Antigen – presentation by vascular endothelium: its role for CTL – mediated vascular injury

Inauguraldissertation

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Abstract

Background: EC express and present MHC class I restricted peptide antigens and due to the exposed position may be targeted by antigen-specific CTL. We wished to explore the type of vascular injury that is mediated by peptide-specific CTL *in vivo* when EC express the source protein from which the antigenic peptide is derived. Methods: We immunized mice that express E.coli β-galactosidase (LacZ) as an antigen specifically under the control of the thrombomodulin promoter, i.e. in vascular endothelial cells, with β-gal$_{(500\text{-}507)}$ pulsed dendritic cells. The peptide specific CTL-response was assessed in splenocytes by intracellular cytokine staining after peptide incubation and histopathological tissue sections were scrutinized for CTL-mediated EC injury. Results: After subcutaneous immunization, on average 1% of the CD8+ splenocytes were β-gal-peptide specific effector cells. Despite of successful immunization we were unable to detect antigen-specific endothelial injury in TM-LacZ mice. A GVHD-like chronic inflammatory, antigen-specific disease characterized clinically by mild wasting was induced in ROSA26-LacZ mice, which ubiquitously express LacZ. Accidentally we observed persistent lung inflammatory infiltrates in mice that received intravenous injections of splenocytes or dendritic cells. These perivascular granuloma-like lesions consisted of highly proliferating CD11c^{+} recipient cells and were not the result of DC trapping. Conclusions: Endothelial cells escape CTL-mediated apoptosis *in vivo*. Intravenous injection of splenocytes and dendritic cells or both, leads to a model of pulmonary granuloma formation and this may represent an early stage of vasculitis.

1.Introduction

Vascular endothelial cells (EC) are important for the maintenance of vascular function. The spectrum of immune-mediated endothelial injury is broad. It can precipitate rapid multi-organ failure [7] or it can result in silent tissue remodeling such as seen in chronic graft versus host disease (GVHD) [8]. Cytotoxic T lymphocytes (CTL) recognize antigen as MHC class I-peptide complexes [9]. EC express MHC class I molecules and can therefore become targets of $CD8^+$ CTL. EC are an exposed target tissue in the course of any immune response. When EC present cognate antigen that activates transmigrating effector CTL rapid EC death may result. However, the outcome of an interaction between effector CTL and EC can be manifold (Figure 1) ranging from ignorance to overt EC death. The aim of this thesis project was to investigate antigen-expressing EC as target cells for antigen-specific CTL *in vivo,* and assess the outcome of this interaction and describe the phenotype of CTL-mediated endothelial injury.

1.1 Cytotoxic T cell-mediated immune responses

Cytotoxic T lymphocytes (CTL) are one of the antigen-specific effector arms of the adaptive immune system [10]. They were originally discovered as mediators of solid organ transplant rejection and GVHD but are physiologically important for immune defense against non-cytopathic intracellular microorganisms and tumors [11]. The highly variable T cell receptor (TCR) of a CTL recognizes antigenic peptide bound to major histocompatibility complex class I receptor (MHC class I) [12]. CTL are usually T lymphocytes that carry the co-receptor CD8. CD8 interacts with the MHC class I molecule and facilitates recruitment and activation of the tyrosine kinase Lck to the TCR complex. Lck phosphorylates CD3 and ζ, two signaling components of the TCR. Efficient signal transduction through the TCR results in various response patterns

ranging from cytotoxic granule release to cytokine gene expression. The maturation and differentiation state of the $CD8^+$ T cell determines the signal strength required for full T cell activation. MHC class I restricted antigens are either derived from endogenous [13] or intracellular proteins that are degraded by the proteasome in the cytoplasma [14]. Cytoplasmic peptides allocated to the MHC class I pathway are transported by the transporter associated with antigen processing (TAP) [15] from the cytosol into the lumen of the endoplasmic reticulum (ER). Peptides are assembled with MHC class I molecules in a complex in presence of chaperones [16]. Finally, peptide-MHC class I complexes are translocated to the surface of a target cell to be presented to the CTL [17,18].

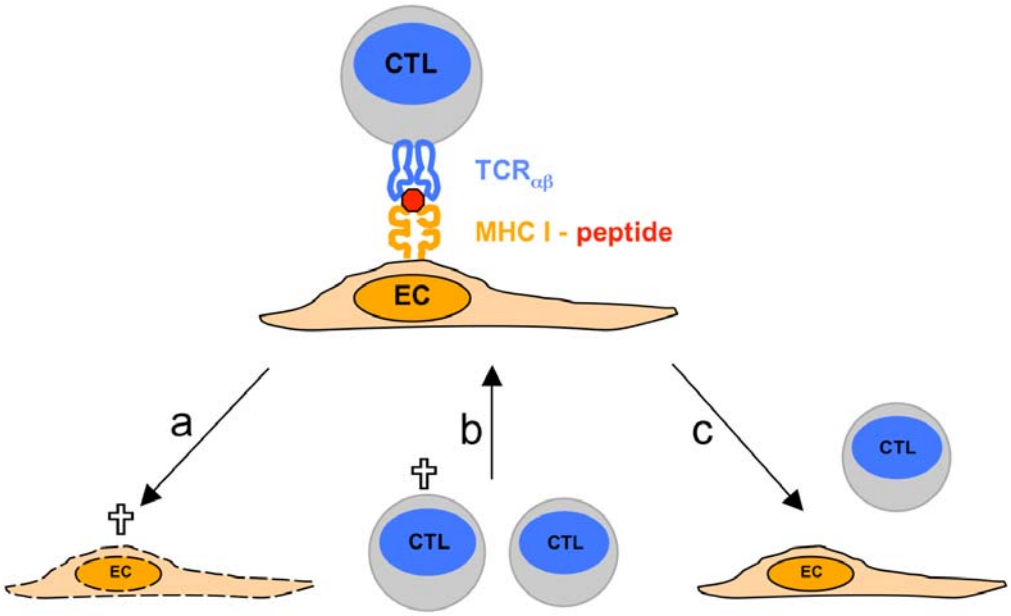

Figure 1. Potential outcome of an interaction between EC and an effector CTL.

Effector CTLs interact with endothelial cells through their T-cell receptor ($TCR_{\alpha\beta}$) and the major histocompatibility complex (MHC) class I molecule – peptide complexes on the endothelial cell surface. The outcome of such an interaction may be: a) EC apoptosis [2,3], b) CTL inactivation by anergy or deletion [4] or c) mutual ignorance [5,6].

Pro-T-lymphocytes arise from stem cells in the bone marrow and migrate to the thymus for maturation. The thymus is the primary lymphoid organ involved in T cell generation. During this process, the T cell precursors somatically rearrange gene segments of the α and β chains of the T-cell receptor (TCR). This process leads to the extraordinary diversity of the TCR that is required for meeting the challenges of antigen recognition. After leaving the thymus, each mature naïve T cell possesses a unique TCR dedicated for antigen recognition and a single co-receptor, either CD4 or CD8 for forming the interaction with MHC class II or I, repetitively. T cells are then homing to the secondary lymphoid organs, the tissue compartment where professional antigen presenting cells (e.g. dendritic cells) can further activate these cells to proliferate and differentiate into effector T lymphocytes.

For the effective activation of naïve CD8 T cells (pre-CTL) into cytotoxic effector CTL, several concomitant signals are required. When a naïve CD8 T cell encounters antigen as a specific peptide-MHC I complex presented by APC, this interaction is further stabilized by the CD8 co-receptor. A second signal needs to be delivered through costimulatory ligands (e.g. CD80 and CD86) that are highly expressed on activated APC. These ligands bind to the costimulatory CD28 receptor, which is expressed on naïve CD8 T cells. The two signals through TCR and costimulatory molecules activating leads to cytokine synthesis and secretion, upregulation of the IL-2 receptor α-chain (CD25) and promotes T cell proliferation and differentiation [19,20]. Once activated, the CTL undergo clonal expansion with the help of T cell growth factors such as interleukin-2 (IL-2). IL-7 or IL-15 are differentiation factors and involved in homeostatic proliferation [21,22]. Recent data also demonstrate an important role of IL-12, interferons (IFNs), IL-10 and TGF-β which are all critical to support T cell survival and growth at different stages of CTL differentiation [21].

The degree of inflammation, precursor frequency, antigenic signal strength and duration determine the efficiency of pre-CTL differentiation [21,23-25]. The majority of reactive effector CTL that emerge after clonal expansion are short-

lived and undergo apoptosis after the task is accomplished, i.e. antigen-bearing target cells are eliminated. Only a small number of antigen-experienced cells survive for a long time as memory T cells. These memory T cells are subdivided into two populations on the basis of their migratory ability [26]: the so-called effector memory T cells migrate to peripheral tissues, whereas central memory T cells express a repertoire of homing molecules similar to that of naïve CD8 T cells and migrate to lymphoid organs. Upon re-encounter with their cognate antigen, memory T cells can very efficiently respond by strong proliferation and undergo a rapid transition into effector CTL [27].

Mature, differentiated effector CTL leave the secondary lymphoid organs and reach peripheral tissues to kill antigen-bearing cells within minutes to hours by at least three distinct pathways [28]: Upon recognizing cognate antigen, CTL release perforin and granzymes from preformed granules. Perforin forms pores in the plasma membrane of target cells allowing granzymes to enter and cause cell death [29]. Second, CTL express membrane-bound CD95L, and this executor molecule is upregulated on the cell surface after TCR activation. CD95L binds to Fas (CD95) on target cells and induces receptor-mediated apoptosis. The third pathway is soluble cytokine-mediated. Activated CTL synthesize and release cytokines, such as tumor necrosis factor-α (TNF-α). TNF-α itself binds to TNFR 1 and 2. The outcome of TNF-α acting on target cells depends on the cell type and cellular activation state. In vascular endothelial cells the TNF response pattern reaches from apoptosis to survival [30]. IFNγ, a second cytokine produced by activated effector CTL upregulates expression of MHC class I and various adhesion molecules in target cells. IFNγ and TNF-α act synergistically on vascular endothelial cells inducing a more than additional response of two signals [31]. Enhanced presentation of endogenous peptides by MHC class I and upregulation of adhesion and costimulatory molecules favor engagement of CTL with target cells. Induced expression of Fas further increases susceptibility to target cell killing [32]. Fully differentiated effector CTL are capable to kill antigen-presenting target cells in the absence of any costimulatory signals or co-receptors.

In the face of the fatal cytotoxic potential of fully differentiated effector CTL, it is crucial that these cells respond only to infected or transformed target cells but ignore self-peptides. This discrimination between self and non-self is maintained by a process called tolerance. Immunological tolerance is achieved by central and peripheral mechanisms. Central tolerance is established during development of pro-T cells in the thymus. During this process, immature T cells that recognize self-peptide-MHC complexes with high affinity are deleted by a process referred to as negative selection, whereas T cells that express TCR with low affinity for self-peptide-MHC ligands are positively selected [33]. T cells that fail to bind thymic self-antigens are eliminated by neglect. Among the T cells that populate the peripheral lymphoid tissues are found cells that display strong reactivity to foreign antigens, but are tolerant towards self-antigens [34-36]. Since many peripheral tissue-specific self-antigens are constitutively expressed in thymic epithelial cells (TECs), central tolerance protects even against tissue-specific peripheral antigens. This function depends on the expression of the autoimmune regulator (ARIE) gene [33,37]. By similar mechanisms, stromal cells in secondary lymphoid organs can present tissue-specific antigens to T cells. Antigen presentation by lymph node stroma is sufficient to induce primary activation and subsequent tolerance among naïve CD8 T cells [38].

Central tolerance mechanisms are not completely protective against self-reactive lymphocytes. In fact, low avidity self-reactive T cells are normally present in peripheral blood of healthy donors [39] and can cause autoimmune disease [40]. Therefore, different peripheral-tolerance mechanisms exist to eliminate, inactivate or suppress self-reactive CTL who survived central tolerance mechanisms in the thymus [41]. Peripheral tolerance is either accomplished by peripheral deletion, by anergy or by ignorance [42]. Activated DCs present self-antigen to naïve $CD8^+$ T cells in peripheral tissues, and this process could provide an opportunity for autoreactive CD8 T cell activation. In the absence of toll-like receptor signals, DCs are quiescent and express low levels of costimulatory molecules [43]. Lack of costimulation does not promote increased levels of antiapoptotic molecules, therefore leads to CTL death [44,45].

Anergy is an active process that is dependent upon the continuous presence of antigen, as the cells regain their ability to respond to antigen once they are allowed to rest in the absence of antigen [46]. High doses of antigen persistently presented promote CD8 T cell anergy and low doses induce cell death. The persistent presence of self antigens leads finally to complete clonal deletion [4,47,48]. Self-reactive T cells may be unable to cause harm by simply ignoring self-antigen [42]. Taken together, tolerance mechanisms normally prevent CTL attacking self-peptides presented on self-MHC class I molecules and only failure of all levels of tolerance leads to overt autoimmune disease.

In summary, an efficient CTL-mediated immune response is initiated if a naïve or memory CTL recognizes specific peptide-MHC I complex on an activated professional APC. Under normal circumstances, central and peripheral tolerance mechanism control autoreactive CD8+ T cells and prevent CTL-mediated autoimmune diseases.

1.2 Endothelial cells as a target of immune mediated injury

Vascular endothelial cells (EC) form the luminal, single-cell pavement of the blood vessel and heart that represents an interface between liquid blood on one and solid tissues on the other side. EC have crucial functions in organ homeostasis by prevention of blood coagulation, regulation of vascular diameter, tone and permeability and controlling cell recruitment. Vascular endothelium is an important checkpoint and amplifier of any immune response, be it antimicrobial, anti-transplant or anti-self [49]. EC are in intimate contact with CTL under two different circumstances. First, when CTL circulate in blood they contact EC by passing through capillaries where the diameter is narrower than a T cell's diameter. Second, transmigrating CTL leave the blood and enter the tissue by squeezing through the endothelial monolayer, thereby having tight cell-cell contact.

There are several potential interactions between EC presenting antigen and CD8+ T cells recognizing antigen. First, EC can activate memory $CD8^+$ T cells to proliferate and differentiate into effector CTL both *in vitro* and *in vivo* [50-52]. Second, EC can induce antigen-specific tolerance. Lung EC negatively regulate CD8 T cell function [53]. Liver sinusoidal EC can induce CD8 T cell tolerance to soluble [54,55] or tumor-derived antigens [56]. By another postulated mechanism, immunological ignorance, vascularized solid organ allotransplants were tolerized by the immune system in *aly/aly* mice, These mice lack secondary lymphoid organs, suggesting that endothelial antigen presentation was not sufficient to initiate an immune response. These experiments demonstrated on the other hand that the primary activation of efficient T cell responses mediating solid organ transplant rejection are dependent on an organized interaction between APC and CD8 T cells within secondary lymphoid tissues [6]. Endothelial ignorance and a certain level of peripheral, antigen specific tolerance by crosspresentation has recently been described in a mouse model of endothelial cell specific and restricted antigen presentation [57].

Third, another important interaction sets EC as possible targets of a CTL-mediated immune response. Many microvascular EC, which are lining capillaries and venules express high level of MHC class I [58]. Therefore they are an exposed target tissue in the course of CTL-mediated immune diseases. Effector T cells transmigrate through monolayers of vascular EC during their passage to peripheral tissues. If EC express and present cognate antigen that activates transmigrating effector CTL they may be recognized as target cells and this contact should result in rapid EC death [59]. CTL-mediated EC injury can be caused by cytotoxic effector molecules such as perforin/granzyme B, Fas ligand or TNF-α [60]. This project will focus on EC as targets of antigen-specific CTL.

In an animal study [3], disseminated CD95-mediated endothelial cell apoptosis was observed in all organs after i.v. injection of Fas-ligating antibody or systemic transfer of allogeneic lymphocytes into recipient mice. When CD95L

knockout lymphocytes were used, no endothelial injury was observed. Two findings were intriguing in these early studies: although widespread endothelial apoptosis was observed by histopathological criteria, no organ dysfunction occurred. Further evidence of a discrepancy between extensive endothelial injury and lack of consequences was that the mice were relatively healthy and no fatalities were observed. Interestingly, there were signs of lymphocyte mediated EC apoptosis but no inflammatory infiltrates. These findings were confirmed in a second animal study performed by the same group of investigators, in a model of acute GVHD. Both EC apoptosis and GVHD were completely dependent on CD95-CD95L ligand interaction [2]. Still, no disseminated microvascular coagulation, no multi-organ failure and no inflammatory infiltrates were observed.

In summary, in their neuralgic position at the interface between blood and tissue, EC are involved in regulating several key physiological functions, both immune and non-immune. EC are positioned to come into contact with circulating and recirculating T cells. EC can activate memory T cells by presenting antigens, can induce the CD8 T cell tolerance and can be targets of an effector CTL-mediated immune response.

1.3 Inflammatory infiltrates and organ injury

Inflammation is the complex biological response of tissue to harmful stimuli. Pathologically, local inflammation is characterized by redness (rubor), hyperthermia (calor), swelling (tumor) and pain (dolor) and at least 3 of 4 of these phenomena involve EC. Inflammation is a protective attempt of the organism to remove injurious stimuli by innate and adaptive immune responses and initiates tissue repair [61]. This response involves tissue microvessels, the immune system and parenchymal and stroma cells within the injured tissue. A fundamental event in the inflammatory reaction is the localized and restricted recruitment of blood leukocyte subsets to tissues through endothelial-dependent mechanisms [62]. Mediators of inflammation can

activate EC by upregulating the expression of adhesion molecules, involved in leucocyte tethering and adhesion which precede both transmigration through the EC and recruitment to peripheral tissues [63]. Among the various effector leucocytes are also differentiated effector CTL which transmigrate through EC into inflamed tissues in order to contribute to an efficient immune response [64].

