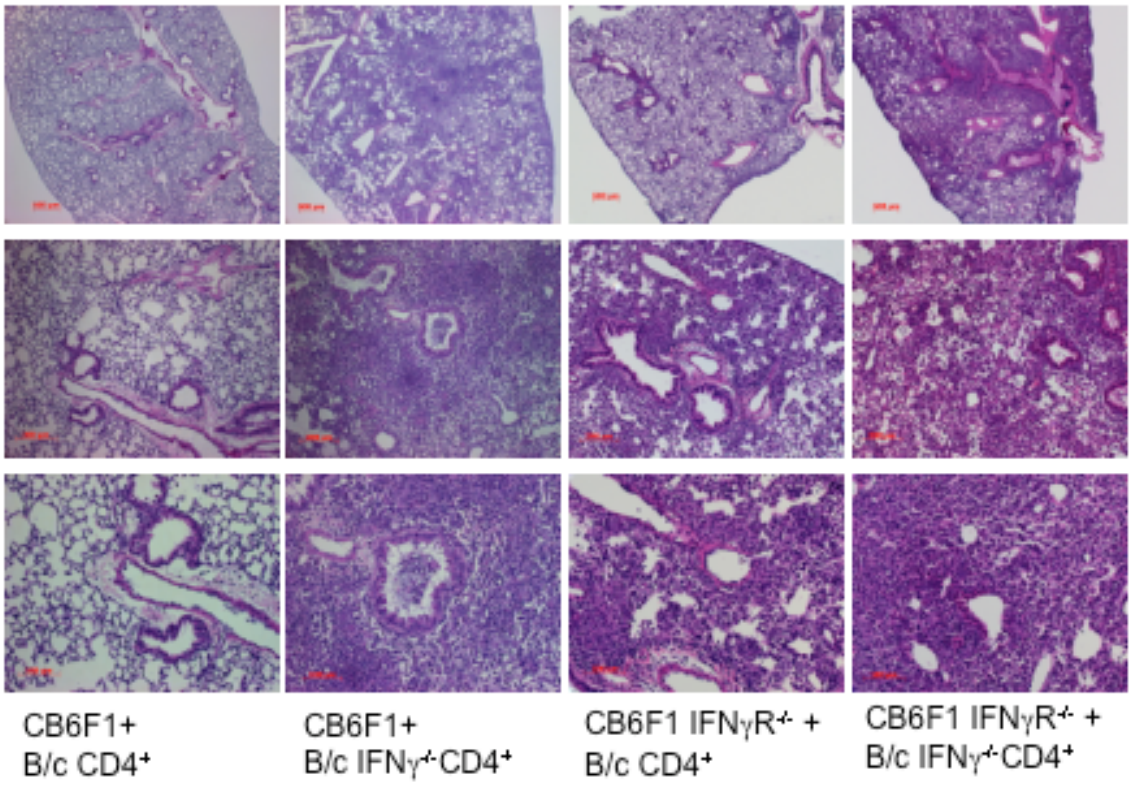


Figure 11b.) Lethal IPS induced by donor T cell IFNγ deficiency or host IFNγ receptor deficiency. Paraffin-embedded lungs of recipient mice were stained with hematoxylin/eosin. Representative photographs of one mouse from designated groups are shown at 25x, 100x and 200x magnifications of original (top to bottom).

FACS analysis of the digested lungs revealed an overall increase in $CD3^+$ T cells in the $IFN\gamma^{-/-}$ T cell treated mice compared to the wild-type T cell treated semi-allogeneic transplanted mice. Relative numbers of CD3/8 positive T cells exceeded numbers of CD3/4 positive T cells in the $IFN\gamma^{-/-}$ T cell treated semi-allogeneic transplanted animals (Fig. 11c). Taken together, pulmonary graft versus host disease is exacerbated and lethal in semi-allogeneic bone marrow transplanted mice in the presence of $IFN\gamma^{-/-}$ donor T cells or absence of $IFN\gamma^{-/-}$-recpetor on either lung APC or parenchymal tissue.

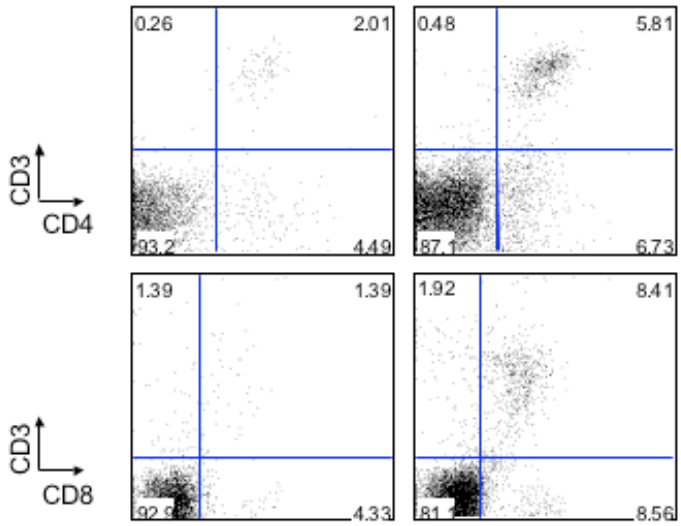


Figure 11c.) Lethal IPS induced by donor T cell IFNγ deficiency or host IFNγ receptor deficiency. Lung parenchym of *CB6F1* mice, reconstituted with bm of BALB/c $Rag2^{-/-}$ and either $5x10^6$ BALB/c wt splenocytes **(left)** or BALB/c $IFN\gamma^{-/-}$ splenocytes **(right)** was enzyme digested (see materials and methods) and single cell suspensions were stained for $CD3^+$ FITC, $CD8^+$ PE and $CD4^+$ APC.

We next analysed T-bet, the T box transcription factor that controls the expression of the hallmark Th1 cytokine, IFNγ. As shown previously in vitro stimulated $T\text{-}bet^{-/-}$ CD4 T cells were not able to produce significant amounts of IFNγ, whereas $T\text{-}bet^{-/-}$ CD8 T were capable to do so [22]. Similar to $IFN\gamma^{-/-}$ splenocytes, transfer of BALB/c $T\text{-}bet^{-/-}$ splenocytes into CB6F1 resulted in massive enhanced pulmonary GvHD underlining the important role of CD4 T cells deficient in IFNγ production (Table 4).

Table 4. Different outcome of IPS by designated reconstitution of bone marrow, T cell or APC populations and/or treatment strategies.

donor bm	donor T cells	donor APCs	recipient	IPS score
BALB/c IFNγR$^{-/-}$	BALB/c wt T cells	-	CB6F1	0, 0, 0
BALB/c RAG2$^{-/-}$	BALB/c wt T cells	BALB/c wt	CB6F1 IFNγR$^{-/-}$	2.5, 3, 3, 3
BALB/c IL12R$^{-/-}$	BALB/c IFNγ$^{-/-}$ T cells	-	CB6F1	3, 3, 3, 3, 3
BALB/c IL12p35$^{-/-}$	BALB/c IFNγ$^{-/-}$ T cells	-	CB6F1	3, 3, 3
BALB/c RAG2$^{-/-}$	BALB/c IFNγR$^{-/-}$ T cells	BALB/c wt	CB6F1	0, 0.5, 0, 0
BALB/c RAG2$^{-/-}$	BALB/c wt T cells	BALB/c IFNγR$^{-/-}$	CB6F1	0, 0.5, 0, 0
BALB/c RAG2$^{-/-}$	BALB/c T-bet$^{-/-}$ T cells	BALB/c wt	CB6F1	1, 2, 1, 1.5
BALB/c RAG2$^{-/-}$	BALB/c wt	BALB/c T-bet$^{-/-}$	CB6F1	0, 0, 0, 0
CB6F1	CB6F1	CB6F1	CB6F1	0, 0, 0
BALB/c RAG2$^{-/-}$	BALB/c IL12R$^{-/-}$	-	CB6F1	0, 0, 0, 0
BALB/c RAG2$^{-/-}$	BALB/c wt 100ug TREM1 peptide bid	-	CB6F1	0.5, 0.5
BALB/c RAG2$^{-/-}$	BALB/c IFNγ$^{-/-}$ T cells 100µg TREM1 peptide bid	-	CB6F1	3, 3, 3, 3
BALB/c RAG2$^{-/-}$	BALB/c IFNγ$^{-/-}$ T cells αGR-1 Ab every 72h	-	CB6F1	3, 3, 3
BALB/c RAG2$^{-/-}$	BALB/c wt αGR-1 Ab every 72h	-	CB6F1	0, 0

Recipient mice were irradiated as described and reconstituted with bone marrow, T cells and APCs from different donor mice summarized in the Table. On day 13 mice were sacrificed and IPS scored semi-quantitativly based on lung histopathology.

Lethal pulmonary graft versus host disease is CD4$^+$ T cell mediated

To further analyse the role of T cell subsets for the induction of the observed phenotype, we either transferred 2x10^6 MACS sorted wild-type or IFNγ$^{-/-}$ CD4 T cells or CD8 T cells into CB6F1. Whereas IFNγ$^{-/-}$ CD4 T cells induced a similar enhanced pulmonary destruction as 5x10^6 whole splenocytes, the inflammation induced by IFNγ$^{-/-}$ CD8 T cells was only minimal (Fig. 12a). The co-transfer of IFNγ$^{-/-}$ CD4 T cells and IFNγ$^{-/-}$ CD8 T cells only minimally enhanced the disease. Again, no or minimal IPS could be detected after transfer of wild-type CD4 or CD8 T cells. Accordingly, *in vivo* BrdU incorporation was clearly enhanced in CD4$^+$ T cells, whereas only few proliferating CD8$^+$ T cells could be detected (Fig.

12b). Taken together, these findings suggest that pulmonary graft versus host disease is mainly donor $CD4^+$ T cell mediated and is negatively regulated by IFNγ release from donor $CD4^+$ T cells.

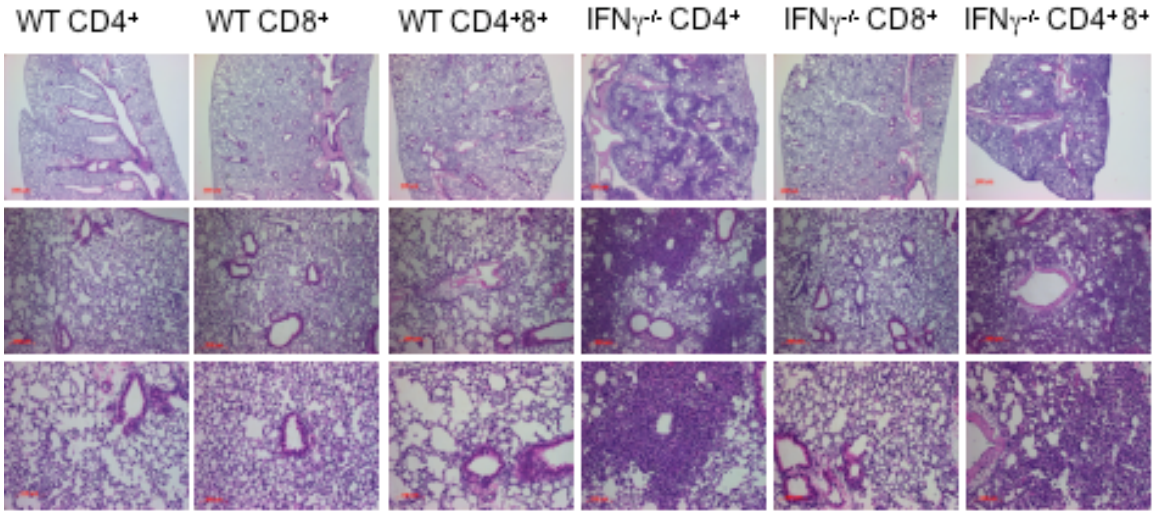


Figure 12a.) IPS is $CD4^+$ mediated. Hematoxylin/eosin stains of lungs from *CB6F1* mice reconstituted with $Rag2^{-/-}$ BALB/c bm and receiving $2x10^6$ sorted wt $CD4^+$, wt $CD8^+$, wt $CD4^+$ $CD8^+$, $IFN\gamma^{-/-}$ $CD4^+$, $IFN\gamma^{-/-}$ $CD8^+$ or $IFN\gamma^{-/-}$ $CD4^+$ $CD8^+$ (all BALB/c background). Mice were sacrificed at day 13, before $IFN\gamma^{-/-}$ $CD4^+$ recipients show mortality due to IPS. Representative photographs from one mouse per designated groups are shown at 25x, 100x and 200x magnifications of original (top to bottom).

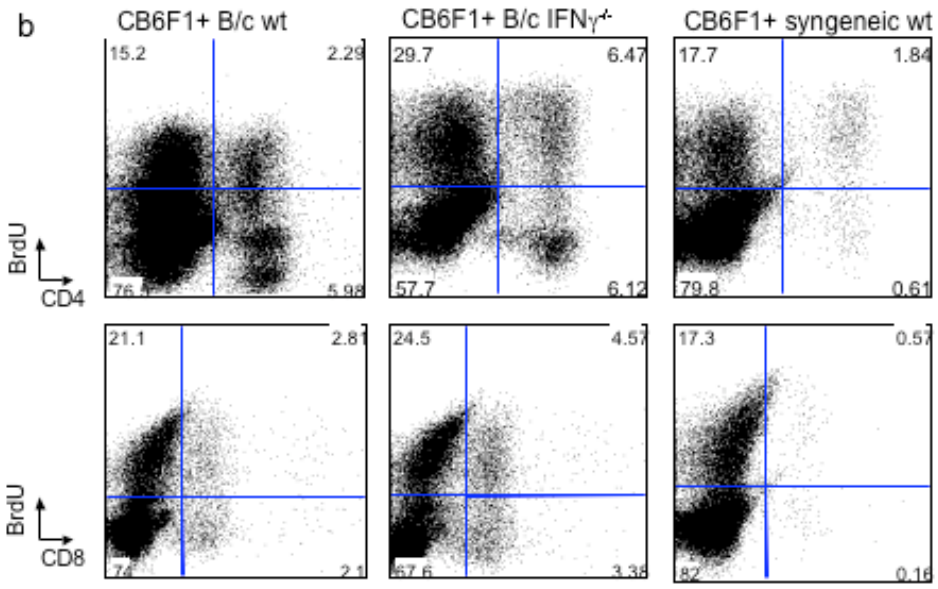


Figure 12b.) $IFN\gamma^{-/-}$ T cells show increased proliferation in the lung. BrdU was administered i.p. every 12h for the last 2 days before sacrifice on day 13, lung tissue was digested as described and stained for BrdU, $CD4^+$ and $CD8^+$. As control, *CB6F1* mice were reconstituted with syngeneic splenocytes.

Next we analysed the time course of the expansion of macrophages and dendritic cells after transfer of either wild-type, or IFNγ$^{-/-}$ CD4 T cells in the digested lung [142]. Six days after T cell transfer no significant difference could be observed between wildtype or IFNγ$^{-/-}$ T cells. However, ten days after transfer a significant amount of myeloid dendritic cells and macrophages were present in the IFNγ$^{-/-}$ situation compared to the wt T cell transfer (Fig. 13).

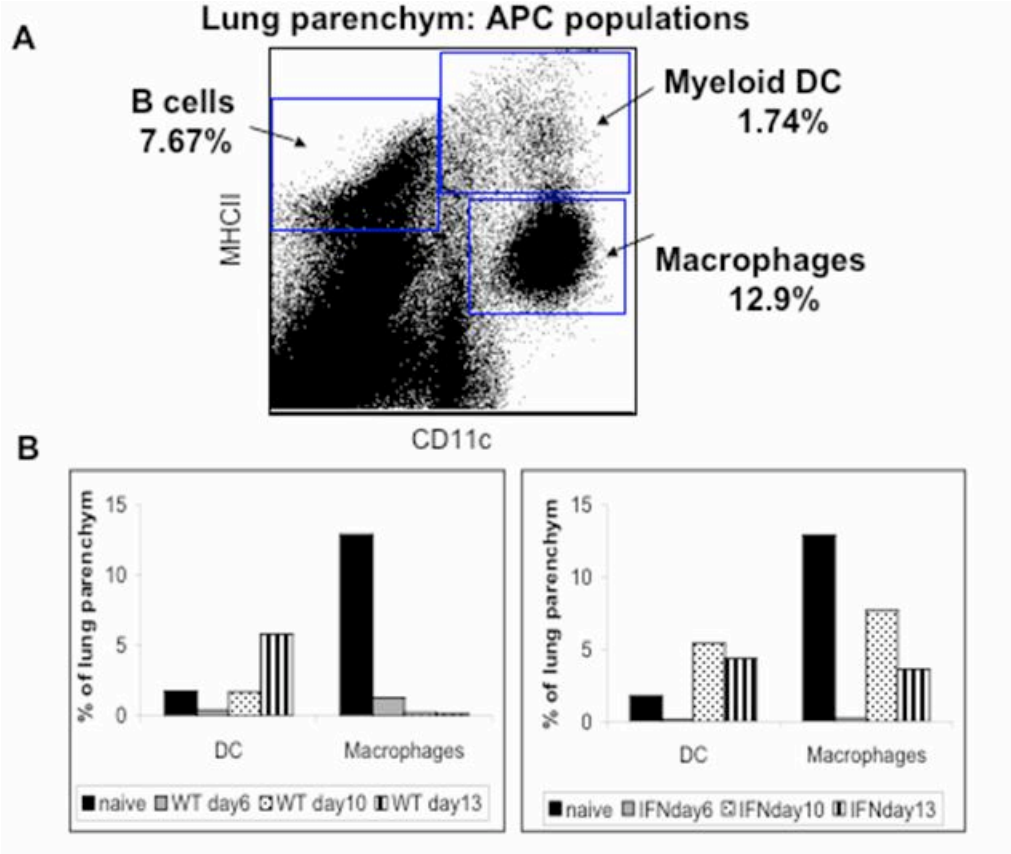


Figure 13.) Characterization of lung APC populations during IPS. A) Lung parenchym of a naïve *CB6F1* mouse was digested and stained for CD11c and MHC-II (I-A/I-E), five populations of antigen presenting cells can be discriminated as described before (reference CvG The Journal of Immunology, 2005, 175: 1609-1618.). B) Lung parenchym from *CB6F1* hosts reconstituted with Rag2$^{-/-}$ bm and either wt (left) or IFNγ$^{-/-}$ CD4^{+} (right) was stained for CD11c and MHC-II after enzymatic digestion at day 6, 10 and 13.