Vasculitis is a group of diseases with inflammation localized to the wall of blood vessel. Vasculitis can affect veins, arteries and capillaries. A hallmark of vasculitis is injury and destruction of the vessel wall. Systemic vasculitides have a predilection for certain organs, and interestingly, most of them involve lung and kidney. They can cause severe disability, such as in pulmonary-renal syndrome and even have a fatal course. Pulmonary vasculitis is a primary idiopathic vasculitis restricted to the lung. Wegener's granulomatosis (WG), which belongs to the ANCA-positive vasculitides, affects small to medium-sized vessels, including venules and arteries. WG is characterized by granulomatous inflammations, necrotizing vasculitis in the respiratory tract and glomerulonephritis. Microscopic polyangiitis (MPA) affects capillaries, venules or arteries, forming the focal, segmental necrotizing vasculitis and a mixed inflammatory infiltrates without granulomata. Pulmonary vasculitis can be primary immune complex-mediated, like Goodpasture's syndrome, which also presents with acute renal failure due to rapidly progressive glomerulonephritis [65]. Focussing on vascular EC as targets of CTL-mediated injury we hypothesized that the type of tissue injury that we would observe could resemble some form of pauci-immune vasculitis [66].

1.4 Aims and approaches of the study

In my project, I wished to focus on CD8 T cell mediated EC injury *in vivo*. In order to address this question I used a mouse model in which a protein antigen, E.coli β-galactosidase, was expressed exclusively by EC. This would allow investigating CTL-mediated injury limited to EC. The MHC class I / $H2k^b$ restricted, immunodominant epitopes are well characterized and therefore I

could work with antigenic peptides both at the level of CTL induction and functional CTL characterization. Currently, a number of transgenic mouse lines are available that exhibit EC-restricted expression of E.coli β-galactosidase: von Willebrand Factor-LacZ mice [67] show transgene expression restricted to yolk sac and brain EC. Tie2-LacZ mice [68] uniformly express transgene in endothelial cells of all organs. In my project, I used TM-LacZ mice [1] on a C57/B6 background. In this model, the β-galactosidase gene is knocked in to be expressed under the control of the thrombomodulin (TM) promoter. Homozygous knock-in mice are embryonically lethal. Therefore, breeding was between heterozygous and wild type animals and 50% of offspring was heterozygous TM-LacZ and 50% wild type (wt). I found these mice to be well-suited to study EC–CD8 T cell interaction *in vivo*, because the internal control of antigen-positive versus antigen-negative EC existed in the same animal.

In order to establish and investigate an anti-EC CTL response, my project addressed the following hypotheses:

1. An anti-EC CTL-mediated autoimmune response can be elicited
   To induce strong sustained autoimmune response against peripheral EC-restricted self-antigen, repetitive injections of high doses of β-gal$_{(500-507)}$- primed DC over a prolonged period were applied. This protocol was shown to break tolerance against vascular smooth muscle cell-restricted β-galactosidase [69].

2. An anti-EC CTL-mediated, mHA restricted alloimmune response can be elicited
   In order to induce β-gal$_{(500-507)}$-specific CTL, we performed bone marrow transplantation using wt mice as donors and TM-LacZ mice as recipients. This mimics MHC-matched transplantation across minor histocompatibility barriers. Under these conditions, β-galactosidase represents a minor histocompatibility alloantigen.

3. An anti-EC CTL-mediated immune response is observed by adoptive transfer of effector CTL

   To investigate the direct interaction between β-gal$_{(500-507)}$-specific effector CTL and EC, antigen-specific effector CTL were adoptively transferred to the TM-LacZ mice. It was recently shown that fully-differentiated effector CTL cells were less effective for adoptive transfer in an anti-tumor immunotherapy model [70]. Therefore we also transferred β-gal$_{(500-507)}$-primed splenocytes to the TM-LacZ mice in certain experiments.

4. Toll-like receptor activation aggravates CTL-mediated EC injury

   Lang [71] showed that among the various of Toll-like receptor ligands, poly(I:C) was able to transform tissue infiltration by autoreactive CTL into overt autoimmune disease through upregulation of MHC class I expression by IFNα. Based on this observation, in selected experiment, mice were poly(I:C) treated.

5. Route of immunization affects the efficiency of anti-EC CTL-mediated immune response

   Labelling studies with radioactive tracer have demonstrated that there are significant differences in the distribution of DC that are administered by different routes [72,73]. Cells injected intravenously (i.v.) were first collected in lung and then redistributed to the liver, spleen and bone marrow. Cells injected subcutaneously (s.c.) or intradermally (i.d.) can migrate to draining lymph nodes with varying efficiencies. We tested whether the route of DC administration is important for the outcome of immunization *in vivo.* Mice were immunized by i.v., s.c. and intraperitoneal (i.p). injection of β-gal$_{(500-507)}$ peptide pulsed DC.

6. During the study of EC as target of antigen-specific CTL, the systematic histological analysis of various organs (liver, lung, kidney and heart) for signs of immune mediated injury revealed an unexpected finding: consistent and reproducible lung inflammatory infiltrates (LII) of

variable severity without involvement of other organs after **intravenous** injection of immune cells. LII were not the result of an antigen-specific anti-EC CTL-mediated immune response as confirmed by control experiments. In order to further elucidate the pathogenesis of lung injury, I injected i.v. dendritic cells, splenocytes, bone marrow, purified CD4+ and CD8+ to the mice, either alone or in combination, and examined LII two weeks after injection.

2. Methods and Materials

2.1 Mice

All mice used in this study were kept under specific pathogen-free condition and in accordance with Swiss federal law. TM-LacZ mice (mouse strain (background C57BL/6-Ly5.2; $H2k^b$) with a knock-in of E.coli β-galactosidase gene under the control of the thrombomodulin promoter) have been described [1] and were obtained from Hartmut Weiler-Güttler through the Animal Resource Center, Medical College of Wisconsin, Milwaukee WI, USA. TM-LacZ mice were crossed with C57BL/6-Ly5.2 mice to keep heterozygous offspring. Genotyping of TM-LacZ mice was performed with the primer ATGGTGCCAATGAATCGT and GTGGTCGGCTTACGGCGGTG, yielding a 513-bp PCR product (Figure 2). All experiments were carried out with age- (about 17 - 20 wk) and sex-matched animals, and littermates were used as wild type controls. For selected experiments, C57BL/6-Ly5.1 mice ($H2k^b$) mice were used as a source of donor cells in order to determine the origin of infiltrating cells in lung inflammatory infiltrates or the degree of chimerism after bone marrow transplantation. Rosa26-LacZ mice [74] (background C57BL/6-Ly5.2; $H2k^b$), which express β-galactosidase ubiquitously in all tissues of the developing embryo and in most tissues of the adult mouse, were used as control animals in certain experiments.

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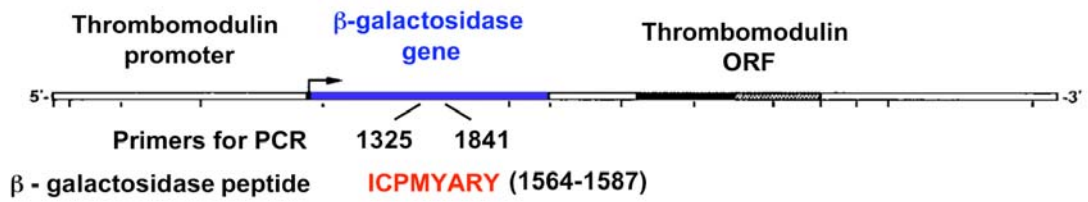


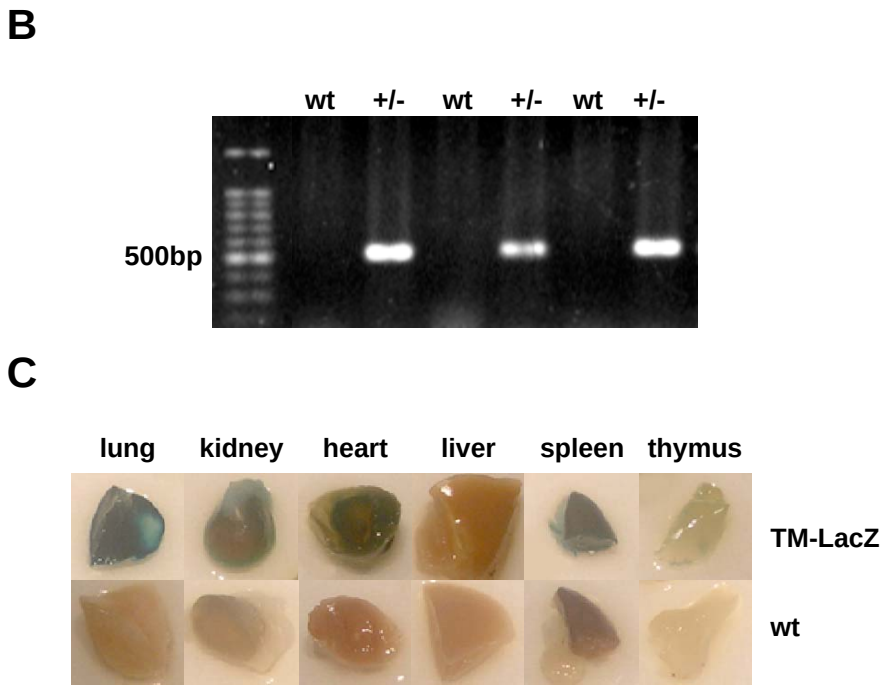


Figure 2. Endothelial cell-specific β-galactosidase expression in TM-LacZ mice.

(A) β-galactosidase is expressed under the control of the thrombomodulin (TM) promoter [1]. (B) Genotyping offspring with β-galactosidase PCR amplifying a 513bp fragment. (C) Whole-mount X-gal staining in organs of TM-LacZ and C57BL/6 (wt) mouse. (D) Organ sections of TM-LacZ mice were stained for β-galactosidase using X-gal as a substrate (blue). β-galactosidase positive cells are vascular endothelial cells.

2.2 Culture media

LCM-10 medium, on the basis of RPMI-1640 (52400025), containing 10 % FCS (10270106), 2 mM L-glutamine (25030024), 100 U/ml penicillin and 100 μg/ml streptomycin (15140122), 5 mM nonessential amino acids (11140035), 5 mM sodium pyruvate (11360039) (all reagents from Invitrogen Life Technologies, Carlsbad, CA, USA) and 50 μM β-mercaptoethanol (M7522, Sigma-Aldrich) was generally used for culturing mouse cells. LCM-2.5 washing medium is based on RPMI-1640, containing 2.5 % FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM nonessential amino acids, 5 mM sodium pyruvate and 50 μM β-mercaptoethanol.

Cloning medium is LCM-10 medium supplemented with 2 ng/ml mIL-2.

DC medium is LCM-10 medium supplemented with 10% of GM-CSF containing cell culture supernatant. For serum-free culture, Cellgro medium [75] (2005, Cellgenix, Freiburg, Germany) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, 10 ng/ml rmGM-CSF and 10 ng/ml rmIL-4 was used.

CTL assay medium is based on Medium-199 (31153026, Invitogen), containing 2% FCS, 5 mM HEPES (15630-056, Invitogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin.

All single cell suspensions were washed with gPBS (1 × Dulbecco's phosphate-buffer saline containing 1 mM glucose) before injected into mice.

2.3 Peptides, cytokines, chemicals and antibodies

The H-2K^{b}-restricted β-galactosidase peptide, ICPMYARV (β-gal$_{(500\text{-}507)}$) was obtained from American Peptides (Sunnyvale, CA, USA). The chicken ovalbumin peptide, SIINFEKL (OVA$_{(258\text{-}265)}$) was a gift from Ed Palmer (Department Biomedicine Basel, Switzerland). Peptides were dissolved in DMSO at 2 mM and stored at 4°C.

The following cytokines were used: recombinant mouse IL-2 (402-ML) was reconstituted at 20 μg/ml stock concentration; recombinant mouse TNFα (410-MT) at 1 μg/ml, recombinant mouse GM-CSF (415-ML/CF) at 100 μg/ml and recombinant mouse IL-4 (404-ML/CF) at 50 μg/ml**.** All recombinant cytokines were from R&D Systems. Stock solutions were stored in aliquots at -70°C.

GM-CSF containing supernatant was generated from transgenically modified producer cell line XG3-GMCSF (gift from Ton Rolink, Department of Biomedicine, University of Basel). For continuous selection, these cells were cultured in 20 ml of LCM-10 medium in the presence of 1 mg/ml of geneticin (10131019, Invitrogen). After washing twice with LCM-2.5 washing medium, 5×10^6 cells were cultured in 20 ml of LCM-10 medium for another two weeks. Then cells were spun down, supernatant was collected, filtered twice, and stored in aliquots at - 20°C.

polyinosinic–polycytidylic acid potassium salt (poly(I:C), P9582) was reconstituted at 1 mg/ml and stored in aliquots at -70°C. Phorphol myristate acetate (PMA, P8139) was reconstituted at 10 μg/ml, ionomycin (10634) at 1 mg/ml and brefeldin A (B6542) at 2 mg/ml. All chemicals were purchased from Sigma-Aldrich and stored in aliquots at -20°C. Calcein-AM (C1430, Molecular Probes, Invitrogen, Basel, Switzerland) was dissolved in DMSO at 5 mM and stored at -20°C. 5-Bromo-2'-deoxyuridine (BrdU, 16880, Sigma-Aldrich) was used for *in vivo* labeling of proliferating cells and was freshly prepared in drinking water at 0.8 mg/ml.

Antibodies were used for FACS and immunohistochemistry. The source, specificity and conjugation are shown in Table 1.

Table 1: Antibodies used for different experiments.

Antigen	Species	Conjugate	Supplier / donor	Cat. No.	Usage	Titer
CD3ε	aHamster IgG	FITC	Miltenyi Biotec	130-092-962	FC	1:25
CD4	rat IgG	PE	Miltenyi Biotec	130-091-607	FC	1:25
CD4	rat IgG	PE	BD Pharmingen	553652	FC	1:100
CD8α	rat IgG	PE	BD Pharmingen	553032	FC	1:100
CD8α	rat IgG	APC	Miltenyi Biotec	130-091-606	FC	1:25
CD11c	aHamster IgG	FITC	BD Pharmingen	557400	FC	1:200
CD11c	aHamster IgG	PE	BD Pharmingen	557401	FC	1:100
CD45.1	mouse IgG	FITC	BD Pharmingen	553775	FC	1:200
CD45.1	mouse IgG	PerCP	eBioscience	45-0453-80	FC	1:100
CD45.2	mouse IgG	FITC	BD Pharmingen	553772	FC	1:400
CD45.2	mouse IgG	APC	BD Pharmingen	558702	FC	1:200
F4/80	rat IgG	PE	eBioscience	12-4801-82	FC	1:100
FasL	aHamster IgG	PE	BD Pharmingen	555293	FC	1:100
H-2Kb	mouse IgG	FITC	BD Pharmingen	553569	FC	1:200
I-Ab	mouse IgG	FITC	BD Pharmingen	553551	FC	1:200
IFNγ	rat IgG	FITC	BD Pharmingen	554411	FC	1:200
BrdU	mouse IgG	FITC	BD Pharmingen	347583	FC	1:10
-	mouse IgG	PE	BD Pharmingen	555748	FC	5ul/tube
-	mouse IgG	FITC	BD Pharmingen	55749	FC	5ul/tube
-	mouse IgG	APC	BD Pharmingen	554681	FC	1:200
CD4	rat IgG	purified	Burkhard Ludewig	YTS 191.1	IHC, FC	1:800
CD8α	rat IgG	purified	Burkhard Ludewig	YTS 169.4.2	IHC, FC	1:400
F4/80	rat IgG	purified	Regine Landmann	-	IHC, FC	1:300
CD11c	aHamster IgG	Bio	BD Pharmingen	553800	IHC, FC	1:200
CD45.1	mouse IgG	Bio	BD Pharmingen	553774	IHC	1:200
CD45.2	mouse IgG	Bio	BD Pharmingen	553771	IHC	1:200
BrdU	rat IgG	purified	Abcam	ab6326	IHC	1:200
-	rat IgG	purified	AbD Serotec	mca1125	IHC, FC	1:200-800
-	aHamster IgG	Bio	BD Pharmingen	553952	IHC, FC	1:200
-	mouse IgG	biotin	BD Pharmingen	33812x	IHC, FC	1:200
rat IgG	goat antiserum	purified	Caltag Laboratories	R40000	IHC	1:500
goat IgG	donkey antiserum	AP	Jackson	705-055-147	IHC	1:2500
rat IgG	donkey antiserum	HRP	Jakson	712-035-153	IHC	1:200
rat IgG	donkey antiserum	Alexa 488	Molecular Probes	A21208	FC	1:400
-	Steptavidin	Cy2	Jackson	016-220-084	FC	1:400

* FC: flow cytometry

IHC: immunohistochemistry

2.4 Isolation of cells from mouse organs

2.4.1 Bone marrow

Mice were sacrificed by cervical dislocation and the hind limbs were removed. Bone marrow was flushed out of dissected hind limb bones into LCM-10 medium. The tissue was passed through a 40 μm nylon mesh (S31109279, Sefar) in order to obtain single cell suspensions. In certain experiments, erythrocytes were depleted by incubating the cells for two minutes in ice-cold ammonium chloride solution (see above 2.4.3). The total yield from the four leg bones of a 8- to 12-week-old C57BL/6 mouse was 3-5 $\times$ 10^7 cells.

2.4.2 Splenocytes

Mice were sacrificed by cervical dislocation and the spleen was removed. Splenocytes were squeezed and filtered through a 70 μm nylon mesh (3053-0201-10000, Sefar, Heiden, Switzerland) in order to prepare single cell suspensions. In certain experiment, erythrocytes were depleted by incubating the cells for two minutes with ice cold ammonium chloride solution (0.02 M Tris-HCl, 0.14 M NH_4Cl, pH7.2). The total yield from the spleen of a C57BL/6 mouse was about 10^8 cells.