13 days after transfer similar amounts of myeloid dendritic cells were present in both situations whereas still almost no macrophages could be detected after wt T cell transfer (Fig. 14a). As shown in Figure 13 transfer of IFNγ$^{-/-}$ CD4 T cells resulted in a rapid repopulation of the CD11c high, MHC class II intermediate population (positive in CD11b, F4/80, CD105; not shown), whereas they almost

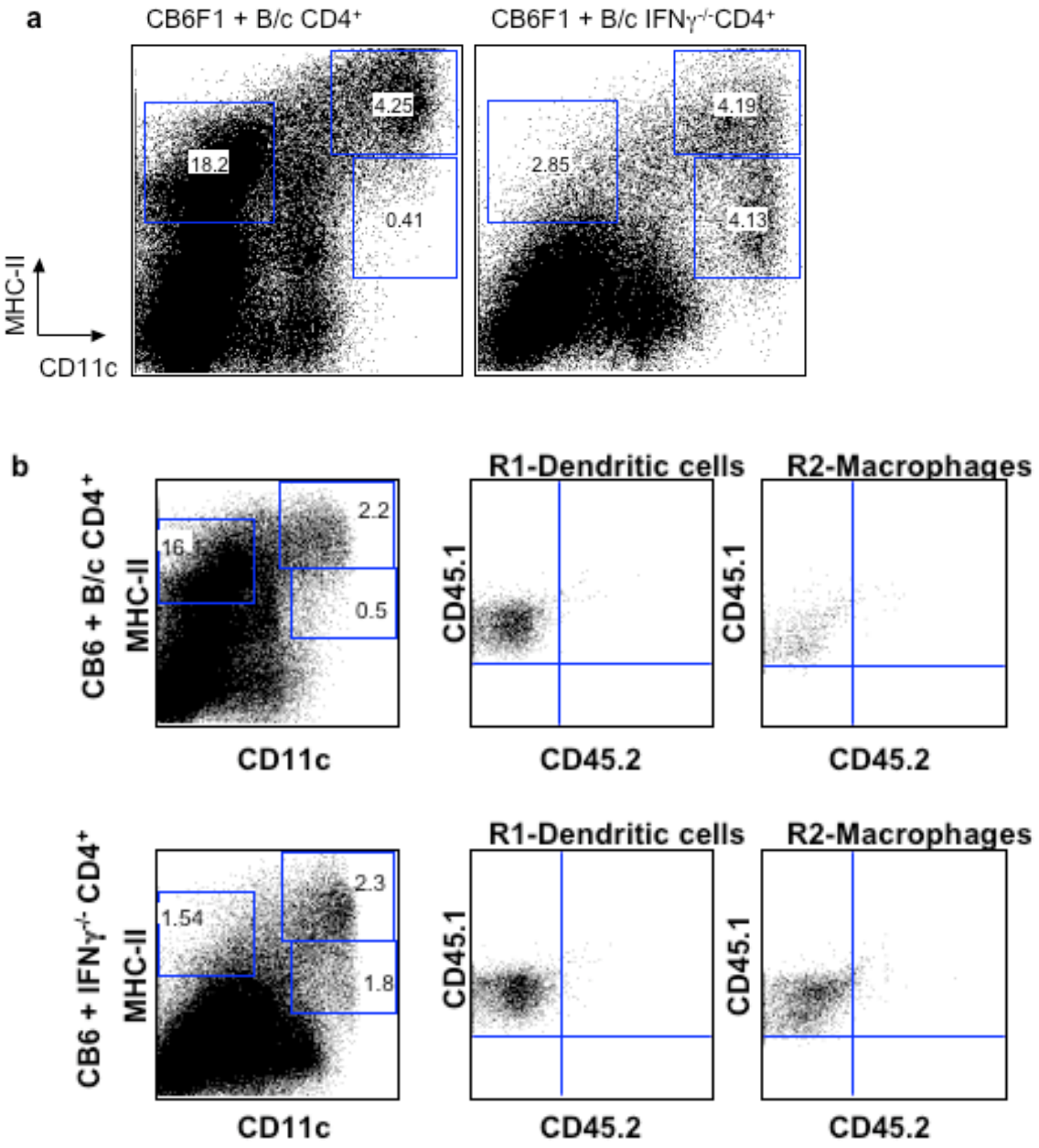


Figure 14.) Lung APC repopulation differs in absence of donor IFNγ and lung APCs are of donor origin. a) Lungs of *CB6F1* mice, reconstituted with Rag2$^{-/-}$ bm and either wt CD4^{+} or IFNγ$^{-/-}$ CD4^{+} (all BALB/c background) were perfused with cold PBS and lung parenchym digested as described before, single cell suspensions were stained for CD11c PE and MHC-II FITC (I-A/I-E). b) *CB6F1* hosts were reconstituted with T cell depleted BALB/c CD45.1^{+} bm plus wt or IFNγ$^{-/-}$ CD4^{+} (CD45.2^{+}). Lung digests of both groups were stained for CD11c PE, MHC-II FITC (I-A/I-E), CD45.1-APC-Cy7 and CD45.2-PerCP-Cy5.5.

lacked the MHC class II high, CD11c low population (CD11b negative; not shown). In contrast to the FACS analysis in lung tissue, in the BALF a significant increase in neutrophils could be observed in IFNγ$^{-/-}$ CD4 T cells transferred mice

and a relative decrease in macrophages (Fig. 15). However, these are relative numbers, where the total BALF cell number was increased by factor 10 in IFNγ$^{-/-}$ T cell transferred mice. Next we analysed the origin of these accumulating macrophages and dendritic cells using the CD45.1/CD45.2 system. T cell depleted bone marrow of CD45.1 BALB/c mice was transferred together with CD45.2 BALB/c IFNγ$^{-/-}$ CD4 T cells into lethally irradiated CD45.2 positive CB6F1 or CD45.1 BALB/c wild-type CD4 T cells. As depicted in Figure 14b, almost all macrophages, dendritic cells and precursor cells were of donor origin. This indicates a rapid turnover of the hematopoietic system after bone marrow transplantation.

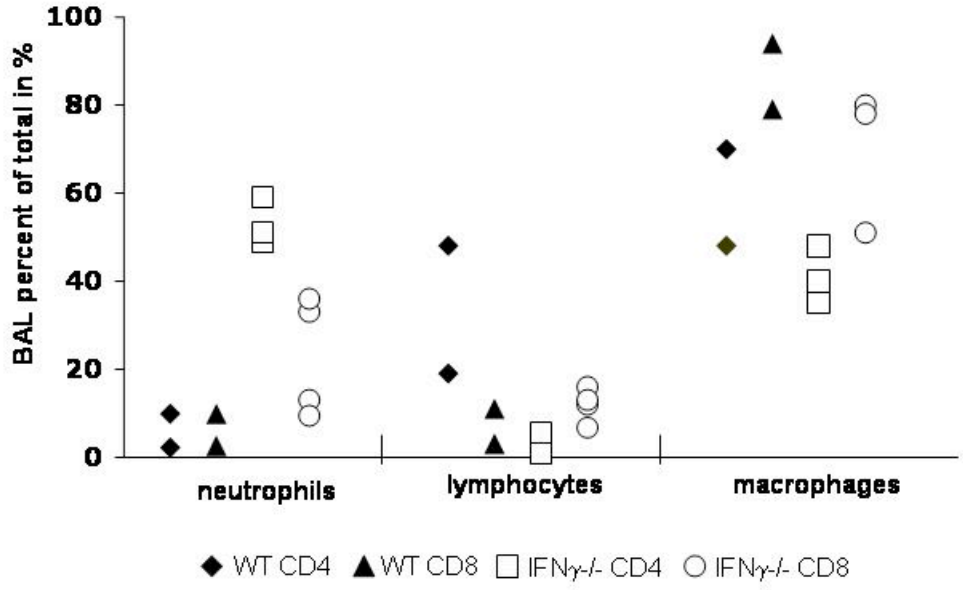


Figure 15.) Influence of donor T cells on BAL cell content. BAL cells in IPS. BAL was performed at day 13 from *CB6F1* host mice reconstituted with Rag2-/- BM and wt CD4^{+}, wt CD8^{+}, IFNγ$^{-/-}$ CD4^{+} or IFNγ$^{-/-}$ CD8^{+}. BAL cells were counted, spun onto glass slides and stained with Diff-Quik®. Neutrophils, lymphocytes and macrophages were counted based on their light microscopical appearance.

We next addressed the question whether different sources of donor bone marrow or donor APC could influence the course of the disease. To this end, we transferred T cell depleted bone marrow of BALB/c IL12R$^{-/-}$, IL12p35$^{-/-}$, IFNγ$^{-/-}$ or IFNγR$^{-/-}$ mice with wildtype T cells. In none of the described situation, however, a relevant pulmonary GvHD was observed (Table 4). Similarly, Rag2$^{-/-}$ bone marrow together with T-bet$^{-/-}$, IFNγ$^{-/-}$ or IFNγR$^{-/-}$ APC (T cell depleted splenocytes) and wildtype T cells was not associated with pulmonary inflammation (Table 4). We therefore conclude that the absence of IFNγ release from donor CD4^{+} T cells or the missing negative feedback loops are the only relevant factors for enhanced disease severity.

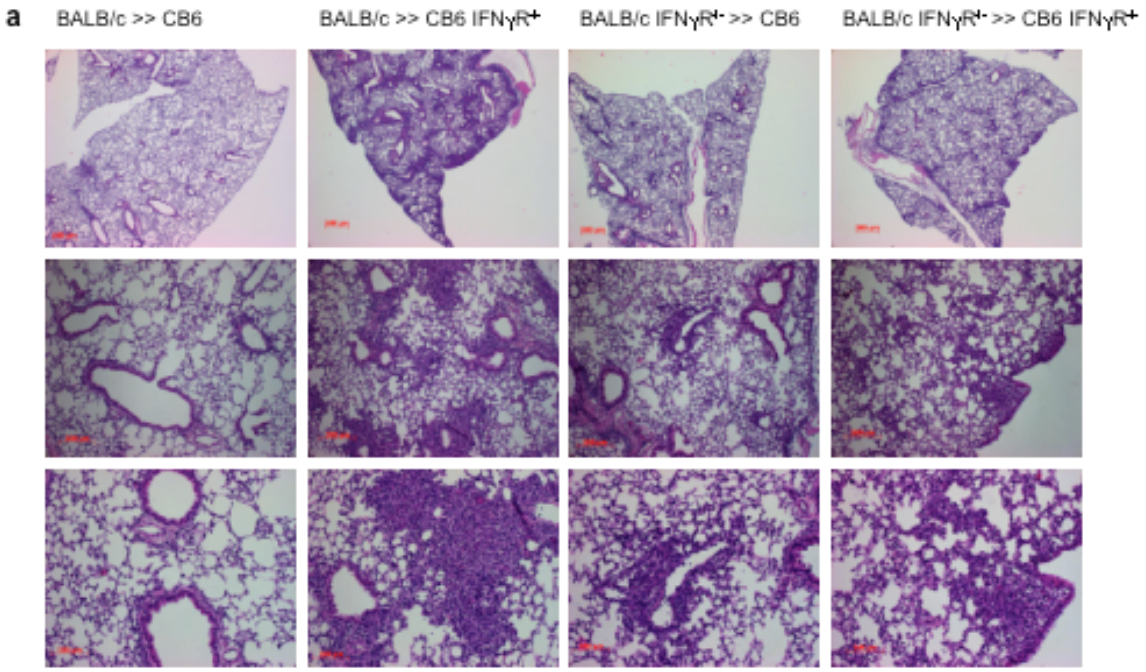
Radio-resistant host parenchymal cells mediate a IFNγ dependent negative feedback on donor CD4^{+} T cells

So far, it is not clear whether the absence of IFNγ receptor signalling on lung parenchymal cells or persisting, radio resistant host APCs is crucial for the observed phenotype. In order to answer this question we generated bone marrow chimeras by adoptive transfer of CB6F1 bone marrow into CB6F1-IFNγR$^{-/-}$ recipients and CB6F1-IFNγR$^{-/-}$ bone marrow into CB6F1 recipients. After 3 months these chimeras were treated with Rag2$^{-/-}$ BALB/c bone marrow and wild-type CD4^{+} T cells. As shown before in Figure 14b, using the CD45.1/CD45.2 system, all host APC are replaced by donor APC, we could exclude relevant contamination of host APCs after 3 months. IPS could only be observed in IFNγR$^{-/-}$ host independent if they have received wild-type bone marrow or IFNγR$^{-/-}$ bone marrow (Fig. 16a). Only minimal disease was detected in wild-type hosts receiving either wild-type bone marrow or IFNγR$^{-/-}$ bone marrow. The phenotype is therefore dependent on the IFNγ signaling on lung parenchymal cells and the resulting feedback to the infiltrating T cells.

Table 5. Long-term bone marrow chimera.

Group	Recipient	First donor BM	Second donor BM	Donor T cells	IPS score
1	CB6F1	BALB/c wt	BALB/c Rag2$^{-/-}$	BALB/c CD4	0, 0.5, 0.5
2	*CB6 IFNγ R$^{-/-}$*	BALB/c wt	BALB/c Rag2$^{-/-}$	BALB/c CD4	2, 3, 3
3	*CB6F1*	BALB/c IFNγR$^{-/-}$	BALB/c Rag2$^{-/-}$	BALB/c CD4	1, 1, 1
4	*CB6 IFNγ R$^{-/-}$*	BALB/c IFNγR$^{-/-}$	BALB/c Rag2$^{-/-}$	BALB/c CD4	2, 2, 2

Recipient mice were irradiated and reconstituted with 1.donor BM for 3 months, followed by a second irradiation and reconstitution with 2.donor BM plus donor T cells. After 12 days, mice were sacrificed and IPS scored semiquantitatively based on lung histopathology.



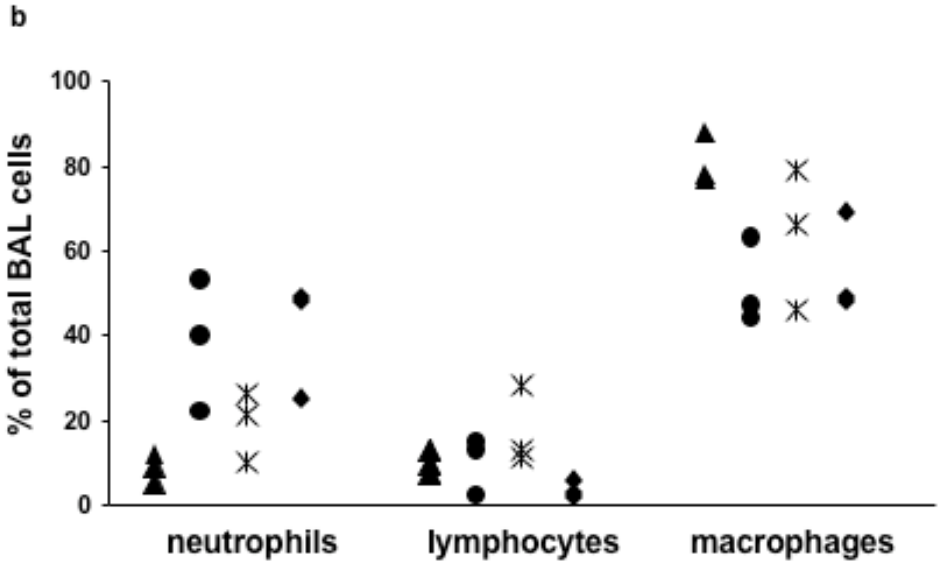


Figure 16.) Long term bm chimera reveal importance of host lung parenchym. Bone marrow chimeric mice were irradiated once (950cGy), were i.v. injected with designated bm ($1x10^7$ cells), then were allowed reconstitution for 3 months (group1: BALB/c >> CB6F1, group2: BALB/c >> CB6F1 IFNγR$^{-/-}$, IFNγR$^{-/-}$ BALB/c >> CB6F1 IFNγR$^{-/-}$, group 4: BALB/c IFNγR$^{-/-}$ >> CB6 IFNγR$^{-/-}$), before receiving a second irradiation (950cGy) and i.v. Rag2$^{-/-}$ bm and wt CD4^{+}. a) Hematoxylin/eosin stains of lungs from one mouse per designated groups are shown at 25x, 100x and 200x magnifications of original (see Table 3.). b) Broncho-alveolar lavage was performed at day 10 after the second irradation / reconstitution of designated groups (group 1 = ▲, group 2 = ●, group 3 = ✳, group 4 = ◆). BAL cells were counted, spun onto glass slides and stained with Diff-Quik®. Neutrophils, lymphocytes and macrophages were counted based on their light microscopical appearance.