2.4.3 Peripheral blood mononuclear cell (PBMC) collection

200 μl of blood was collected from the tail artery in a glass tube (12 $\times$ 75mm, 2787/6, DURAN, Germany) containing heparin (final concentration 500 U/ml, B. Braun Medical AG, Emmenbrücke, Switzerland). 2 ml of PBS was added, mixed and the diluted blood was layered over 2ml Ficoll-Paque^{TM} Plus (17-1440-02, GE Healthcare Bio-Sciences AB, Sweden). Blood was spun at 2400 rpm for 30 minutes at RT. After carefully removing the diluted plasma, PBMC

were harvested and washed in 3ml PBS (1400 rpm for 10 minutes). The PBMC pellet was then used for further experiments. About 2 × 10^6cells /ml of peripheral blood was obtained.

2.4.4 Purified CD4 and CD8 cells from spleen

Erythrocyte-depleted splenocytes were washed two times with AutoMACS buffer (PBS, pH7.2, supplemented with 0.5% FCS and 2mM EDTA) and filtered through a 40 μm strainer. $CD4^+$ and $CD8^+$ populations were purified by negative selection using the mouse $CD4^+$ T cell isolation kit (130-090-860) and mouse $CD8\alpha^+$ T cell isolation kit (130-090-859, Miltenyi Biotec Inc., Auburn, CA, USA), according to the manufacture's instruction. Cell purity of > 80% for all populations was confirmed by flow cytometry (Figure 3). Purified lymphocytes were then transferred intravenously into TM-LacZ mice. About 1.5 × 10^6 $CD4^+$ and 10^6 $CD8^+$ cells / 10^7 splenocytes were obtained.

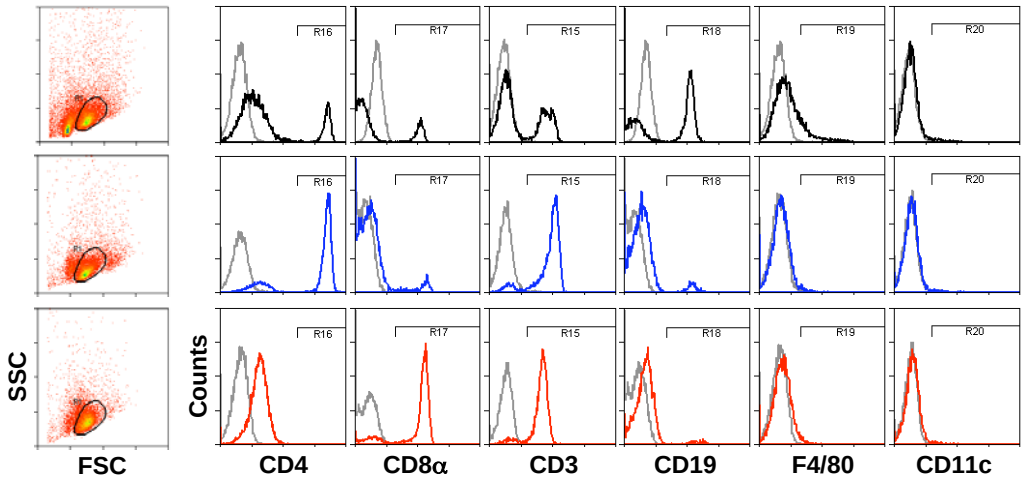


Figure 3. Phenotypical characterization of total splenocytes and purified lymphocyte populations.

Generally, unfractionated splenocytes were used for adoptive transfer. For selected experiments, $CD4^+$ and $CD8^+$ populations were purified from total splenocytes by using the mouse $CD4^+$ and $CD8\alpha^+$ T cell isolation kit. Cell composition was detected by FACS stained with fluorescently labelled (either FITC, PE or APC) anti-CD4, anti-CD8α, anti-CD3, anti-CD19, anti-F4/80 and anti-CD11c mAbs. Gray line is isotype control, black line is

unfractionated splenocytes, blue line is purified CD4 population and red line is purified CD8 cell population.

2.5 Bone-marrow derived dendritic cells (DC)

Bone marrow derived DC were generated as described [76]. The BM single cell suspensions were washed once with LCM-10 medium, and 0.5 × 10^6 cells /cm^2 were plated in DC medium. After 3 days of culture, 80% of culture supernatant was carefully replaced by fresh medium. For serum-free culture, Cellgro medium was used. After 6 days in culture, the resulting bone marrow-derived DC population showed that more than 70% of the cells were $CD11c^+$ and showed strong expression of MHC class I and II antigens (Figure 4).

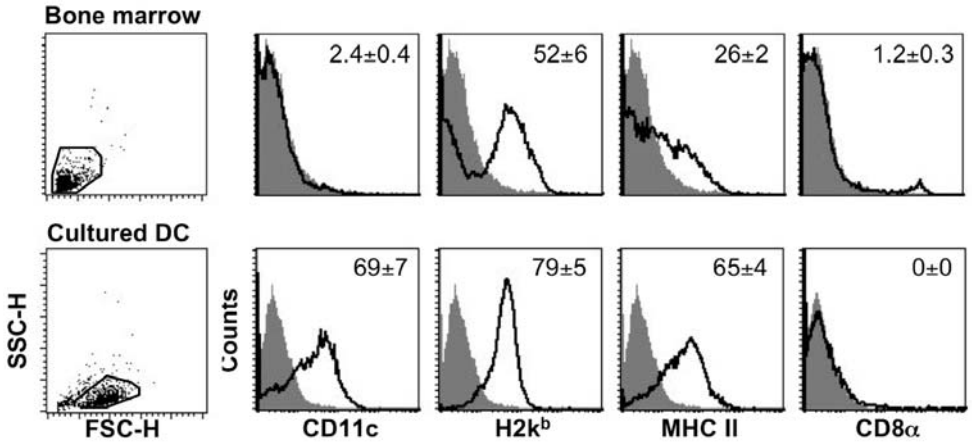


Figure 4. Phenotypical characterization of bone marrow-derived DC.
Bone marrow (BM) cells were cultured in GM-CSF conditioned medium. After 6 to 8 days, non-adherent cells were collected and analyzed by FACS using fluorescently labelled mouse antibodies against CD11c, MHC I ($H2k^b$), MHC II and CD8α. (A) BM cells. (B) Cultured DC on day 7.

2.6 Immunization of mice

After 6 to 8 days in culture, non-adherent C57BL/6 bone-marrow derived DC were collected, suspended at 5 × 10^6 cells/ml and pulsed for 60 minutes at

37°C in DC medium containing 10^{-5} M β-gal$_{(500-507)}$ or OVA$_{(258-265)}$, respectively. After washing the cells three times with gPBS, 5×10^5 DC in 0.25ml gPBS were injected intravenously into the tail vein (i.v.), subcutaneously in the neck (s.c.) or intraperitoneally (i.p.). Unless otherwise indicated, immunization with peptide pulsed DC was repeated three times on consecutive days.

2.7 In vivo primed, peptide-specific splenocytes

Seven days after peptide-pulsed DC immunization, *in vivo* primed splenocytes were obtained. These cells were either used for adoptive transfer or for the generation of peptide-specific CTL lines. The fraction of peptide specific $CD8^+$ cells was determined by ICS (Figure 8).

2.7.1 Splenocytes adoptive transfer

For adoptive transfer of DC/β-gal$_{(500-507)}$ primed splenocytes, erythrocytes were depleted by incubating the splenocytes for two minutes with ammonium chloride solution. The cells were then washed three times with gPBS, and 25×10^6 cells in 0.5 ml gPBS were injected intravenously on day 0. In addition, the mice were immunized with β-gal$_{(500-507)}$ pulsed DC on day 1, 2 and 3 as described [76]. In selected experiments, mice were treated with 200 μg poly(I:C) i.p., a ligand for TLR3, one day before splenocytes adoptive transfer [71].

2.7.2 Generation of peptide-specific effector CTL

Seven days after DCs/β-gal$_{(500-507)}$ or DC/OVA$_{(258-265)}$ immunization, C57BL/6 mice splenocytes were plated at 5×10^6 cells/ml in a 24-well plate in 1 ml LCM-10 medium and 5×10^6 cells/ml β-gal$_{(500-507)}$ or OVA$_{(258-265)}$ peptide pulsed splenocytes as feeder cells were added. Every 10 days, about 3×10^6

cultured responder were restimulated with 6 × 10^6 peptide-pulsed, irradiated C57BL/6 splenocytes in cloning medium. The total cell number per well did not exceed 10^7. After 4 – 5 weeks, the effector CTL were phenotyped by FACS for CD8 expression (Figure 5A), and were tested functionally for peptide-specific lysis in a calcein-release cytotoxicity assay (Figure 5B). When the effector CTL were > 80% CD8 positive, and demonstrated peptide specific lysis > 70% at E/T ratio of 10:1, they were used for i.v. adoptive transfer into recipient mice.

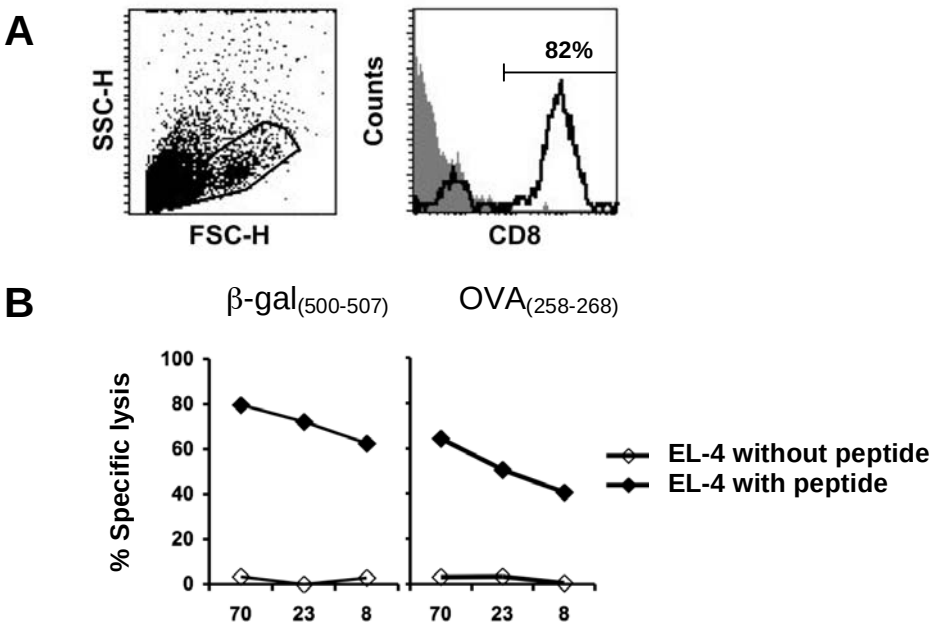
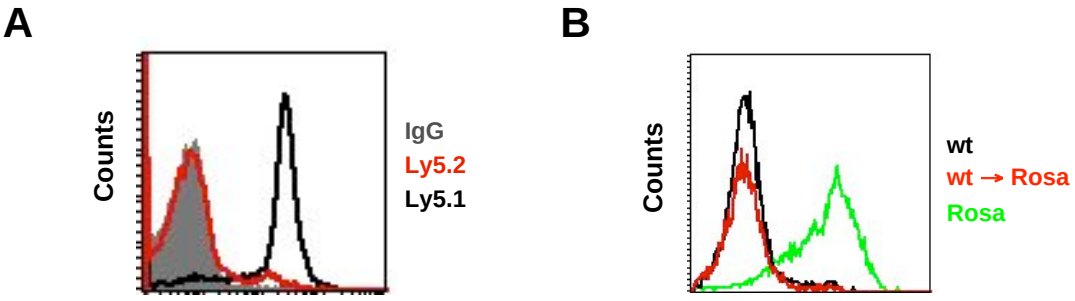


Figure 5. Phenotypical and functional characterization of peptide-specific effector CTL expanded *in vitro*.

Primed splenocytes harvested seven days after immunization with β-gal$_{(500-507)}$ or OVA$_{(258-265)}$ peptide-pulsed DC, respectively. They were cultured *in vitro* in the presence of IL-2 and restimulated every 10 days with β-gal$_{(500-507)}$ or OVA$_{(258-265)}$ peptide pulsed, irradiated wt splenocytes for 5 weeks. (A) All effector CTL lines were > 80% CD8 positive (black line). Gray shade is isotype control. (B) Peptide specific cytotoxic activity of CTL lines was tested in a calcein-release cytotoxicity assay. The close squares indicate peptide-pulsed target cells, and the open squares indicate non-pulsed target cells.

2.8 Bone marrow transplantation

Recipient mice were lethally irradiated with 11Gy at fractionated doses (6Gy and 5Gy with 4 hours interval). Within 24 hours after whole body irradiation of recipients, donor mice were killed, bone marrow was flushed from femurs and tibias, erythrocytes were depleted and single cell suspensions were prepared in gPBS. Recipient mice then received 10^7 bone marrow cells in 0.5 ml gPBS via tail vein injection. Survival and the degree of clinical GVHD after BMT was monitored daily, using a scoring system that sums changes in six parameters: weight loss, posture, activity, fur texture, skin integrity and diarrhea, as described [77,78]. Animals were sacrificed when they lost more than 10% of their original body mass. Chimeric mice were maintained on water containing sulfadoxin and trimethoprin as antibiotics (Mepha Pharma AG, Basel, Switzerland) for 10 days post-transplant. Mice were used for experiments 8 to 10 weeks after bone marrow reconstitution. To check chimerism of the recipient, C57BL/6-Ly5.1 bone marrow was transplanted into Ly5.2 mice, or C57BL/6 BM into Rosa26-LacZ mice. Recipient mice were bled on day 35 and 60 after BMT. PBMC were stained with anti-CD45.1 and CD45.2 mAbs respectively. Alternatively, β-galactosidase activity was detected with fluorescein di-D-galactopyranoside (FDG) as a substrate (see below 2.16.2) and leukocyte reconstitution was checked in FACS (Figure 6A, 6B). Blood smears were used for differential blood counts (normal range of cell component of C57BL/6: neutrophils are 3.7-21%, lymphocytes are 68-92%, monocytes are 0.2-3%) (Figure 6C).



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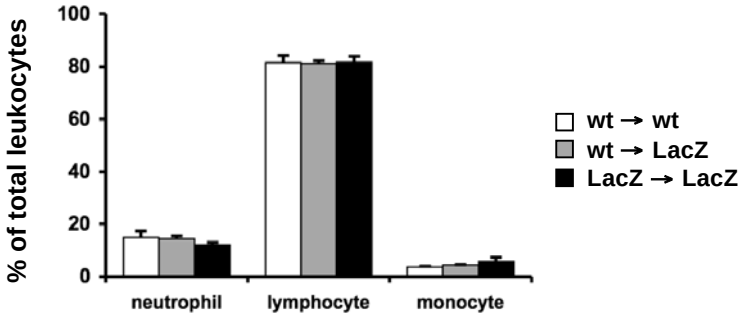

Figure 6. Blood reconstitution and donor chimerism after BMT.

(A) C57BL/6-Ly5.1 BM was transplanted into C57BL/6-Ly5.2 recipient. After eight weeks, chimerism was tested by FACS in mouse PBMC. PBMC were stained with anti-CD45.1 (black line) and CD45.2 (red line) mAbs. Gray shade is isotype control (B) C57BL/6 (wt) BM was transplanted into Rosa26-LacZ (Rosa) recipient. After eight weeks, chimerism was tested by FACS in mouse PBMC. PBMC were stained with FDG as a substrate of β-galactosidase (see methods). Black line is C57BL/6 mouse PBMC, green line is Rosa26-LacZ mouse PBMC, red line is chimera mouse PBMC (wt → Rosa). (C) Completed leukocyte reconstitution in the peripheral blood eight weeks after BMT.

2.9 Cell proliferation assay

2.9.1 in vivo BrdU labeling

In order to determine proliferation rate of lung inflammatory infiltrates, BrdU was administrated in drinking water (0.8 mg/ml) for 7 days before sacrifice the animals [79]. BrdU-containing drinking water (light free) was made up fresh and changed daily.

2.9.2 in situ tissue staining for proliferating cells

The recipient mice were sacrificed at indicated time point. Cryosections of 10 μm thickness were fixed with 4% paraformaldehyde in PBS at RT and washed twice with 1× PBS, incubated with 1N HCl at 37°C for 30 minutes. Then

quenched with methanol containing 0.3% of fresh H_2O_2, blocked with 1% FCS, 2% BSA, 0.1% Tween-20 in PBS for 30 minutes. Anti-BrdU antibody was incubated in PBS containing 1% FCS, 1% BSA, 10% DNAse buffer and DNAse (50 units/ml, M610A, Promega) for 2 hours, followed by goat anti-donkey HRP and visualized by DAB (K3468, DAKO North America Inc., Carpinteria, CA, USA). The percentage of $BrdU^+$ cells was calculated as: BrdU stained positive nuclei in the lesion area / total nuclei in same area × 100 (Figure 24A).

2.10 Measurement of IFNα serum levels

In order to confirm biological activity of poly(I:C) treatment we measured serum IFNα levels after the i.p. injection of 200 μg poly(I:C). 1, 6 and 24 hours after poly(I:C) treatment, 20 to 30 μl of blood was obtained into a 0.5 ml Eppendorf tube from the mouse tail vein. Whole blood was kept for 10 minutes at RT to allow blood clotting, then 30 minutes at 4°C. The clotted blood was centrifuged at 2500 g for 20 minutes at 4°C, the supernatant transferred into a new tube and spun again at 1500 g for 10 minutes. The final supernatant was clear serum, stored in aliquots at -70°C before use. Serum was diluted 1:15 with dilution buffer and IFNα concentration determined with the mouse interferon alpha ELISA kit (42100-1, PBL Biomedical Laboratories, Piscataway, NJ, USA) according to the manufacturer's instruction (Figure 7).

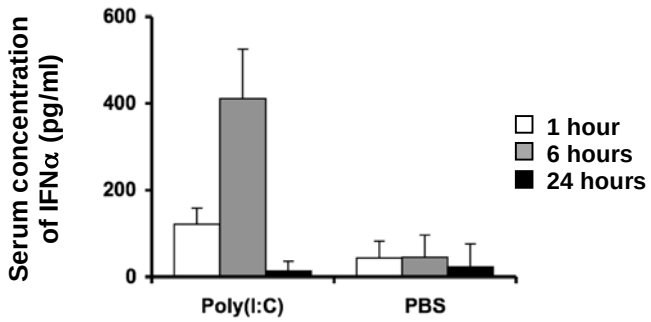

Figure 7. Biological effect of poly(I:C) treatment as measured by IFNα plasma levels.
Mice were treated with poly(I:C) or PBS i.p. and blood was drawn from tail vein after 1, 6 and 24 hours. IFNα level was determined in the serum by ELISA (±SEM, n=4 in each group).

2.11 Leukocyte surface phenotyping

Generally, all FACS staining procedures were carried out at 4°C. 10^5 cells/tube were washed once with 1% BSA-PBS and incubated with the respective conjugated antibodies for 30 min at 4°C. Cells were washed again, fixed in 2% paraformaldehyde in PBS and analyzed on a flow cytometry. For each quantitative analysis a cumulative number of 10^4 cells were collected in the live cell gate as defined by the typical pattern in the forward (FSC) and sideward scatter (SSC) display. The acquired fluorescence intensity data were further analyzed with BD CellQuest™ Pro software (Becton Dickinson). For unconjugated or biotinylated primary antibodies, Alexa Fluor® 488-conjugated donkey-anti-rat IgG or Cy™2-conjugated streptavidin was used as a secondary antibody.

2.12 Intracellular IFNγ staining for the detection of peptide specific CD8 T cells

10^5 splenocytes were resuspened in 200 μl of LCM-10 medium containing 20 μg/ml brefeldin A and 2 ng/ml recombinant mouse IL-2. To activate peptide specific CD8 T lymphocytes, cells were incubated for 5 hours at 37°C in the presence of 10^{-5} M of β-gal$_{(500-507)}$ or OVA$_{(258-265)}$ peptide, respectively. As a positive control, cells were treated by PMA (final concentration, 10 ng/ml) and ionomycin (final concentration, 0.5 μg/ml). After T cell activation, the cells were washed and stained for 30min at 4°C with anti-mouse CD8α-PE Ab. Then the cells were washed twice with 1% BSA-PBS, fixed in 4% paraformaldehyde-PBS for 10 min, permeabilized with 0.1% saponin, 1% BSA in PBS for 10 min, and blocked with 1% FCS for 10 min. After washing twice, anti-mouse IFNγ-FITC Ab was added for 30 min at 4°C. The fraction of IFNγ positive CD8 T cells was determined by FACS (FACS Calibur flow cytometer, BD Biosciences, San Jose, CA, USA). The percent age of peptide specific

CD8 T cells was calculated as (stimulated IFNγ $^{+}$CD8^{+} cells/ total CD8^{+} cells × 100) – (non-stimulated IFNγ $^{+}$CD8^{+} cells/ total CD8^{+} cells × 100) (Figure 8).

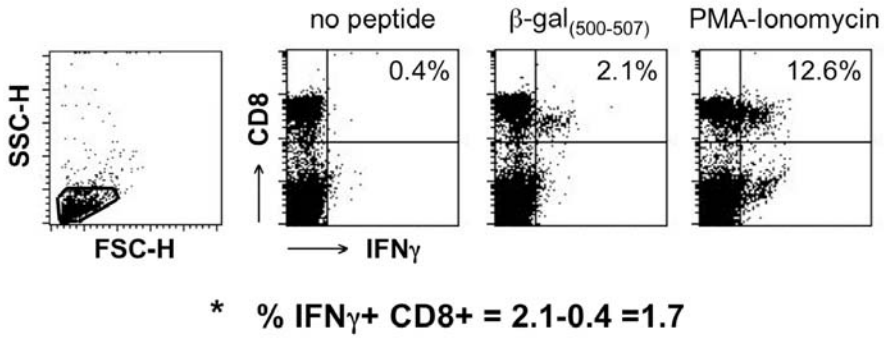

Figure 8. Quantification of peptide-specific CD8 positive splenocytes with ICS.
After immunization with peptide-pulsed DC, wt mice splenocytes were isolated and activated for 5 hours with or without peptide or with PMA (10 ng/ml) and ionomycin (0.5 μg/ml) as positive controls. The percent age of peptide specific CD8 T cells was calculated as: % IFNγ $^{+}$ CD8^{+} cells = 2.1 - 0.4 = 1.7%.

2.13 Calcein release-based cytotoxicity assay

Target cell lysis was assessed *in vitro* by a calcein release assay as described [50]. Briefly, EL-4 cells (gift from Ton Rolink), a H2k^{b} positive thymoma cell line used as targets were loaded for 30 min at 37°C with 20 μM calcein-AM in HBSS, washed with LCM-10 medium, and pulsed with indicated peptide (10^{-5} M) for 1 hour in RPMI complete medium at 37°C. After washing twice with CTL assay medium, EL-4 cells were counted and transferred to 96-well, round-bottom plate at 10^{4} cells/well. Peptide specific CTL were washed with CTL assay medium, counted and added to the calcein-loaded target cells (final volume, 200 μl/well) at titrated cell numbers resulting in decreasing **E**ffector : **T**arget ratios. EL-4 cells without peptide served as controls to determine non-specific cell lysis. Spontaneous release (SR) of calcein from EL-4 cells was determined by adding assay medium; maximum release (MR) was obtained in the presence of detergent by the addition of lysis buffer (50 mM sodium borate in 0.1% Triton X-100 (both from Sigma-Aldrich), pH 9.0). After 2 hours, 75 μl supernatant was carefully removed and transferred to a

96-well, flat bottom plate. Released calcein was measured in a fluorescence multiwell plate reader (SpectraMax GeminiXS, Molecular Devices; excitation wavelength, 485 nm; emission wavelength, 538 nm). The percent specific lysis was calculated as (sample release – SR)/(MR – SR) × 100. SR was < 20% in all experiments (Figure 5B).

2.14 Necropsy and organ embedding

Mice were intraperitoneally euthanized with 30 mg/kg of pentobarbital. Before asystole, 200 μl blood was collected from the left ventricle in heparin treated polypropylene tube (365966, BD Microtainer™ tube) for the isolation of plasma. When used for intracellular cytokine staining or FACS, spleen, lymph node and bone marrow were collected. The mice were perfused with 15 ml of PBS (37°C) followed by 30 ml of PIPES-buffered paraformaldehyde (0.1 M PIPES (pH 6.9), 2 mM $MgCl_2$, 2% paraformaldehyde, and 1.25 mM EGTA). Subsequently, organs were removed and incubated in PIPES-buffered paraformaldehyde for 2 hours, rinsed twice in PBS and equilibrated in 30% sucrose-PBS (wt/vol.) overnight. Tissues were embedded in OCT compound (4583, Tissue-Tek, Medite, Burgdorf, Germany) and snap frozen at -50°C. Frozen samples were stored at -70°C until further use. 10 μm tissue sections were cut with a cryotome (HM560, Microm International GmbH, Germany) onto Superfrost® plus glass slides (J1800AMNZ, Thermo Scientific, Menzel GmbH & Co. KG, Braunschweig, Germany). Sections were air dried for 30 minutes and either stained immediately or stored at -70°C until further use.

2.15 Histology and immunohistochemistry

2.15.1 Hematoxylin-eosin (H&E) staining

OCT was removed from tissue and tissue was rehydrated through graded ethanol: 100%, 95%, 75% and 50%, then washed with tap water. Slides were incubated with hematoxylin for 1 minute at RT, washed with tap water and warm water, then incubated with eosin for 30 second at RT. After washing with tap water, slides were dehydrated through graded ethanol: 50%, 75%, 95% and 100% and mounted in Pertex (41-4010-00, Medite). Lung inflammation was quantified according to a semiquantitative score: 0 = no inflammatory infiltrates, 1 = mild perivascular inflammatory infiltrates, 2 = severe vascular and perivascular inflammatory infiltrates (Figure 9). All slides were coded and evaluated independently by two observers, one of which was blinded for the experimental conditions (BCB, XW). Generally, agreement between the two readers was >95%.

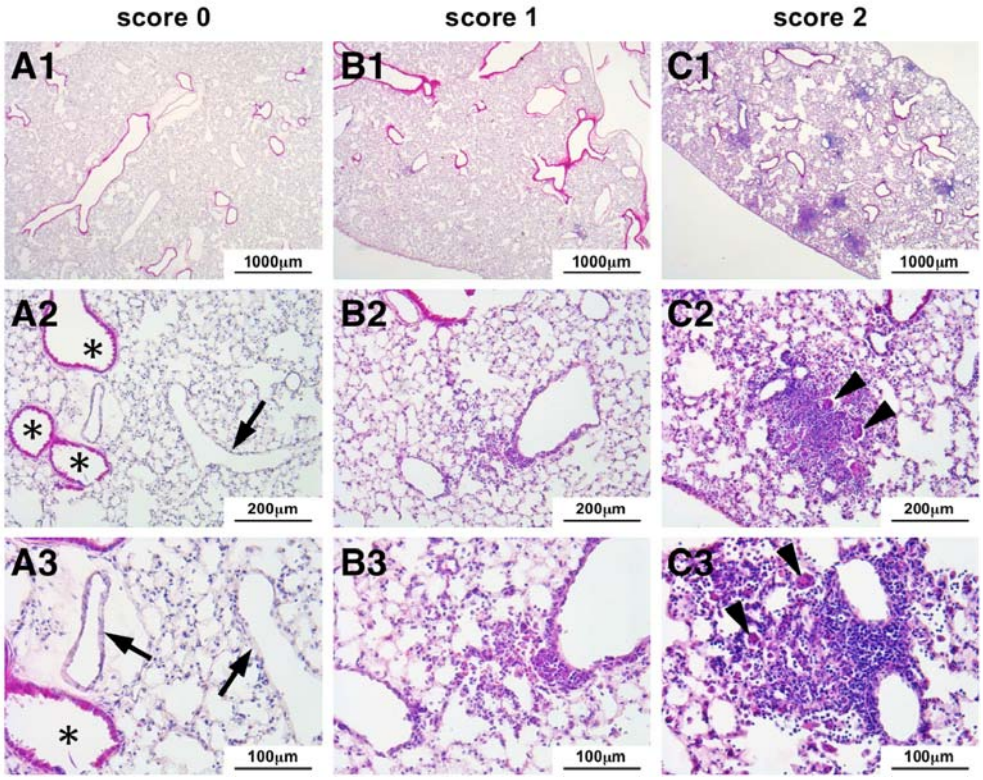

Figure 9. Assessment of lung injury by a semiquantitative histopathological score.

Murine lungs were perfused, fixed and embedded in OCT. 10μm thick cryosections were stained with hematoxylin/eosin (H&E). (A) score 0 = no inflammatory infiltrates (B) score 1= mild perivascular inflammatory infiltrates and vasculitis (C) score 2 = severe perivascular inflammatory infiltrates and vasculitis, giant cell formation. The black stars show bronchi, black arrows show blood vessels and black arrowheads show giant cells.

2.15.2 Immunohistochemistry (IHC)

To further characterize the cellular composition of the inflammatory infiltrates, immunohistochemistry was performed. Frozen tissue sections were fixed in acetone for 10 minutes, and non-specific binding sites were blocked for 30 minutes with PBS containing 1% FCS, 2% BSA and 0.1% Tween-20. Sections were incubated with the first antibodies specific for CD4 (clone YTS191.1), CD8 (clone YTS169.4.2) (both gift from Burkhard Ludewig, Kantonsspital St. Gallen) or inflammatory macrophages (F4/80, gift from Regine Landmann, Department of Biomedicine, Basel) followed by goat anti-rat IgG. Finally, alkaline phosphatase-labeled donkey anti-goat IgG was added. The third enzyme conjugated antibody was diluted in Tris-HCl (0.1 M, pH 7.4) containing 5% normal mouse serum. Alkaline phosphatase was visualized by using AS-BI phosphate/New Fuchsin (SK-5100, Vector Laboratories, Inc., Burlingame, CA, USA). Endogenous alkaline phosphatase was blocked by Levamisole solution (SP-5000, Vector). Tissue staining with biotinylated anti-CD11c, biotinylated anti-CD45.2 or biotinylated anti-CD45.1 was visualized by avidin-biotin-enzyme complex (PK-6100, Vector) and DAB. To block endogenous peroxidase, methanol containing 0.3% of H_2O_2 was used. Rat IgG2b, biotinylated Armenian hamster IgG1 and biotinylated mouse IgG were used as isotype controls at the same concentration as the first antibodies. Normally, antibodies were prepared in PBS containing 1% FCS and 1% BSA. Between the steps, slides were washed with PBS containing 0.1% Tween-20. All sections were counterstained with hematoxylin, dehydrated through graded ethanol: 50%, 75%, 95% and 100% and mounted in Pertex. Images were acquired using an OLYMPUS BX61 microscope equipped with ColorView and F-View cameras. Digital images were processed using Imaging software Cell^P (OLYMPUS Imaging Europa GmbH, Hamburg,

Germany) and Adobe Photoshop. The percentage of positive cells / total cells in area was calculated as: positive stained cells in lesion area / total cells in lesion area × 100 (Figure 20D, 24C, 24D).

2.16 β-Galactosidase detection

2.16.1 in situ tissue staining

X-gal (V394A, Promega, Madison, WI, USA) was used as a substrate for β-galactosidase to detect enzyme expression in vascular endothelial cells as described [1]. In brief, the frozen tissue sections were allowed to air dry, were postfixed with 2% paraformaldehyde in PBS for 10 minutes at RT. Then they were washed twice with PBS. Slides were incubated for 8-16 hours at 37°C in PBS (pH 7.4) containing NP-40 (0.02%), SDS (0.01%), $MgCl_2$ (2 mM), 10 mM $K_3Fe(CN)_6$ and 10 mM $K_4Fe(CN)_6$ along with chromogenic β-galactosidase substrate X-gal (1 mg/mL). Stained sections were rinsed with PBS and tap water, counterstained with Eosin, and dehydrated through graded ethanol: 50%, 75%, 95% and 100% and mounted in Pertex (Figure 2). Whole mount staining of perfusion-fixed organs of tissue fragments was performed by incubation with X-gal overnight as described above. The frequency of X-gal stained blood vessels was calculated as: number of positive stained blood vessels in inflammatory infiltrates of whole lung area / all vessels involved in inflammatory infiltrates of whole lung area × 100 (Figure 25D).

*2.16.2 β-ga*l activity detected by FACS

Single cell suspension of splenocytes, PBMC, lymph node and bone marrow cells (3×10^5 cells / tube) were washed once with 1% BSA-PBS. Cells were then resuspended in 25 μl of 1% BSA-PBS and incubated for hypotonic loading with 25 μl of FDG (diluted 1:10, F1179, LUBIO) at 37°C for 30

minutes, returned to isotonicity at 4°C by adding 500 μl of ice-cold 1% BSA-PBS, and stained with antibodies for specific surface proteins as described [74]. For antibody staining of cells ‘‘loaded’’ with FDG, the cells were kept at 4°C at all steps in the staining procedure, including centrifugation in prechilled rotors and adapters. Antibody stains and the staining mediums used in the procedure also were kept on ice throughout the duration of the procedure. To exclude dead cells, propidium iodide (PI, 5 μg/ml, 70335, Sigma-Fluka, Buchs, Switzerland) was used prior to data acquisition (Figure 5B). Cells were generally analyzed without fixation.

2.17 Statistical data analysis

To compare means of different groups, the Mann-Whitney U test was used. *p* values < 0.05 were considered to indicate a statistically significant difference between groups. Value of *p* representation: < 0.05 is represented by one asterisk; < 0.01 by two asterisk; < 0.001 by three asterisk Statistical data analysis was performed using SPSS 13 version for Mac OS X (SPSS Inc., Chicago, IL, USA). Unless indicated otherwise, mean ± SEM are shown.

3. Results

3.1 Antigen – presentation by vascular endothelium: its role for CTL – mediated vascular injury

3.1.1 Endothelial β-galactosidase as an autoantigen

In this study, TM-LacZ mice with beta-galactosidase (β-gal) expression confined to the vascular endothelium were used to study the interaction of CTL with EC. For TM-LacZ mice, β-galactosidase is an autoantigen. In order to test the level of tolerance, the TM-LacZ mice were immunized repetitively i.v. with β-gal$_{(500-507)}$-pulsed DC [54] and the peptide-specific CTL response was determined two weeks later (Figure 10A). The intracellular IFN-γ production by CD8 T cells upon *in vitro* stimulation with or without β-gal$_{(500-507)}$ peptide was examined in splenocytes. There were no peptide specific CD8 T cells detectable in immunized TM-LacZ mice, under conditions, under which wt mice developed a strong immune response against β-gal$_{(500-507)}$ (Figure 10B, 10D). When mice were immunized with OVA$_{(258-265)}$-pulsed DC, no difference in the differentiation expansion of OVA-specific CD8 T cells was observed between wt and TM-LacZ mice. As a general sign of health, body weight of the mice was assessed. No significant weight change occurred during the course of the experiment (Figure 10C). There was no tissue injury detectable, in particular no vascular injury, in TM-LacZ mice.