Mechanism of enhanced pulmonary GvHD in absence of IFNγ in donor T cells

We next measured the production of nitric oxide and TNFα in the BALF of either wt or IFNγ$^{-/-}$ T cell treated mice. Not surprisingly, in absence of IFNγ in donor T cells no nitric oxide but enhanced levels of TNFαwas present ten days after transfer (Fig. 17a/b). Semiquantitative RT-PCR of the digested lung 13 days after transfer of either IFNγ$^{-/-}$ or wild-type CD4 T cells showed comparable iNOS mRNA levels in both situations, but no nitric oxide was found in BAL of IFNγ$^{-/-}$ CD4 T cell recipients (Fig. 17c). No IDO mRNA could be detected after transfer of IFNγ$^{-/-}$ CD4 T cells (Fig. 17c).

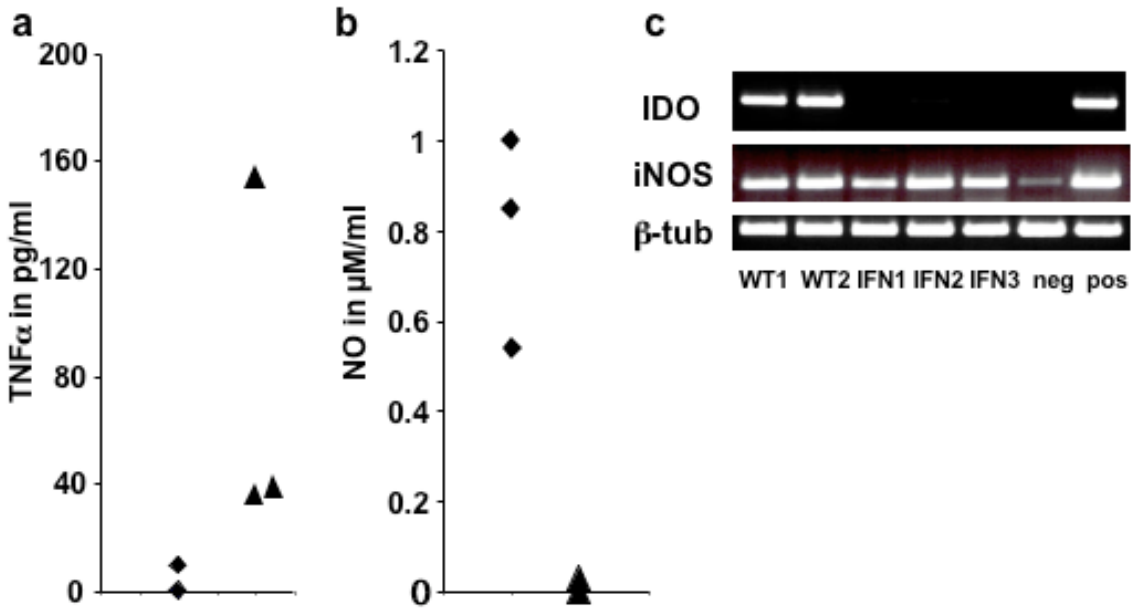


Figure 17.) IFNγ dependent factors in IPS diseased lung. a) TNFα concentration in BAL from perfused lungs of *CB6F1* hosts with wt or IFNγ$^{-/-}$ CD4^{+} was determined by ELISA. b) Nitric oxide was measured in BAL fluid of *CB6F1* hosts with wt or IFNγ$^{-/-}$ CD4^{+} by Griess Reagent. c) *CB6F1* hosts with wt (samples WT1 and WT2) or IFNγ$^{-/-}$ CD4^{+} (samples IFN1-3) were sacrificed at day13, RNA was isolated from cells after lung digestion using Trizol™. Reverse Transcriptase - PCR was performed for IDO, iNOS and β-tubulin. cDNA of MC22 (negative) and MC24 (positive) were used as IDO controls [76]. cDNA of non- or IFNγ- stimulated M-CSF derived bm-macrophages served as iNOS controls, respectively.

As a therapeutical approach we neutralized TNFα using 40µg i.v. of the commercial available anti-TNFα (Etanercept) antibody four, eight and eleven days after transfer of either wt or IFNγ$^{-/-}$ T cells. Mice treated with wt T cells and eternacept had increased nitric oxide levels in their BALF, whereas IFNγ$^{-/-}$ T cells treated mice were not able to increase their nitric oxide levels (Fig. 18a). However, eternacept administered to IFNγ$^{-/-}$ treated mice resulted in reduced levels of neutrophils and compensatory higher macrophages in BAL (Fig. 18b). The severity of pulmonary GvHD was slightly reduced and more localized by neutralizing TNFα, arguing for a role in the patho-mechanism of pulmonary GVHD (Fig. 18c).

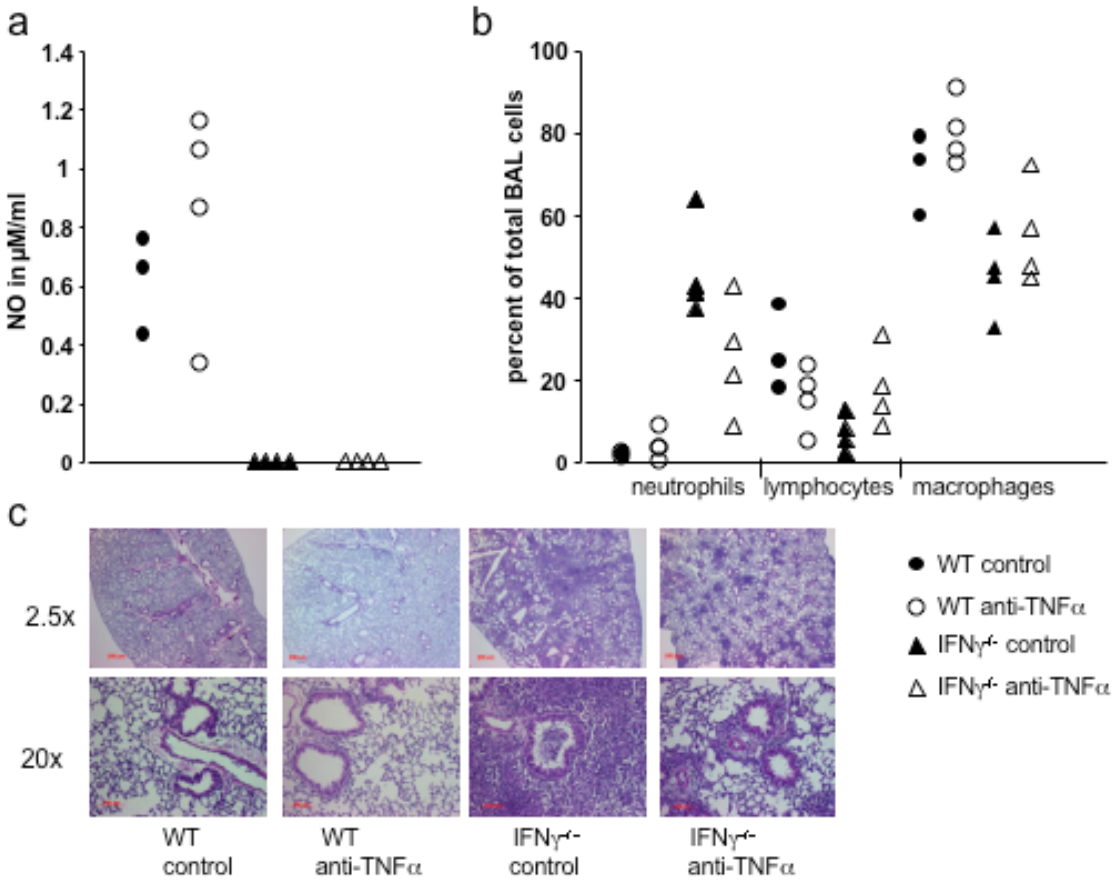


Figure 18.) Anti-TNFα treatment has beneficial effect on severe IPS. *CB6F1* hosts were reconstituted with Rag2$^{-/-}$ bm and wt or IFNγ$^{-/-}$ CD4^{+} and received i.v. 40µg anti-TNFα (Enbrel, Wyeth Pharmaceuticals AG, Zug) on days 4, 8 and 11, control mice received vehicle alone (PBS). Mice were sacrificed on day 13, after perfusion of lungs, BAL was obtained. a) Nitric oxide levels in BAL fluid of untreated and anti-TNFα treated mice was determined by Griess reagent. b) BAL cells of untreated and anti-TNFα treated mice were counted, spun onto glass slides and stained with Diff-Quik®. Neutrophils, lymphocytes and macrophages were counted based on their light microscopical appearance. c) Histopathology of lungs, shown are 25x and 200x magnifications of original Hematoxylin/Eosin stains of one representative mouse per designated group (each group n=4).

Given the histological picture showing massive infiltrations of granulocytes in the destroyed lung tissue, we first asked whether these cells are directly and critically involved in the pathogenesis of the observed disease phenotype. To address this question, we depleted granulocytes in groups of semi-allogeneic bone marrow transplanted and IFNγ$^{-/-}$ donor T cell co-transferred mice with a Gr-1 antibody. No relevant changes in the disease severity could be observed in depleted mice compared to controls (Table 4). Similar results were obtained after treatment with TREM1 (triggered receptor on myeloid cells) peptide [143]. This peptide has

been shown to inhibit both granulocytes and macrophages functions (Table 4). These findings clearly show that neither donor nor host granulocytes are required for disease development.

Lack of IFNγ in donor CD4$^+$ T cells promotes the IL-17$^+$CD4$^+$ subset expansion

As we could observe a clear increase in infiltrating granulocytes and macrophages after transfer of IFNγ$^{-/-}$ T cells, we analyzed the role of CD4$^+$IL-17$^+$ T cells in the pathogenesis of IPS [144, 145]. This subset of CD4 T cells, characterized by IL-17 production, has been described to mediate tissue inflammation in a mouse model of autoimmune disease [110]. Furthermore, *in vitro* data suggest that IFNγ negatively regulates autoimmune pathogenicity of CD4$^+$IL-17$^+$ T cells [146]. We therefore asked whether the generation of IL-17$^+$ alloreactive CD4$^+$ T cells is affected in the absence of IFNγ.

We first measured IL-17 production in supernatants of *in vitro* co-cultures of CB6F1 dendritic cells and naive BALB/c IFNγ$^{-/-}$ T cells vs. wild-type T cells. In fact, IL17 was detectable in supernatants of IFNγ$^{-/-}$ T cell only after 96 hours of culture. Next, we sacrificed IFNγ$^{-/-}$ T cell or wild-type T cell treated mice 13 days after bone-marrow transplantation and donor T cell transfer and analyzed the capacity of the lung infiltrating cells to produce IL-17 after overnight re-stimulation with C57BL6 dendritic cells. Again, lung infiltrating cells from IFNγ$^{-/-}$ donor T cell treated mice produced significant amounts of IL-17, whereas no or minimal IL-17 was detectable in mononuclear pulmonary infiltrates of wild-type donor T cell treated controls (Fig. 19a). In accordance with this finding, FACS analysis of pulmonary infiltrates showed markedly enhanced numbers of IL-17 producing CD4$^+$ T cells in lung infiltrating CD4 T cells 13 days after transfer of donor IFNγ$^{-/-}$ CD4 T cells compared to wild-type donor CD4 T cells (Fig. 19b). To directly address the role of IL-17 in the pathogenesis of the enhanced IPS after co-transfer of donor IFNγ$^{-/-}$ T cells in the semi-allogeneic bone marrow transplantation setting, we treated groups of mice with an IL-17 depleting antibody. Control groups received an isotype control antibody. IL-17 depletion in

fact resulted in less weight loss compared to isotype treated transplanted/donor IFNγ$^{-/-}$ T cell-reconstituted mice. Furthermore, histology revealed a reduced but not absent IPS scores in IL-17 treated mice. Taken together, lack of IFNγ production in donor T cells promotes the marked expansion of the IL-17^{+}CD4^{+} T cell subset, which is one major co-factor in the aggravation of graft versus host mediated pulmonary disease.

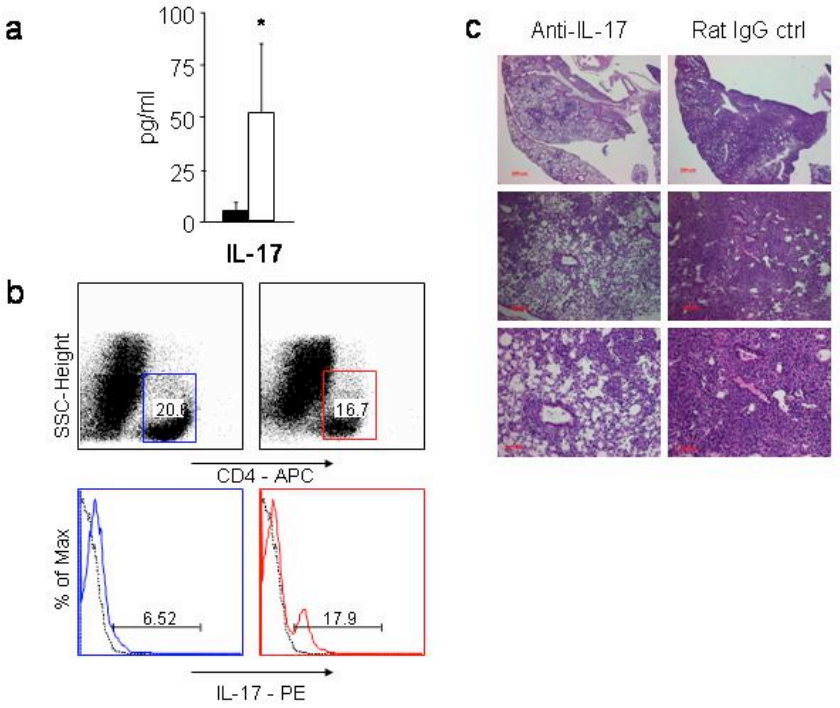


Figure 6.) IL-17 up-regulation in severe IPS. a) Lung infiltrating cells of *CB6F1* hosts reconstituted with Rag2$^{-/-}$ bm plus wt or IFNγ$^{-/-}$ CD4^{+} were restimulated for 48h on C57BL6 BM-DCs. Culture supernatants were analyzed for IL-17 by ELISA. Mean per group ± SD is shown. b) Lung infiltrating cells were stained for CD4 (APC) and intracellular IL-17 (PE), histograms shown are gated on CD4^{+} cells, (wt CD4^{+} recipients – left = blue colour, IFNγ$^{-/-}$ CD4^{+} recipients – right = red colour). c) Anti-IL17 mAb (100µg/mouse) was administered i.p. on days 0, 3, 6, 9 and 12 to *CB6F1* hosts reconstituted with Rag2$^{-/-}$ bm and IFNγ$^{-/-}$ splenocytes. A control group, *CB6F1* hosts reconstituted with Rag2$^{-/-}$ bm and IFNγ$^{-/-}$ splenocytes, received normal Rat IgG (100µg/mouse). Hematoxylin/eosin lung stains from representative animals are shown. Pictures shown are 25x, 100x and 200x magnifications of originals (from top to bottom).

Given the histological picture and the FACS data showing massive infiltrations of granulocytes and macrophages in the destroyed lung tissue and the fact that IL-17 induces recruitment of granulocytes and macrophages, we asked whether

these cells are directly and critically involved in the pathogenesis of the observed disease phenotype. To address this question, we depleted granulocytes in groups of semi-allogeneic bone marrow transplanted and IFNγ$^{-/-}$ donor T cell co-transferred mice with a Gr-1 antibody. No relevant changes in the disease severity could be observed in depleted mice compared to controls (Table 4). Similar results were obtained after treatment with TREM1 (triggered receptor on myeloid cells) peptide [143]. This peptide has been shown to inhibit both, granulocytes and macrophages functions (Table 4). These findings clearly show that neither donor nor host granulocytes are required for disease development.

Discussion

Accordingly, mice lacking IFNγ show high mortality due to persistent T cell activation after infection with non-cytopathic viruses [147-149] . Similarly, CD4$^+$ T cell mediated autoimmune disease models [13, 60, 150], show exacerbated disease and persistence of activated, self antigen-specific CD4$^+$ T cells in the absence of IFNγ.

In addition, IFNγ is also released from activated CD8 T cells and critically controls viral and intracellular bacterial infections [151]. In contrast, recent findings suggest that IFNγ negatively regulates the generation of a specific subset CD4$^+$ T cells characterized by IL-17 [22, 146]. The view, that IFNγ also exerts anti-inflammatory properties is further supported by the observation that IFNγ regulates the extent of CD4 and CD8 T cell expansion and contraction upon antigen challenge. In fact, lack of IFNγ in CD8 T cells results in decreased contraction of activated T cells [152, 153]. In our experiments we found lethal lung destruction after co-transfer of IFNγ deficient donor CD4 T cells in a setting of semi-allogeneic bone marrow transplantation in mice. The observed disease phenotype was IFNγ$^{-/-}$ CD4$^+$ T cell associated with the expansion of the CD4$^+$IL-17$^+$ T cell subset. Of note, the observed enhanced phenotype was restricted to the lungs and not observed in other organs usually affected by graft versus host disease.

Our findings provide evidence that $CD4^+$ but not $CD8^+$ T cells are critical for pulmonary graft versus host disease and clearly show that severe disease developed only in the absence of IFNγ in CD4 T cells or in absence of IFNγR on lung parenchymal cells. Neither $IFN\gamma^{-/-}$ donor APCs reconstituted with donor syngeneic T cells nor IFNγ receptor deficient donor T cells or APCs were relevant for the degree of lung destruction.