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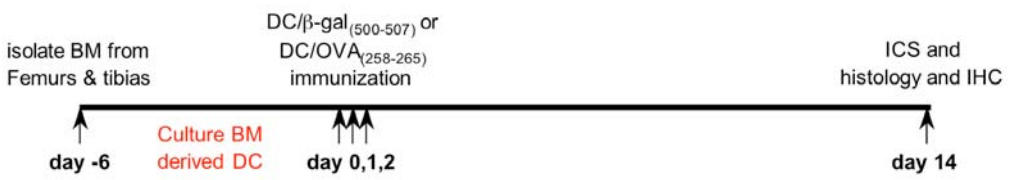


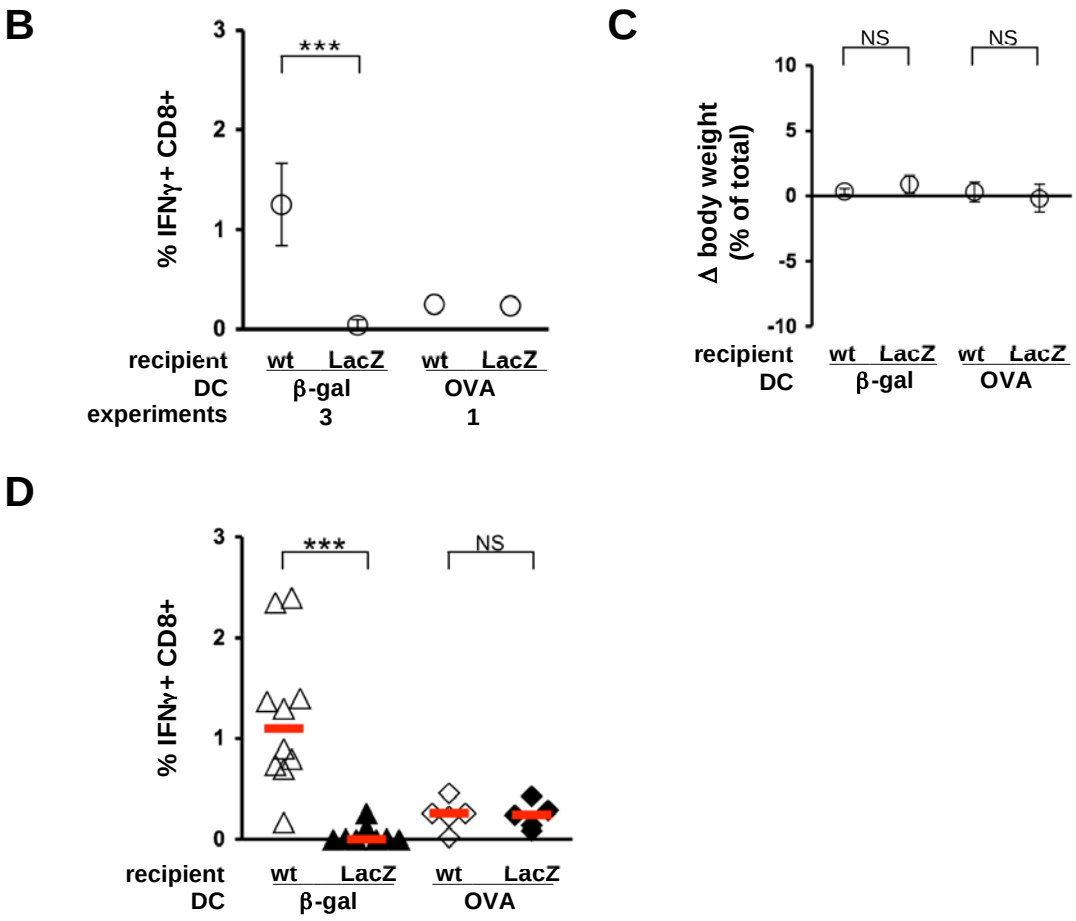
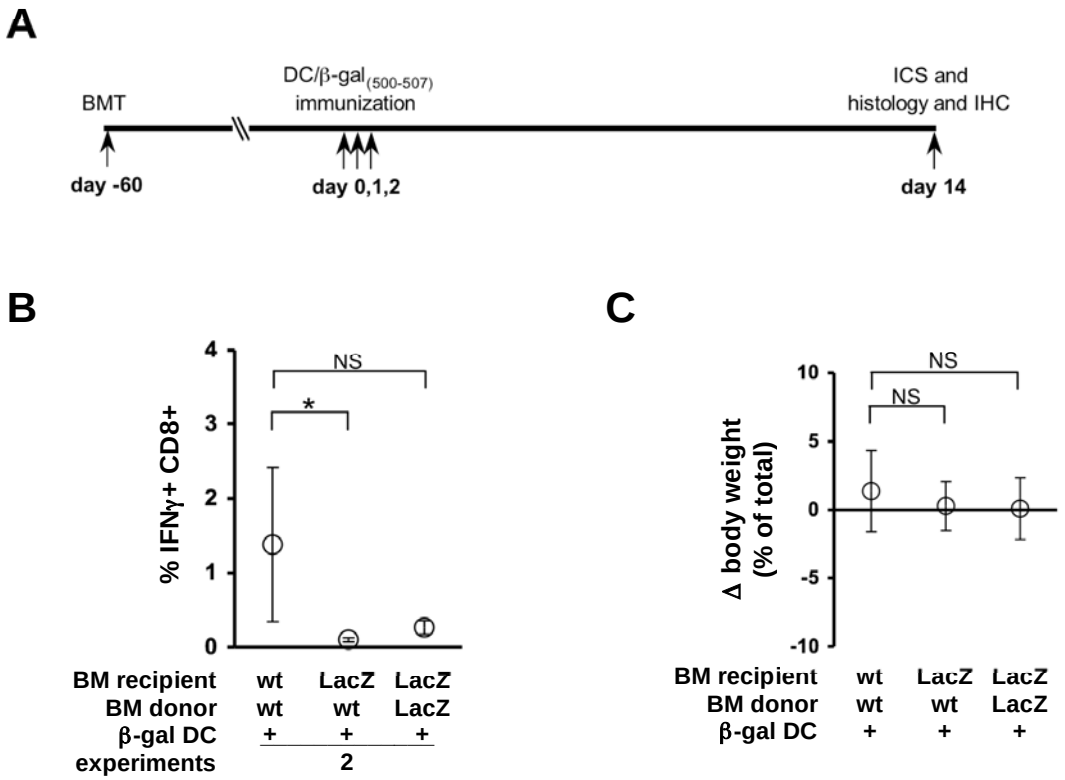


Figure 10. i.v. immunization of TM-LacZ mice with β-gal$_{(500-507)}$-pulsed DC.

(A) *Outline of the experimental design:* TM-LacZ and wt mice were immunized with 5 × 10^5 β-gal$_{(500-507)}$ or OVA$_{(258-265)}$ peptide pulsed bone-marrow derived DC, repetitively. (B) Two weeks after immunization, splenocytes from immunized mice were tested for peptide-specific $CD8^+$ T cells by intracellular production of IFNγ upon *in vitro* stimulation with or without β-gal$_{(500-507)}$ or OVA$_{(258-265)}$ peptide. Values represent mean (± SEM of three independent experiments with similar outcome). (NS = not significant). (C) Changes in the body weight of mice in representative groups (pooled data) within two weeks, calculated as follows: Δ body weight (% of total) = (weight (day 14) – weight (day 0)) / weight (day 0) × 100. Shown is mean ± SEM. (D) Intracellular cytokine staining of individual mice was pooled from all experiments. Red lines represent median values.

3.1.2 Endothelial β-galactosidase as a minor histocompatibility antigen

In order to mimic vascular disease as observed during GVHD after MHC-matched bone marrow transplantation, we performed bone marrow transplantation with wt mice as donors, and TM-LacZ mice as recipients. Under these conditions, β-galactosidase serves as a minor histocompatibility alloantigen. The following BM chimeric mice were generated: wt → wt, wt → LacZ and LacZ → LacZ. All TM-LacZ recipients remained healthy (Figure 11C), even after repetitive immunization with β-gal$_{(500-507)}$-pulsed DC (Figure 11A). No signs of graft-versus-host-disease were observed. This immunization process induced strong CTL expansion in wt → wt mice (Figure 11B, 11D). At the same time, expansion of peptide specific CTL was reduced in both wt → LacZ and LacZ → LacZ recipients. Taken together, these data indicate that antigen expressed on EC alone is sufficient to restore alloantigen-specific tolerance in TM-LacZ mice.



D

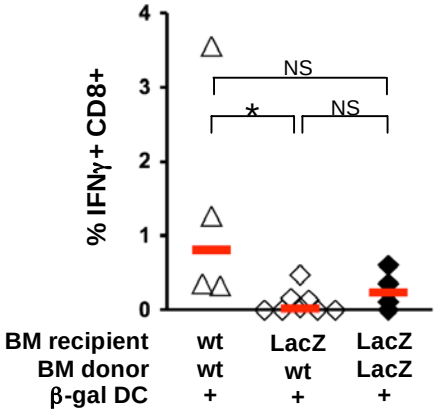


Figure 11. i.v. immunization of C57BL/6 → TM-LacZ bone marrow chimeric mice with β-gal$_{(500-507)}$-pulsed DC.

(A) *Outline of the experimental design:* Bone marrow chimeric mice C57BL/6 → C57BL/6 (wt→wt), C57BL/6 → TM-LacZ (wt →LacZ) and TM-LacZ → TM-LacZ (LacZ→LacZ) were immunized with 5 × 10^5 β-gal$_{(500-507)}$-pulsed DC. (B) On day 14, splenocytes were isolated for intracellular cytokine staining of IFNγ. Data shown mean ± SEM of peptide-specific $CD8^+$ T cells in spleen from two independent experiments with similar outcome. (C) The body weight loss or gain of mice in representative groups within two weeks. Shown is mean ± SEM. (D) Intracellular cytokine staining of individual mice was pooled from two experiments. Red lines represent median values.

3.1.3 Endothelial β-galactosidase as a "foreign" or "third party" antigen (adoptive transfer of effector CTL or primed splenocytes)

To directly expose EC to effector CTL, 10^6 or 10^7 β-gal-specific CTL that were expanded and tested *in vitro* (Figure 5), were injected i.v. to wt or TM-LacZ mice, followed by repetitive immunization with peptide-pulsed DC (Figure 12A). No peptide-specific CD8 T cells were detectable in TM-LacZ mice regardless of the number of effector CTL that was injected (Figure 12B). No obvious health problems or body weight change was observed in the recipients (Figure 12C). These results indicate that adoptively transferred peptide-specific effector CD8 T cells fail to precipitate disease, and obviously do not home to the spleen, even after the injection of 10^7 CTL (corresponding to 10% of average total splenocyte number).

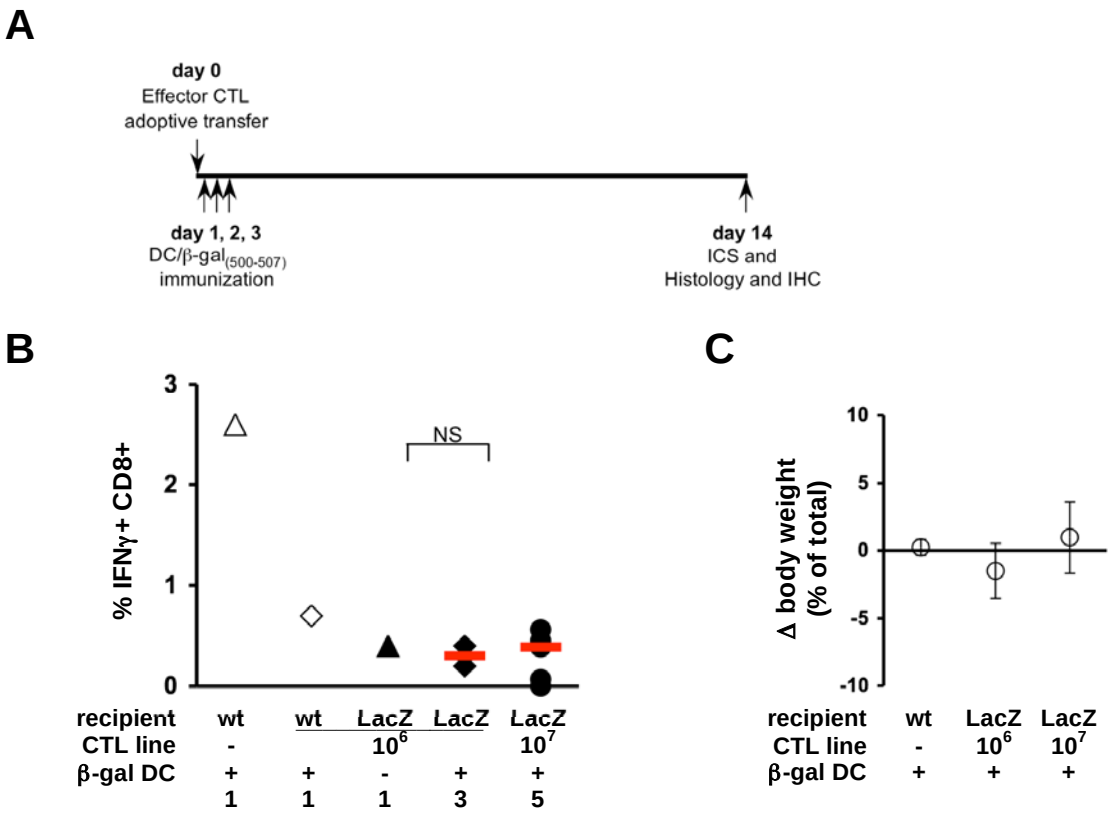


Figure 12. i.v. immunization of TM-LacZ mice with β-gal$_{(500-507)}$-pulsed DC after adoptive transfer of β-gal specific effector CTL.

(A) *Outline of the experimental design:* 10^6 or 10×10^6 cultured effector CTL were adoptively transferred i.v. into TM-LacZ and wt mice. In order to activate adopted T cells, mice were immunized with 5×10^5 β-gal$_{(500-507)}$-pulsed DC intravenously on day 0, 1 and 2, repetitively. (B) Two weeks after immunization, splenocytes of recipient mice were analyzed for intracellular cellular cytokine staining. Each symbol represents an individual mouse, and red lines represent median values. (C) Changes in the body weight of mice in representative groups (pooled data) within two weeks.

It was recently study shown that fully-differentiated effector CTL cells were less effective for adoptive transfer in an auti-tumor immune-therapy model [62]. Therefore instead of effector CTL, primed-effector splenocytes were used for adoptive transfer into the mice, followed by immunization of peptide-pulsed DC. Wt mice were able to mount a detectable peptide-specific immune response, but in TM-LacZ mice, a β-gal$_{(500-507)}$-specific CTL were not detected in the spleen (Figure 13B, 13D). Again, as for the previous experiments, no significant body weight change indicating general disease was observed

(Figure 13C). These experiments demonstrate that overt CTL mediated vascular injury cannot be induced by adoptive transfer of mature effector CTL or primed splenocytes into TM-LacZ mice.