Several factors seem to be involved in absence of $IFN\gamma^{-/-}$ and the thereby missing autocrine/paracrine loop. Nitric oxide production is impaired and the induction IDO is missing. Additionally, it has been recently shown, that IFNγ negatively regulates the development of IL-17 producing CD4 T cells [110]. The generation of IL-17 producing CD4 T cells is critical dependent on IL23 [18, 19] and seems to represent a different T helper cell lineage apart from type 1 and 2 [14, 110]. IL-17 producing CD4 T cells are pathogenic for experimental allergic encephalitis (EAE) [17, 18] and collagen-induced arthritis [19]. In addition, IL-17 may play a direct role in allograft rejection [154, 155]. Our data provide *in vivo* evidence that $IFN\gamma^{-/-}$ $CD4^+$ T cells promotes the expansion of IL-17 producing donor $CD4^+$ T cells in graft-versus-host disease. Furthermore, we have shown that $IL\text{-}17^+CD4^+$ T cells accumulate in the lung. A direct pathogenic role for IL-17 was suggested from the observation that IL-17 depletion ameliorates IPS severity after semi-allogeneic bone marrow transplantation and $IFN\gamma^{-/-}$ donor T cell co-transfer. However, blocking IL17 production could not completely inhibit IPS, either because our antibody is not efficient enough or because IL17 is only one out of several important players in disease pathogenesis.

Interestingly, the lungs are highly susceptible to $CD4^+IFN\gamma^{-/-}$ T cell mediated effects or to absence of IFNγR on lung tissue. This seems to be a unique and organ-specific feature of the pulmonary endothelia or epithelia. Nevertheless, transgenic over-expression of IL-17 in lung epithelial cells leads to severe lung inflammation [14]. In this context, we must also ask, which cytokines other than IFNγ are involved in the lung-specific accumulation of $CD4^+IL\text{-}17^+$ T cells. Cytokines of interest might involve TGF-β and IL-6, which might be critical for the local expansion of $CD4^+IL\text{-}17^+$ T cells in the tissue [156].

IL-17 release in tissues results in recruitment of granulocytes and macrophages. Granulocytes release enzymes and highly reactive oxygen species aggravating tissue destruction and inflammation. In IPS, however, depletion of granulocytes does not prevent severe disease after semi-allogeneic bone marrow transplantation and IFN$\gamma^{-/-}$ donor T cell co-transfer. Obviously, IL-17 mediated effects are far more complex and might involve recruitment of many other inflammatory cells as well as alternative pathways of tissue destruction.

The exact mechanism how IFNγ mediates contraction of the activated T cell compartment is not clear yet. IFNγ dependent induction of inducible NO synthetase [13, 90] and/or indoleamine 2,3-dioxygenase (IDO) [83, 157] in antigen-presenting cells (APC) via lung parenchymal cells or in themselves that results in release of NO or IDO metabolites mediating growth arrest and/or apoptosis of activated T cells is only one mechanism under discussion. However, which other protective factors are induced by IFNγ signalling on pulmonary parenchymal cells must be further investigated.

Conclusions

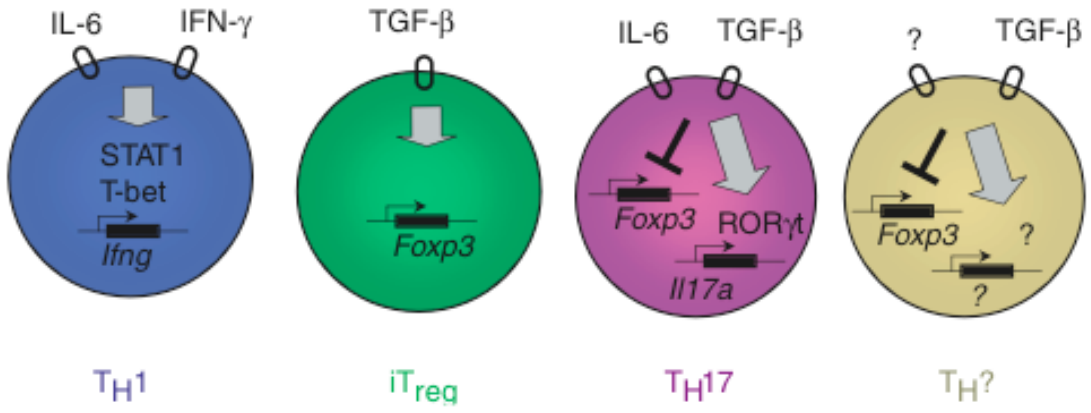
In the here presented thesis, the functions and interplay of two cytokines, Interferonγ and IL-17 were studied in a model for heart specific autoimmunity and in a bone marrow transplantation model that affects the lung.

In mice deficient for the Th1 defining transcription factor T-bet, it could be shown that autoimmune myocarditis is mediated by IL-17 producing T cells. Using several cytokine deficient mice, it became evident that neither typical Th1 cytokines like IFNγ or IL-12, nor typical Th2 cytokines, like IL4, are needed for disease pathogenesis. But rather, IL-23, which shares one subunit of the ligand and the receptor with IL-12 (IL-12p40 and IL12Rβ1) was absolutely crucial for disease induction, as the double ko mice T-bet$^{-/-}$ IL-12Rβ1$^{-/-}$, which cannot respond to IL-23, were totally protected from EAM. In wildtype mice, CD8$^+$ T cells act via bystander suppression by secretion of IFNγ and hence suppressing local IL-17 production, but T-bet deficient CD8$^+$ T cells in the target organ loose their ability to produce IFNγ.

In the second part of the graft versus host mediated idiopathic pneumonia syndrome, we could show that severe lung inflammation develops after bone marrow transplantation, when donor CD4$^+$ T cells are IFNγ deficient. CD4$^+$ T cells are transfering the observed phenotype, as CD8$^+$ T cells did not mediate lung inflammation by themselves. Interestingly, when the host could not respond to IFNγ (by deficiency of the IFNγ receptor), the same IPS phenotype develops, irrespective of the donor T cell IFNγ secretion. We identified that host parenchymal cells and donor T cells stand in an important interplay to suppress allo-antigeneic immune responses after bone marrow transplantation. The mechanism of IPS disease development is likely combined mechanistically of four IFNγ dependent factors; Indoleamine 2,3-dioxygenase, nitric oxide, IL-17 and TNFα.

Both models of autoimmune myocarditis and idiopathic pneumonia syndrome shed new light on the important function of IFNγ. After it was seen mainly in having a pro-inflammatory role, we know now that several

immunosuppressive effects are regulated by it. First of all, IFNγ is crucial to suppress the induction of IL-17 [110]. For the decision of a naïve T cell to differentiate into the Th17 direction, two main cytokines are needed; TGF-β and IL-6. TGF-β gained a double edge side through its involvement in immunosuppression by in vivo induced regulatory T cells and on the other hand its involvement in inducing the main pro-inflammatory Th17 cell subset. Depending on the micro milieu, TGF-β in combination with IL-6 results in Th17 induction, as well as IL-23R expression on T cells, which further sustains and strengthens the Th17 phenotype [21]. In the context of a more anti-inflammatory cytokine milieu, TGF-β induces Foxp3 (induced T_{reg}) and therefore cooperates to create an immunosuppressive effect on inflammatory responses.



from: Bettelli E, Oukka M, Kuchroo VK. Th17 cells in the circle of immunity and autoimmunity, Nat Immunol 2007; 8:345-50 [21]

IFNγ can further positively modulate an immunosuppressive response by inducing IDO and NO (through iNOS). We show the complete lack of IDO on the mRNA level in lungs of mice that received IFNγ$^{-/-}$ CD4^{+} T cells. And although iNOS expression in lung parenchym of mice with severe IPS was similar, no NO was detectable in BAL of IFNγ deficient T cell recipients.

Several other cytokines are known to effect and synergistically act together with IL-17. TNFα and IL-1 were shown to increase IL-17 production in vitro [16]. IL-1R1 deficient mice do not respond with a strong Th17 response after immunization, and IL-1R1 deficient T cells cultured with IL-23 do not produce IL-

17 [158]. In EAM, IL-1R1 deficient mice are protected and do not elicit a $CD4^+$ T cell response nor do they produce any IL-17 and IFNγ upon restimulation with MyHC-α (N.Mauermann, U. Eriksson, unpublished results). In both our EAM and IPS models, the pro-inflammatory cytokine TNFα was enhanced, when the IL-17 response was dominating due to IFNγ or T-bet deficiency in the $CD4^+$ T cell compartment. IL-1β and TNFα both are both capable to induce an inflammatory response by activating the key transcription factor NF-κB. A recent publication reports the interaction of TGFβ signalling with TNFα and IL-1 responses [159]. The anti-inflammatory function of TGFβ is partly mediated by inhibitory adaptors called Smad proteins, some of them are induced by TGFβ. In this report, Smad7 is shown to block TNFα - induced NF-κB activation, by binding TAB2 and TAB3 (they regulate the activity of kinase TAK1, the key in activating NF-κB in response to TNFα and other proinflammatory cytokines). Another member of the Smad family, Smad6, was shown previously to inhibit IL-1R and Toll like Receptor signalling [160]. It can be concluded that TGFβ can suppress inflammation by inducing the inhibitory molecules Smad6 and Smad7, which inhibit the IL-1R, TLR and TNFR pathway, respectively.