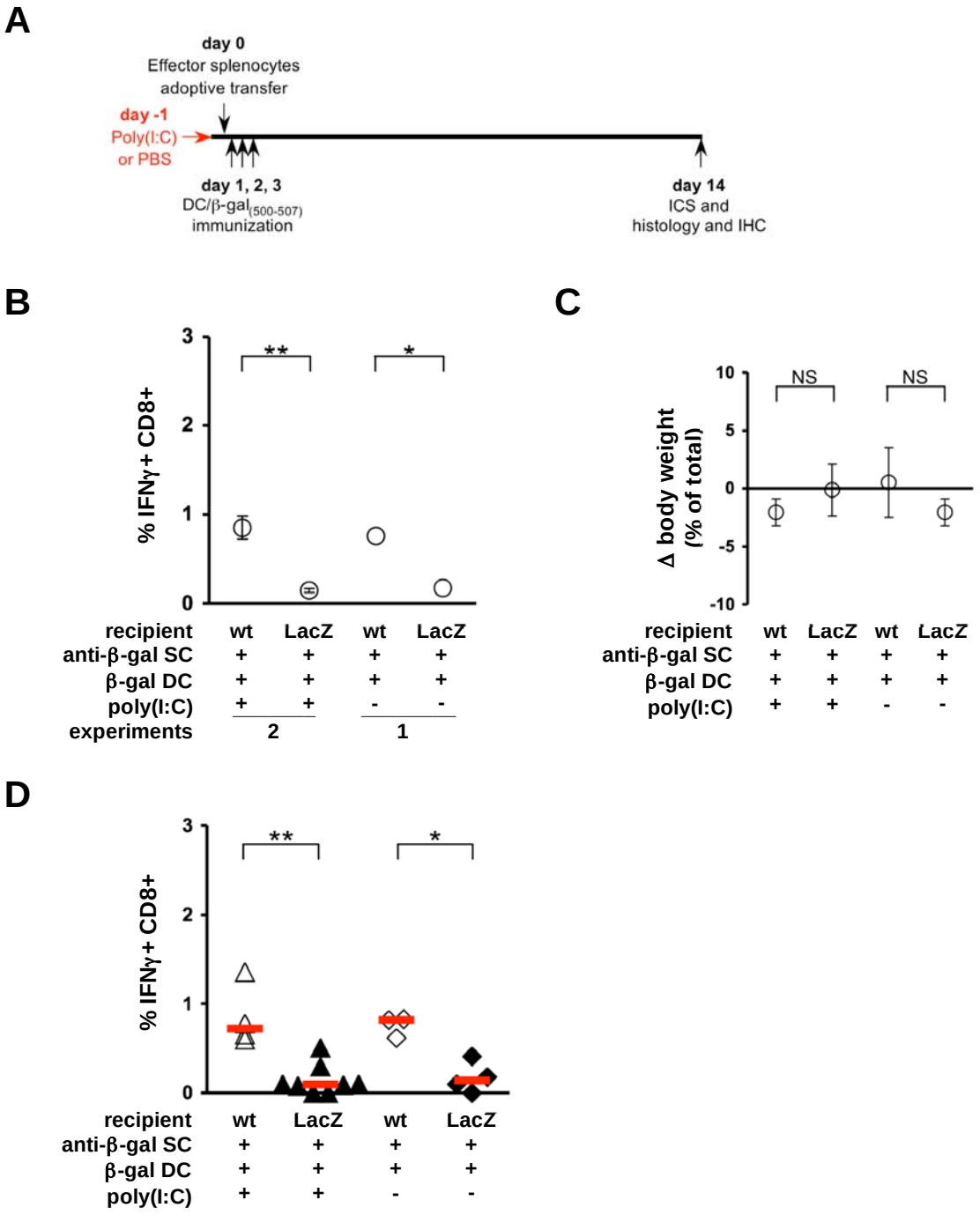


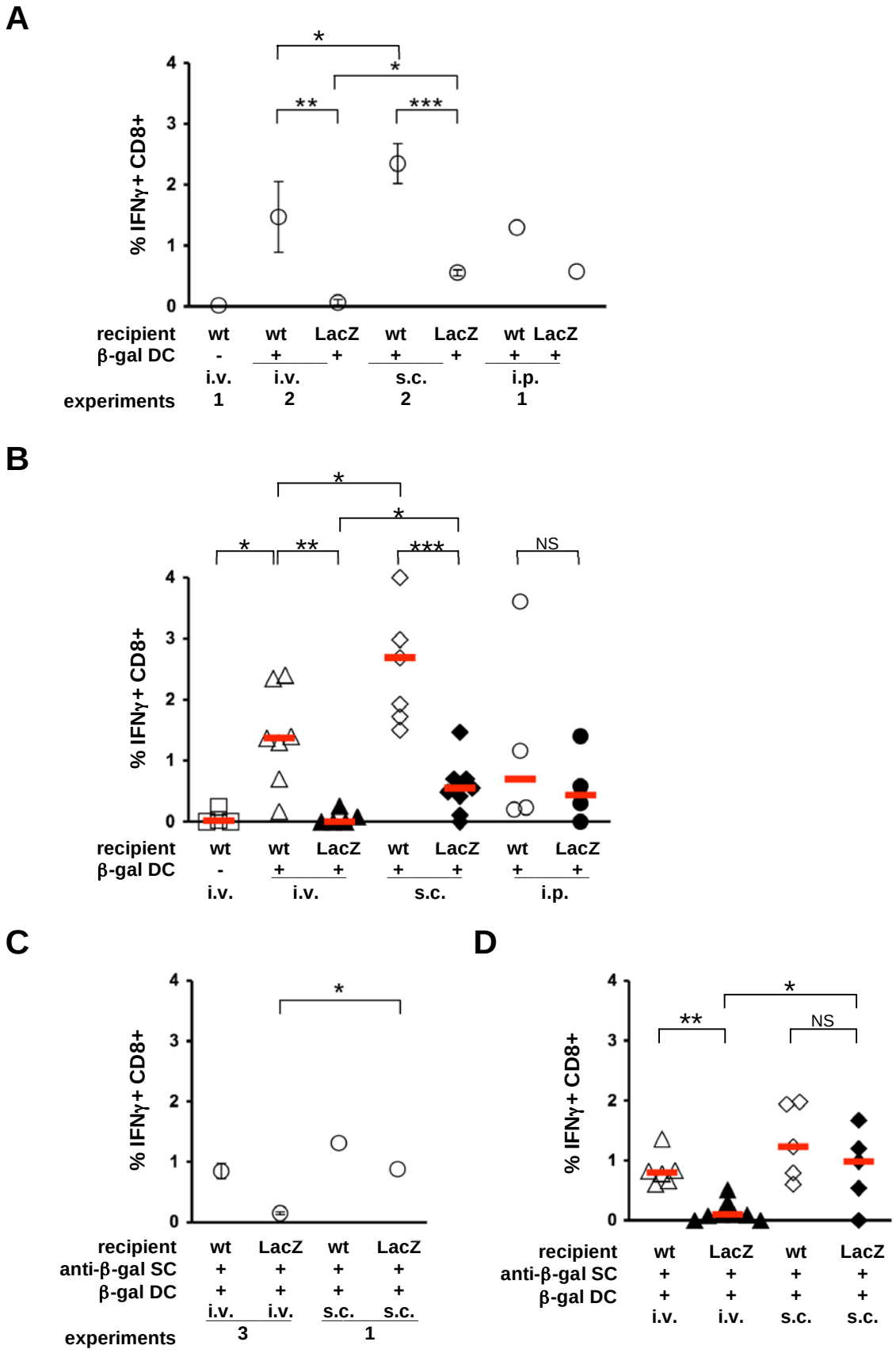
Figure 13. i.v. immunization of TM-LacZ mice with β-gal$_{(500-507)}$-pulsed DC after adoptive transfer of β-gal specific splenocytes.

(A) *Outline of the experimental design:* 25 × 10^6 splenocytes (containing about 4 × 10^5 peptide specific CTL) from β-gal$_{(500-507)}$ immunized wt mice were adoptively transferred to TM-

LacZ and wt mice. On day 1, 2, 3, the mice were immunized with 5×10^5 β-gal$_{(500-507)}$-pulsed DC. Mice were treated with Poly(I:C) or PBS i.p. one day before splenocytes adoptive transfer. (B) On day 14, splenocytes of recipient mice were isolated for intracellular cellular cytokine staining. Data is the mean (± SEM of two independent experiments with similar outcome). (C) The body weight loss or gain of mice in representative groups within two weeks. (D) Intracellular cytokine staining of individual mice was pool from all experiments. Red lines represent median values.

3.1.4 Route of immunization affects the number of β-gal$_{(500-507)}$ specific CD8 T cells in TM-LacZ mice

We next tested whether the route of DC administration is important for the outcome of immunization. Wild type and TM-LacZ mice were immunized intravenously (i.v.), subcutaneously (s.c.) or intraperitoneally (i.p.) with β-gal$_{(500-507)}$-pulsed DC. Subcutaneous immunization increased the number of peptide specific CTL both in wt and TM-LacZ mice, but no significant increasing in both mice after i.p. immunization (Figure 14A, 14B). When β-gal$_{(500-507)}$-primed splenocytes were adoptively transferred before immunization, the peptide-specific CTL population was more pronounced raising to an average of 1% of the $CD8^+$ T cells from spleen in TM-LacZ mice (Figure 14C, 14D). Under these conditions, the difference between wt and TM-LacZ mice disappeared. These results suggested that subcutaneous immunization with peptide-pulsed DC combined with primed splenocytes adoptive transfer was able to overcome tolerance of β-galactosidase and enabled unrestricted expansion of peptide-specific CTL in TM-LacZ mice. However, no antigen-specific, CTL-mediated vascular injury was observed even when anti-β-gal $CD8^+$ T cells were detectable in TM-LacZ mice (Figure 14E). This suggests that β-galactosidase antigen expression by endothelial cells is not recognized or ignored by peptide-specific CTL.


A
% IFNγ+ CD8+
recipient wt wt LacZ wt LacZ wt LacZ
β-gal DC - + + + + + +
i.v. i.v. s.c. i.p.
experiments 1 2 2 1
B
% IFNγ+ CD8+
NS
recipient wt wt LacZ wt LacZ wt LacZ
β-gal DC - + + + + + +
i.v. i.v. s.c. i.p.
C
% IFNγ+ CD8+
recipient wt LacZ wt LacZ
anti-β-gal SC + + + +
β-gal DC + + + +
i.v. i.v. s.c. s.c.
experiments 3 1
D
% IFNγ+ CD8+
NS
recipient wt LacZ wt LacZ
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E

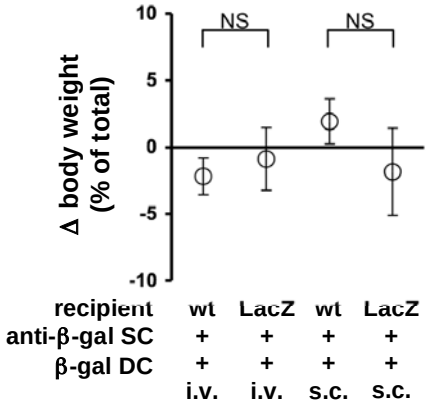


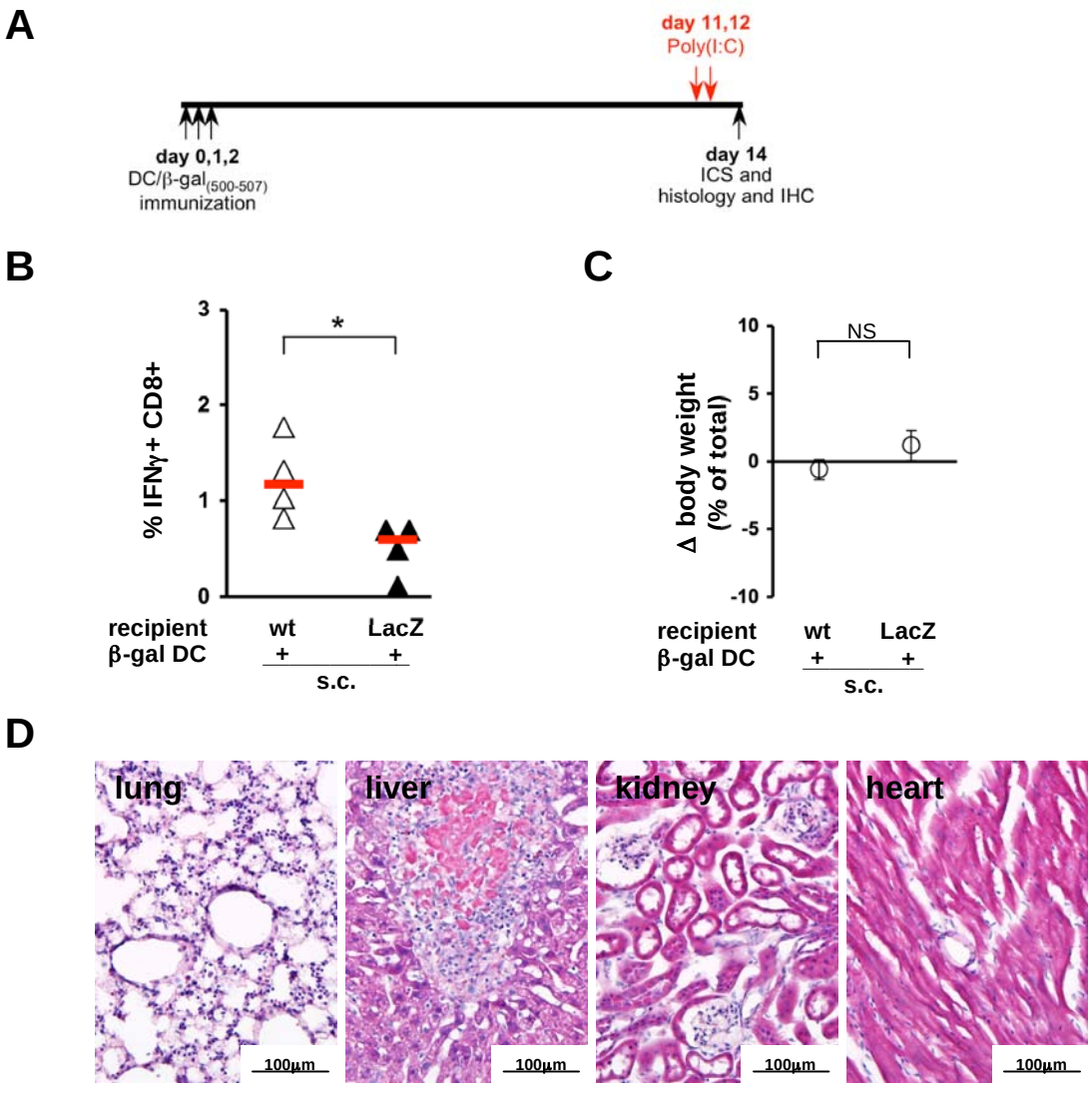
Figure 14. Route of immunization affects the level of peptide-specific CD8$^+$ splenocytes.

(A) TM-LacZ and wt mice were immunized i.v., s.c. or i.p. with 5 × 10^5 β-gal$_{(500-507)}$-pulsed DC, repetitively. As control, wt mice were immunized i.v. with un-pulsed DC. Two weeks after immunization, splenocytes from immunized mice were tested for peptide specific CTL. Values represent mean (± SEM of two independent experiments with similar outcome). (B) Intracellular cytokine staining of individual mice from (A). Red lines represent median values. (C) TM-LacZ and wt mice received 25 × 10^6 β-gal$_{(500-507)}$-primed splenocytes. On day 1, 2, 3, the mice were immunized either i.v. or s.c. with 5 × 10^5 β-gal$_{(500-507)}$-pulsed DC. On day 14, splenocytes of recipient mice were isolated for intracellular cytokine staining. Values represent mean (± SEM of three independent experiments with similar outcome). (D) Intracellular cytokine staining of individual mice was pool from (C). Red lines represent median values. (E) Changes in the body weight of mice within two weeks after received 25 × 10^6 β-gal$_{(500-507)}$-primed splenocytes and DC/β-gal$_{(500-507)}$ immunization.

3.1.5 Toll-like receptor activation does not precipitate immune-mediated injury

Lang [63] showed that among the various of Toll-like receptor ligands, Poly(I:C) was able to transform tissue infiltration by autoreactive CTL into overt autoimmune disease. The mechanism was hypothesized to be upregulation of MHC class I molecules by IFNα. Therefore mice were immunized subcutaneously by peptide-pulsed DC in order to generate CTL; 9 and 11 days after immunization, mice were treated with poly(I:C) in order to activate antigen presenting cells (Figure 15A). Both TM-LacZ mice and wt mice remained healthy (Figure 15C). When we examined several organs, we found

that 1/4 of mice had signs of liver injury, but this finding was non-specific, i.e. independent of antigen expression by endothelial cells (Figure 15D, 15E). This finding may be caused by the activation of innate immune cells in the liver through poly(I:C).



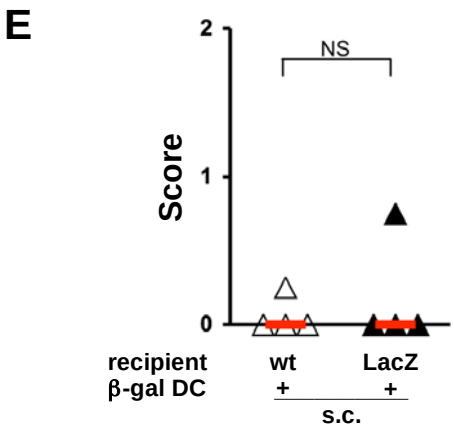


Figure 15. Subcutaneous immunization of wt, TM-LacZ mice with β-gal$_{(500-507)}$-pulsed DC.

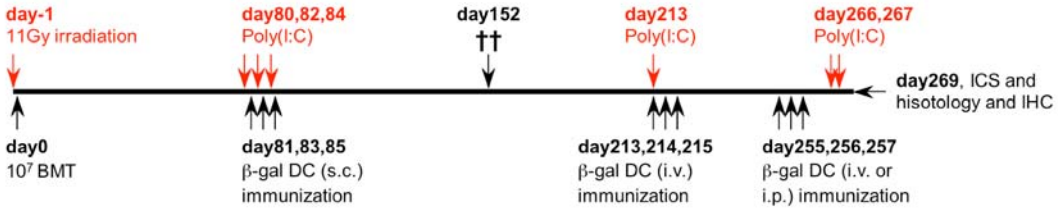
(A) *Outline of the experimental design:* wt, TM-LacZ and Rosa26-LacZ mice were immunized s.c. with 5 × 10^5 β-gal$_{(500-507)}$ peptide pulsed bone-marrow derived DC on day 0, 1 and 2, repetitively, and treated with Poly(I:C) i.p. on day 11 and 12. (B) Two weeks after immunization, splenocytes from immunized mice were tested for peptide-specific $CD8^+$ T cells by intracellular production of IFNγ upon *in vitro* stimulation with or without β-gal$_{(500-507)}$ peptide. Each symbol represents an individual mouse. Red lines represent median values. (C) The body weight loss or gain of mice in representative groups within two weeks. (D) H&E staining of lung, liver, kidney and heart. Black star shows abnormal. (E) Mean inflammatory infiltrates severity scores in lung, kidney, liver and heart of recipients. Each symbol represents an individual mouse. Mean inflammatory infiltrates severity scores were calculated as following: (score of lung + score of liver + score of kidney + score of heart) / 4. Red lines represent median values. Score definitions see Figure 9.

Similar outcome was observed, when we treated mice with Poly(I:C) before peptide-primed splenocytes adoptive transfer and peptide-pulsed DC immunization (Figure 13A). No difference of peptide-specific $CD8^+$ T cells expansion in spleen was detected between Poly(I:C) and PBS treated wild type and TM-LacZ mice (Figure 13B, 13D). During the whole experimental process, no significant body weight change corresponding to general disease was observed (Figure 13C), although administration of Poly(I:C) to the mice resulted in substantial production of IFNα in circulating blood (Figure 7) and precipitated liver disease in some of the mice.

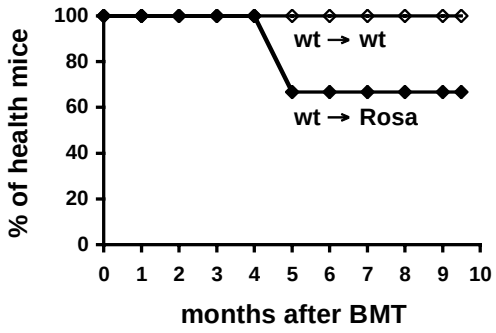
3.1.6 Antigen-specific graft versus host disease in Rosa26-LacZ mice with ubiquitous expression of β-galactosidase

To test whether the ubiquitous antigen expression on target cells would precipitate GVHD like disease in this animal model, Rosa26-LacZ mice, which express β-galactosidase ubiquitously in most tissues of the adult mouse were used as BM recipients. BM chimeric mice (wt → wt and wt → Rosa) were repetitively immunized with 5×10^5 β-gal$_{(500\text{-}507)}$-pulsed DC in different administration route, meanwhile treated with Poly(I:C) i.p. at different time points (Figure 16A). During this long lasting experiment, 100% of wt, but only 66.7% of Rosa26-LacZ mice survived (Figure 16B). Two Rosa26-LacZ mice were sacrificed 10 weeks after s.c. immunization with 5×10^5 β-gal$_{(500\text{-}507)}$-pulsed DC combine poly(I:C) treatment, because of signs of GVHD. The mice developed inflammatory ulcerative skin disease around the s.c. injection site (Figure 16C). No other organ injury was observed. Two weeks after the last immunization, when the mice were sacrificed, about 20 times more peptide specific $CD8^+$ T cell were detected in wild type spleen (5.8%) than in Rosa26-LacZ spleen (0.3%) (Figure 16D). Organ immunohistochemical staining showed that this experiment setting induced chronic fibrotic inflammation in lung, liver and kidney, but not in heart of all recipients (Figure 16F). There was a trend to more sever tissue injury in Rosa26-LacZ mice, but this was not significantly different between wt and Rosa26-LacZ (Figure 16G). The only sign suggesting antigen specific response is body weight change. During the nine months, the weight of wt → wt mice increased by 15 ± 4 %, whereas in wt → Rosa mice the weight remained stable (Figure 16E). The results of this long term experiment suggest that a certain level of antigen dependent tissue injury and disease can be observed in wt → Rosa mice, but this is not reflected by the number of peptide specific CD8 T cells in spleen.

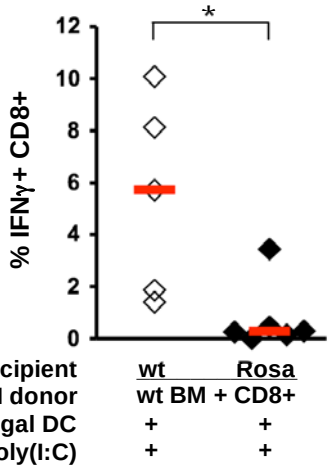
A


day-1
11Gy irradiation
day80,82,84
Poly(I:C)
day152
††
day213
Poly(I:C)
day266,267
Poly(I:C)
day269, ICS and
hisotology and IHC
day0
10⁷ BMT
day81,83,85
β-gal DC (s.c.)
immunization
day213,214,215
β-gal DC (i.v.)
immunization
day255,256,257
β-gal DC (i.v. or
i.p.) immunization

B


% of health mice
100
80
60
40
20
0
wt → wt
wt → Rosa
0 1 2 3 4 5 6 7 8 9 10
months after BMT

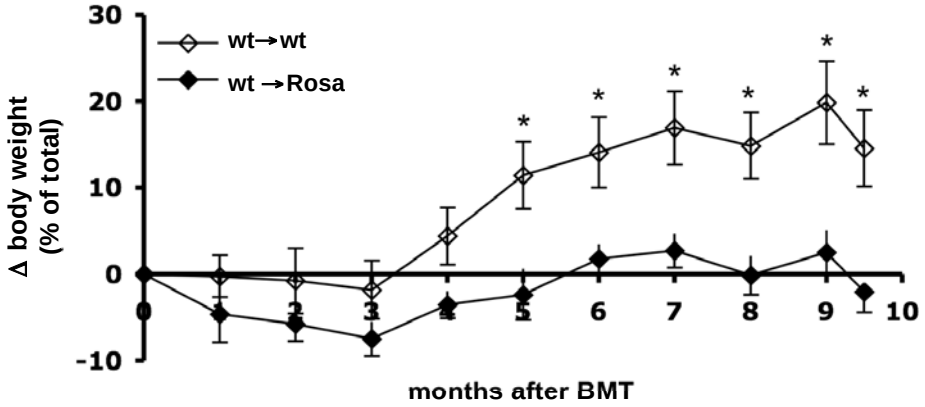
D


*
% IFNγ+ CD8+
12
10
8
6
4
2
0
recipient
BM donor
β-gal DC
poly(I:C)
wt
Rosa
wt BM + CD8+
+
+
+
+

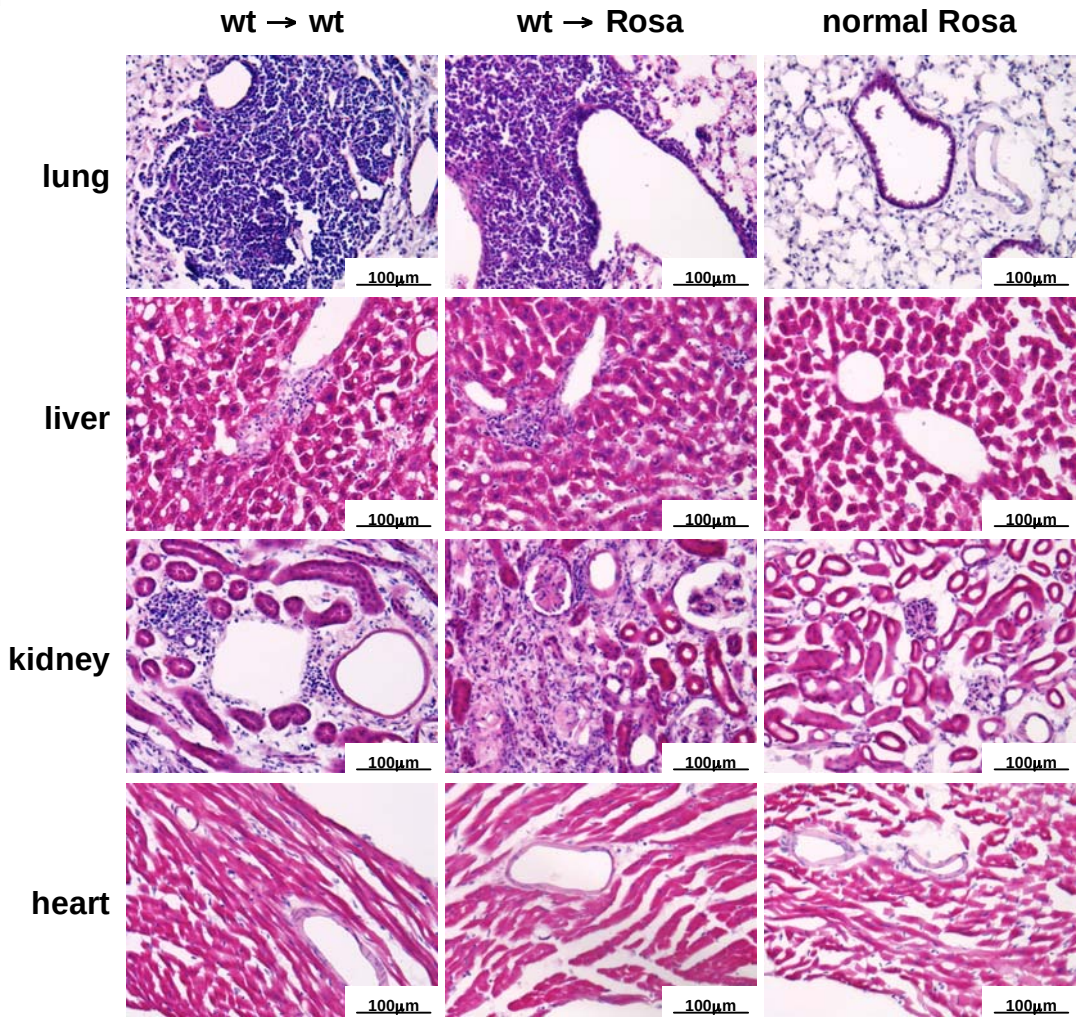
C


L
R

E


Δ body weight
(% of total)
30
20
10
0
-10
wt→wt
wt →Rosa
*
0 1 2 3 4 5 6 7 8 9 10
months after BMT

F


wt → wt
wt → Rosa
normal Rosa
lung
liver
kidney
heart
100μm

G

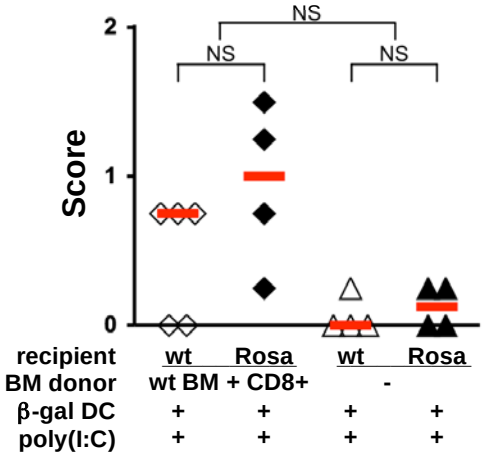

NS
NS
NS
Score
2
1
0
recipient
wt
Rosa
wt
Rosa
BM donor
wt BM + CD8+
-
β-gal DC
+ + + +
poly(I:C)
+ + + +

Figure 16. Organ injury occurred in chimeric mice after long-term treatment.

(A) *Outline of the experimental design:* Bone marrow chimeric mice C57BL/6 → C57BL/6 (wt → wt) and C57BL/6 → Rosa26 (wt → Rosa) were immunized with 5×10^5 β-gal$_{(500\text{-}507)}$-pulsed DC in different administration route, meanwhile treated with Poly(I:C) i.p. at different time points. (B) Survival curve of mice after bone-marrow transplantation. (C) Mice were killed on day 152 showed GVHD-like skin lesion on the ears and upper back. L and R indicate left side and right side of mouse. Black stars show subcutaneous injection site. (D) On day 14 after last immunization, splenocytes of recipient mice were isolated for intracellular cellular cytokine staining. (E) Changes in body weight of mice in representative groups within nine months. (F) Lung, liver, kidney and heart of chimeric mice and normal Rosa26-LacZ were analyzed by H&E staining. (G) Mean inflammatory infiltrates severity scores in lung, kidney, liver and heart of chimeric mice and normal mice after immunization and Poly(I:C) treatment. Each symbol represents an individual mouse. Red lines represent median values.

3.2 Lung inflammatory infiltrates (LII) after intravenous injection of leucocytes

3.2.1. Intravenous injection of DC and splenocytes, but not BM cells induce lung inflammatory infiltrates

The systematic histological examination of various organs (liver, lung, kidney and heart) for signs of immune mediated injury revealed an unexpected finding: after intravenous injection of leucocyte suspensions, we found consistent and reproducible lung inflammatory infiltrates (LII) of variable severity.

In the second part of my thesis, I investigated the pathogenesis of these LII, which did not severely affect the mice but histologically resembled perivasculitis. This perivascular inflammation was limited to the lung, and not observed in other organs.

We initially discovered the inflammatory infiltrates in lung two weeks after immunization with 0.5×10^6 β-gal$_{(500\text{-}507)}$ peptide pulsed DC. In all recipient mice variable degrees of lung inflammatory infiltrates were detected but kidneys appeared normal at the same time (Figure 17A, 17B). To quantify the histological alteration in the lung, a scoring system ranging from 0 to 2 was established (see method and Figure 9). Similar LII were also observed two weeks after intravenous adoptive transfer of 25×10^6 β-gal$_{(500\text{-}507)}$-primed splenocytes in mice (Figure 18A, 18B). The combined injection of DC and splenocytes, i.e. 25×10^6 β-gal$_{(500\text{-}507)}$-primed splenocytes intravenously injected on day 0, and 0.5×10^6 β-gal$_{(500\text{-}507)}$-pulsed DCs for i.v. immunization on day 1, 2 and 3, both wild type and TM-LacZ mice developed the most severe inflammatory infiltrates of the lungs. The LII in combination-injected recipients were scored as 2 (Figure 19B), and in simple DC and splenocytes injected recipients were scored as 1, respectively. This infiltrates were predominantly localized around blood vessels. Giant cells were observed in

some lesion areas, but no pathological findings were observed in other organs of these mice (Figure 19A, 19B). The giant cells suggested a foreign body reaction to be involved in the phenomenon. However, when bone marrow cells were prepared essentially with the same reagents as splenocyte suspension and used for injection, no infiltrate was observed (Figure 18A). Taken together, we found that DC or splenocytes i.v. injection, but not BM, are sufficient to induce LII. Splenocytes combined with DC exerted an additive effect on the inflammatory infiltrates.

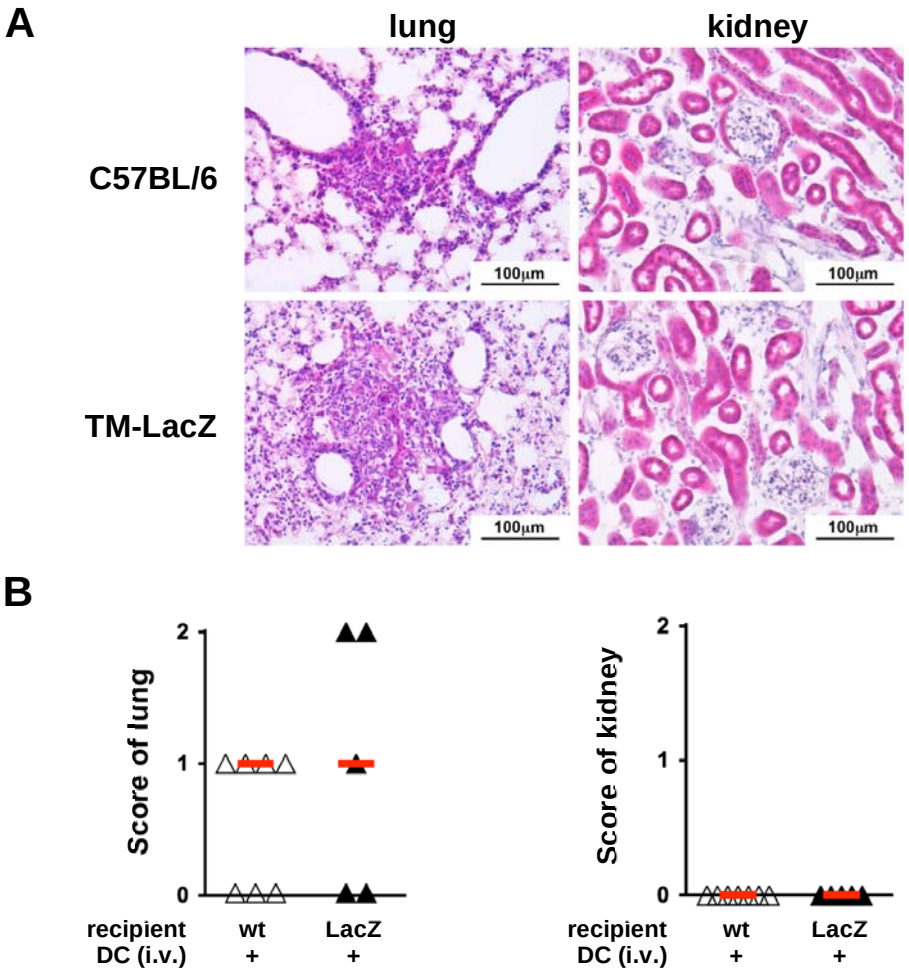


Figure 17. Intravenous injection of DCs induces lung inflammatory infiltrates in mice. 0.5×10^6 β-gal$_{(500\text{-}507)}$-pulsed DCs were adoptively transferred i.v. into wt and TM-LacZ mice on day 0, 1and 2, and organs were analyzed on day 14 (see Figures 10A). (A) H&E stained section of lung and kidney from wt and TM-LacZ mice. (B) Inflammatory infiltrates severity scores in lung and kidney from wt (open symbols) and TM-LacZ (close symbols) mice after immunization.

A

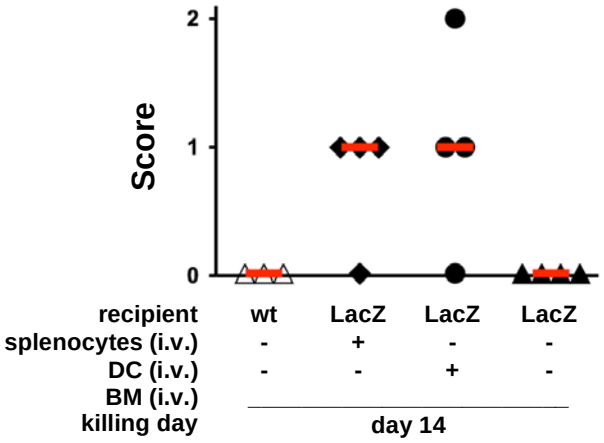
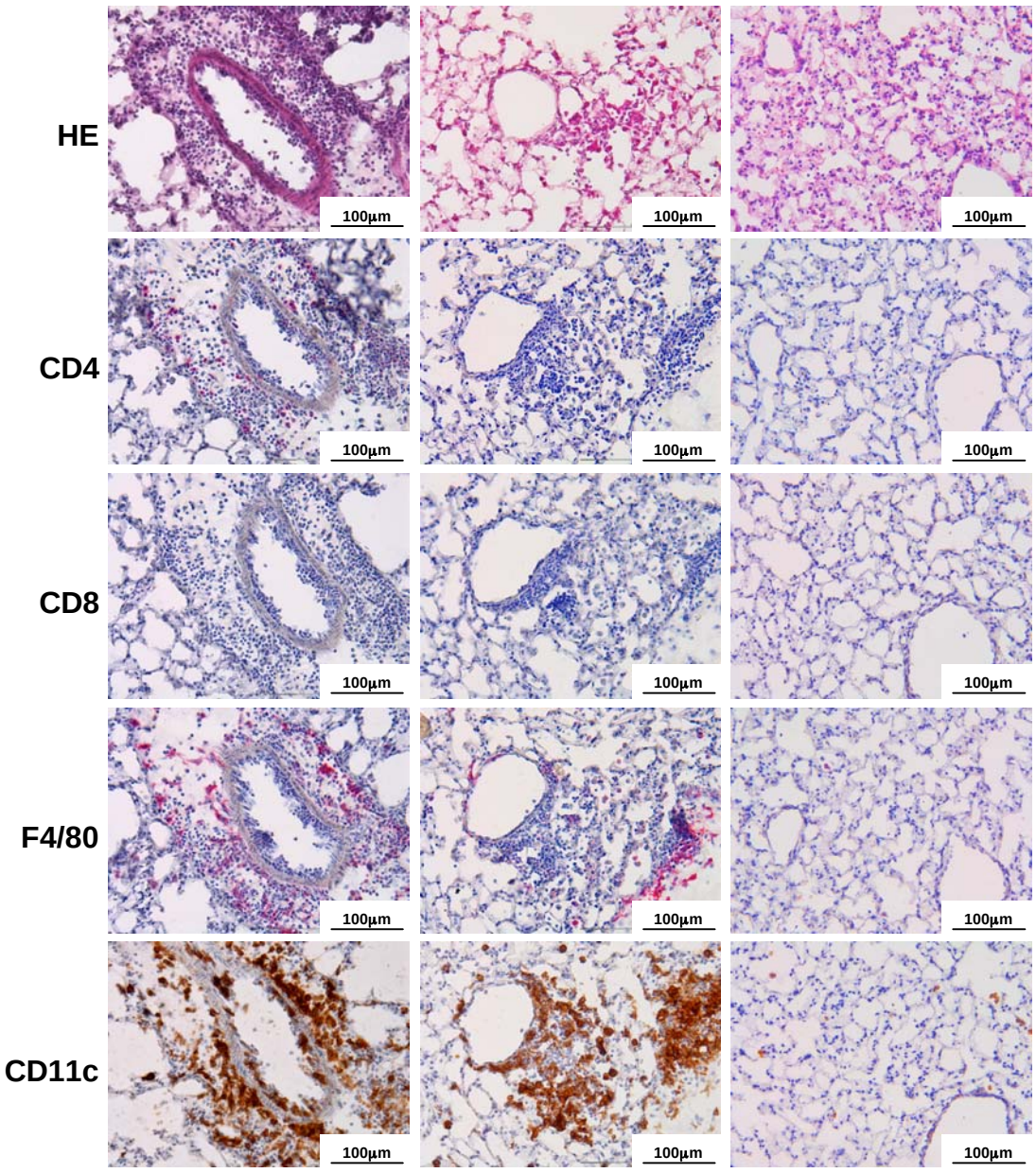


Figure 18. Intravenous injection of splenocytes and DC, but not BM cells induces lung inflammatory infiltrates in mice.