We showed that blocking TNFα in IPS had several benficial effects, it could partly ameliorate severity of inflammation in the lung and neutrophil recruitment to the lung was decreased in BAL. TNFα blockade is already used as a third line therapy option in patients with IPS in the clinic, when steroids and immunosuppressive drugs are failing. Studies in TNFα deficient mice revealed that donor, rather then host-derived TNFα is crucial for lung injury after BMT. Donor macrophages, monocytes and T cells secreting TNFα contribute significantly to this toxicity [139].

The higher neutrophil infiltration seen in severe IPS mediated by IFNγ deficient T cells is a consequence of the up-regulated secretion of IL-17. IL-17 acting on resident lung cells induces the secretion of many cytokines, chemokines and MMPs [161, 162], which results in increased influx of neutrophils to the broncho-alveolar space. Neutrophils can be either helpful or destructive in the lung, as

they secrete many factors that have desastrous effects in the tissue, eg. elastase, reactive oxygen species and MMPs among others, which are critically needed upon invading pathogen, but not in an inflammatory situation without microorganisms [163]. Depletion of granulocytes/neutrophils by a monoclonal anti-Gr1 antibody did not have a beneficial effect in IPS, though.

Another observation in the lung during the course of IPS was the change in antigen presenting cell populations depending on the transfer of either wt or IFNγ deficient T cells. Lung DCs and macrophages play opposing roles in the initiation and maintenance of lung inflammation. Dendritic cells activate T cells and thereby promote inflammation, pulmonary macrophages suppress these processes [164-167]. Dendritic cell populations did reconstitute the lung space with similar levels after irradiation and transfer of bone marrow and T cells in both groups, with faster kinetics in the IFNγ$^{-/-}$ CD4^{+} T cell group though. This might explain additionally the observation of more severe inflammation, as the appearing donor derived DCs can activate donor T cells. In the macrophage repopulation we find a controversy, mice that received wt T cells did not have macrophages appearing in the lung, but IFNγ$^{-/-}$ CD4^{+} T cell recipients showed a fast increase already by day 10. If those macrophages really enhance or suppress disease severity remains unknown, but should be addressed at later stages.

Patient conditioning in the clinical practice of bone marrow transplantation nowadays is more and more drawn towards a non-myeloablative preparation of the patient. There are several advantages and disadvantages for such a “lighter” conditioning that uses less chemo- and/or radio-therapy and it depends largely on the underlying disease of the patient, which regimen is used. Grafts are better tolerated, there are clearly less side effects due to the toxic nature of chemo- and radio-therapy and older people or patients with reduced heart- , kidney- or lung-functions, which would not be acceptable as myeloablative transplant candidates, can still receive a transplant with non-myeloablative conditioning. Concerning some aspects of tissue damage due to irradiation and induction of inflammation by endogenous danger signals as proposed in the danger model of Polly

Matzinger, we can draw some conclusions from our IPS model. Mice receiving wt T cells together with bone marrow, but undergoing the same irradation regimen like IFN$\gamma^{-/-}$ CD4^{+} T cell recipients, developed only minor signs of IPS with no lethality. If irradiation would induce such major proposed endogenous danger signals, we would have seen a strong inflammatory response even in wt T cell recipients. Perhaps this is a mouse phenomenon or other suppressor mechanisms effective in wt T cell transplanted mice? Clinical studies from a report by Fukuda et al. [68] showed that the incidence of IPS was much lower in patients undergoing non-myeloablative preparation.

A recent publication on IPS and the importance of IFNγ to suppress the development of lung inflammation confirm our findings [168]. Interestingly, they showed that IFNγ is pathogenic in GVHD of the gastro-intestinal tract.

As a conclusion from our findings in EAM and IPS we can postulate, depending on the target site of the inflammatory response, IFNγ has several protective effects in an immune response but can also be pathogenic. Most likely every organ or tissue and its specialized cytokine milieu relies differently on IFNγ and factors that are regulated by it. IL-17 is suppressed by IFNγunder normal conditions, but when this suppression is impaired, IL-17 itself enhances inflammatory processes by induction of other pro-inflammatory cytokines, chemokines and neutrophil attraction. Novel therapeutic options for the treatment of unwanted inflammatory processes should be developed to target pathogenic IL-17 production in autoimmunity or chronic inflammation. As it was shown that Th17 are differentiating through the interplay of TGFβ and IL-6, a likely candidate could be IL-6, as a dominating TGFβresponse would preferentially induce regulatory T cells.

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Education

03/2004-05/2007:	PhD student in the group of Prof. Urs Eriksson, Experimental Critical Care Medicine at the University Hospital Basel, Switzerland
03/2003-01/2004:	Visiting student in the group of Prof. Edna Mozes, Department of Immunology at the Weizmann Institute of Science, Israel
10/1999-03/2003:	Graduation at the University Greifswald, Germany ; diploma in human biology (immunology, molecular biology and virology)

05/2001-08/2001:	Visiting Student at the Weizmann Institute of Science, Rehovot, Israel
10/1998-09/1999:	Biology studies at University of Goettingen, Germany
09/1996-09/1998:	Biology studies at University of Erlangen, Germany
08/1991-06/1995:	secondary school, Gymnasium Anklam, Germany, Abitur 1995
08/1983-08/1991:	primary school, Polytechnic School Anklam, Germany

Theses

03/2004-04/2007	**PhD Thesis in the lab of Prof. Urs Eriksson, Dep. of Research, University Hospital Basel, Switzerland** "The opposing role of IFNγ and IL-17 in inflammation"
04/2002-03/2003:	**Diploma Thesis in the lab of Prof. Edna Mozes, Dep. of Immunology, Weizmann Institute of Science, Israel** „Characterization of the mechanism by which a peptide based on the complementarity determining region (CDR) of a pathogenic autoantibody immunmodulates SLE associated responses"

Experience and Skills

Biological Methods:	**-** profound knowledge of mouse disease models, Experimental Autoimmune Myocarditis, Systemic Lupus Erythematosus and Graft versus Host disease - various immunological techniques - flow cytometry, ELISA - DNA and RNA work - profound knowledge in cell culture handling, eg. cell lines, primary organ cultures, stem progenitor cells - mouse heart manipulations with the Langendorff System, immunological assays on heart infiltrating cells - mouse lung manipulations, broncho-alveolar lavage, immunological assays on lung infiltrating cells - ability to direct and supervise animal experiments under swiss and european legislation
Course work during graduate studies	Advanced Immunology I and II (Prof. Antonius Rolink), Transcriptional Regulation (Prof. Roger Clerc), Cellular Signalling (Prof. Kurt Ballmer Hofer), Signalling in Inflammation (Prof. Cecile Arriemerlou), participation at institutional Seminar "Immunommeting"

Language skills:	Excellent spoken, written and presentation skills in English.
02/2000-02/2002:	supervision of student courses in Molecular Immunology at the University of Greifswald
10/1999-12/2001:	student assistant of Prof. Robert Jack, Immunology, University of Greifswald, Germany
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10/1996-10/1998:	undergraduate courses in zoology, botany, chemistry, physics, University of Erlangen, Germany
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Publications

1.) **N.Mauermann**, Sthoeger Z., Zinger H., Mozes E: „Amelioration of lupus manifestations by a peptide based on the complementarity determining region 1 of an autoantibody in severe combined immunodeficient (SCID) mice engrafted with peripheral blood lymphocytes of systemic lupus erythematosus (SLE) patients“
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2.) Sela U, **Mauermann N**, Hershkoviz R, Zinger H, Dayan M, Cahalon L, Liu JP, Mozes E, Lider O: „ The inhibition of autoreactive T cell functions by a peptide based on the CDR1 of an anti-DNA autoantibody is via TGF-beta-mediated suppression of LFA-1 and CD44 expression and function“
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5.) **N.Mauermann**, Burian J, von Garnier C, Dirnhofer S, Bingisser R, Tamm M, Eriksson U$^{\times}$, Hunziker L$^{\times}$: “Interferon gamma negatively regulates idiopathic pneumonia syndrome, an IL-17+ CD4+ T cell-mediated graft versus host disease"manuscript in preparation. $^{\times}$Shared last authors

Poster presentations at scientific meetings

1.) **Nora Mauermann**, Michael Kurrer, René Marty, Stephan Dirnhofer, Steffen Jung, Urs Eriksson „ In vivo depletion of $CD11c^+$ cells induces severe T-cell independent myocarditis" Keystone Symposia 2006 on Tolerance, Autoimmunity and Immune Regulation, Breckenridge, CO

2.) René R. Marty, Stephan Dirnhofer, **Nora Mauermann**, Urs Eriksson "MyD88 but not TLR4 or TLR9 deficiency protects from Experimental Autoimmune Myocarditis" Keystone Symposia 2006 on Tolerance, Autoimmunity and Immune Regulation, Breckenridge, CO

3.) Manu Rangachari, **Nora Mauermann**, René Marty, Stephan Dirnhofer, Michael O. Kurrer, Josef M. Penninger and Urs Eriksson "T-bet is a negative regulator of autoimmune heart disease" Keystone Symposia 2006 on Tolerance, Autoimmunity and Immune Regulation, Breckenridge, CO

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