(A) PBS, 25 × 10^6 splenocytes, 10 × 10^6 bone marrow cells or 0.5 × 10^6 cultured DC were adoptively transferred i.v. into the mice, and lungs were analyzed on day 14. Red lines represent median values. (B) **see next page.** Immunohistochemical analysis of lung inflammatory infiltrates for CD4 (helper T cells), CD8 (cytotoxic T cells), F4/80 (macrophages) and CD11c (dendritic cells) positive cells after i.v. injection of splenocytes, bone marrow and DC.

B



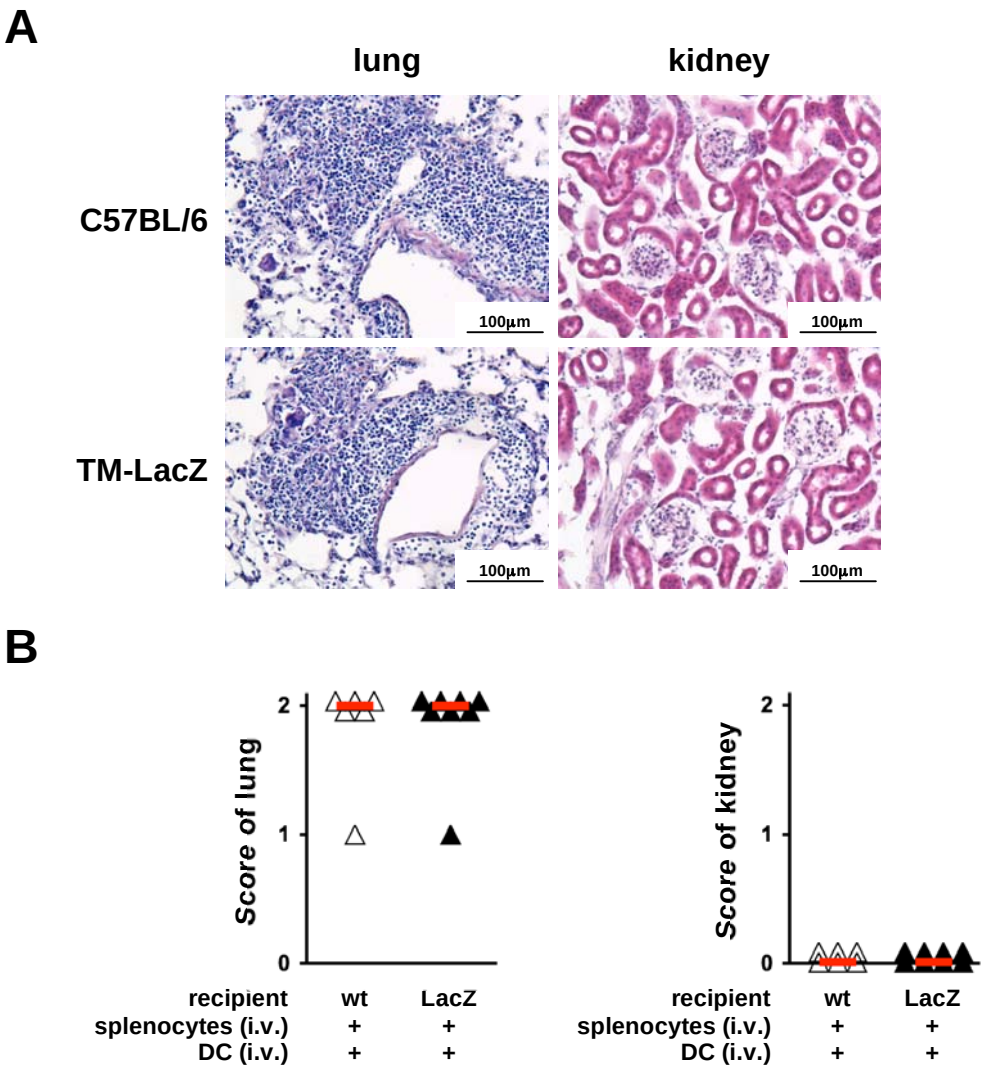
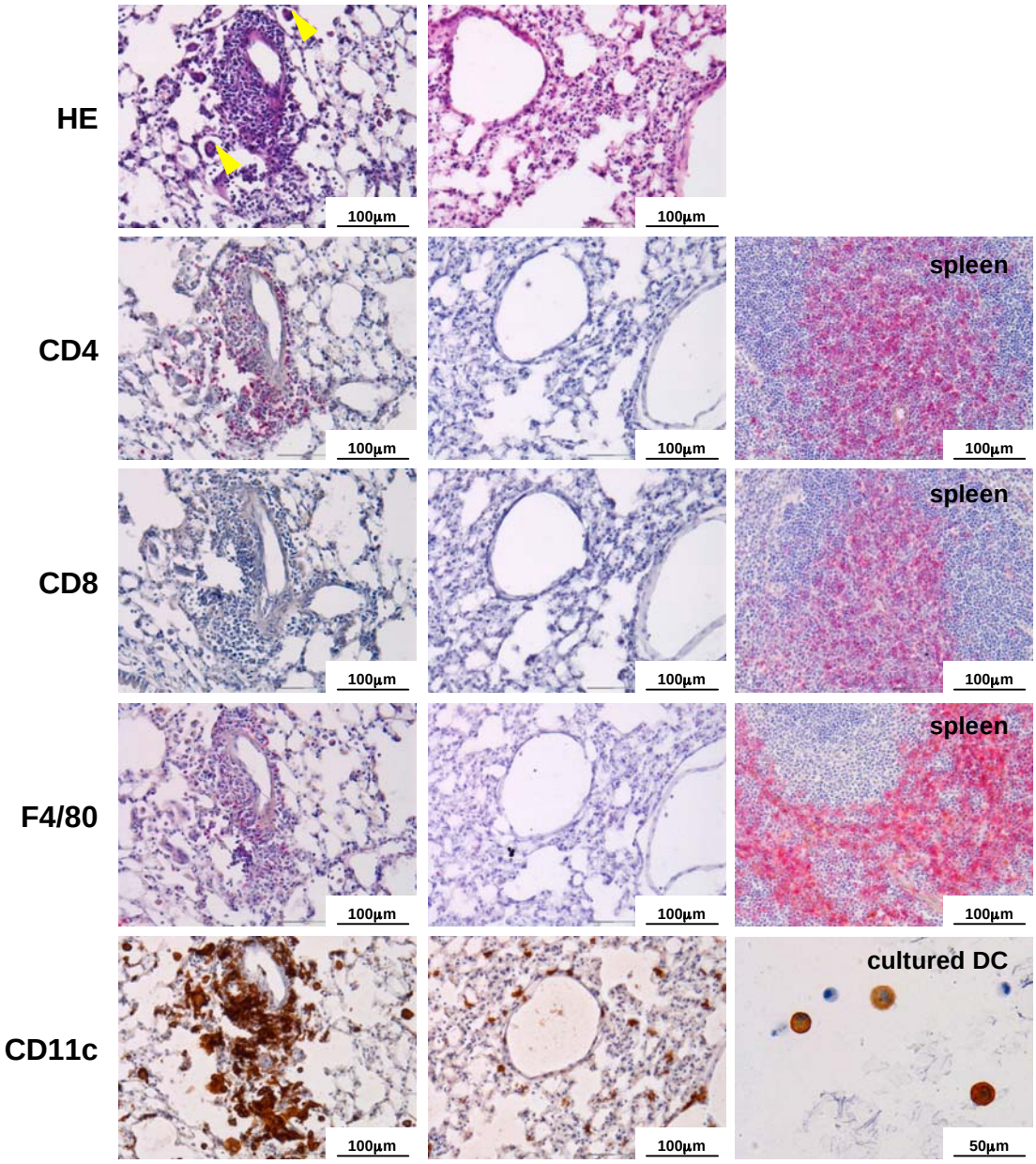


Figure 19. Intravenous injection of splenocytes and DCs induce the most sever lung inflammatory infiltrates in mice.

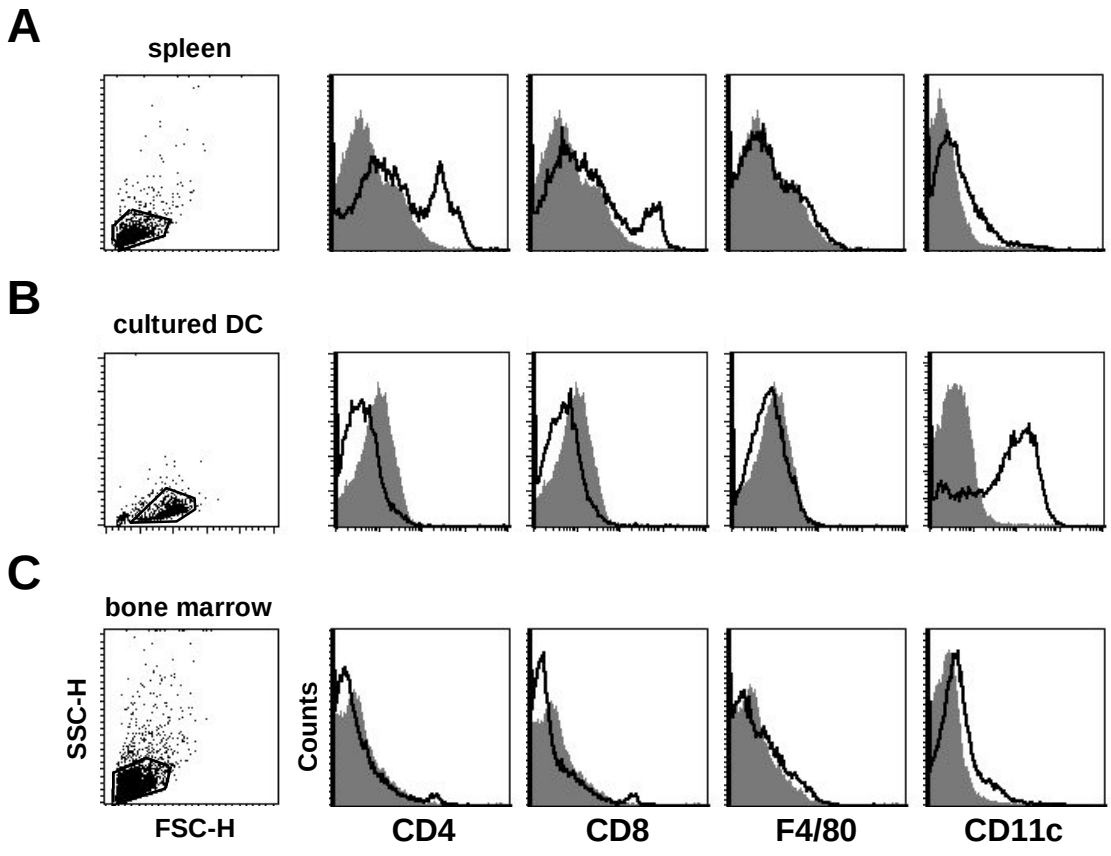
25 × 10^6 β-gal$_{(500-507)}$-primed splenocytes on day 0 and 0.5 × 10^6 β-gal$_{(500-507)}$-pulsed DCs were adoptively transferred i.v. into the mice on day 1, 2 and 3, and organs were analyzes on day 14 (see Figure 13A). (A) H&E stained section of lung and kidney from wt and TM-LacZ mice. (B) Inflammatory infiltrates severity scores in lung and kidney of wt (open symbols) and TM-LacZ (close symbols) mice after cells adoptive transfer. Red lines represent median values. (C) **see next page.** Immunohistochemistry analysis of lung inflammatory infiltrates for CD4, CD8, F4/80 and CD11c (right panel). PBS-treated lung (middle panel) was used as control. Spleen (left panel for CD4, CD8 and F4/80) and cultured DC (left panel for CD11c) were stained as control. Yellow arrowheads indicate giant cells.

C


HE
CD4
CD8
F4/80
CD11c
100μm
spleen
cultured DC
50μm
Splenocytes (i.v.)
+ DC (i.v.)
PBS (i.v.)
staining control

3.2.2. CD4, CD8 and CD11c$^+$ cells are involved in the pathogenesis of lung inflammatory infiltrates

The three cell suspensions (splenocytes, DC and BM) were further characterized by FACS (Table 2). Splenocytes contain about 25.3 ± 1.2 % CD4$^+$, 11.5 ± 1.2 % CD8$^+$, about 2.4 ± 0.7 % macrophages (F4/80$^+$) and 5.3 ± 1.2 % CD11c$^+$ cells under the gate of leucocytes (Figure 20A); cultured dendritic cells contain 73.2 ± 3.4% CD11c positive, nearly no CD4, CD8, nor macrophages (Figure 20B); BM contains only about 8.9 ± 1.2 % CD11c$^+$, 4.8 ± 3 % F4/80$^+$ and 1.3 ± 0.1% CD4$^+$ and 1.1 ± 0.2 % CD8$^+$ (Figure 20C). This suggests that CD11c$^+$ are sufficient for the induction of LII and macrophage may not be involved in the pathogenesis.



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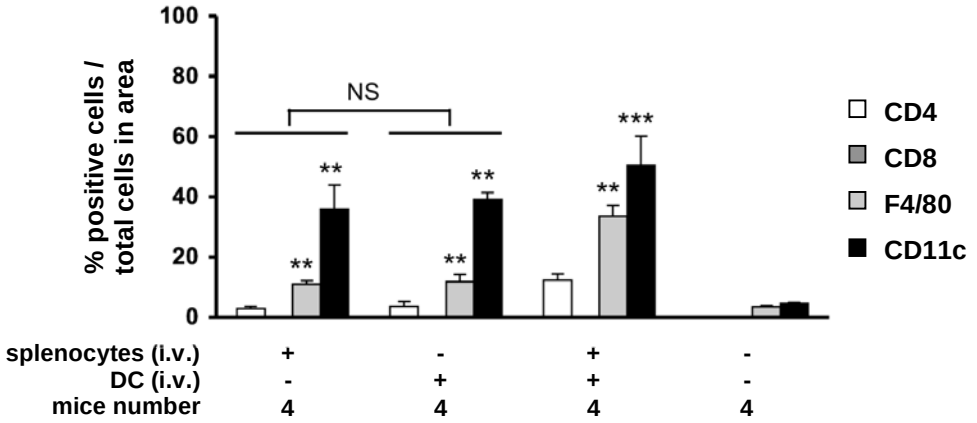


Figure 20. Comparison of leukocyte component in splenocytes, DC and BM for adoptive transfer.

C57BL/6-Ly5.1 (A) splenocytes, (B) DC or (C) BM were adoptively transferred intravenously to the recipients. The donor cells were analyzed by FACS with same antibodies as used for IHC against CD4, CD8, F4/80, CD11c. Data shown mean ± SEM.

We wished to test the role of CD4 and CD8 T cells in the pathogenesis of LII. CD4 and CD8 T cells were enriched from splenocytes using magnetic beads isolation. The purity of $CD4^+$ and $CD8^+$ T cell population were 82.8 ± 2.3 % and 86.6 ± 1.9 %, respectively. There were still 1.1 ± 0.3 % and 1.8 ± 0.9 % of macrophages, 0.7 ± 0.5 % and 1.1 ± 0.9 % of $CD11c^+$ cells in purified CD4 and CD8 cell population, respectively (Table 2).

Table2. Comparison of leukocyte component in splenocytes, DC, BM, purified CD4 and purified CD8 using for adoptive transfer

	CD4	CD8	F4/80	CD11c
splenocytes	25.3±1.2	11.5±1.2	2.4±0.7	5.3±1.2
DC	0±0	0±1	0.4±0.4	73.2±3.4
BM	1.3±0.1	1.1±0.2	4.8±3.0	8.9±1.2
purified CD4	82.8±2.3	7.0±0.1	1.1±0.3	0.7±0.5
purified CD8	0.2±0.1	86.6±1.9	1.8±0.9	1.1±0.9

However, intravenously injected $CD4^+$ (Figure 21B, 21G) or $CD8^+$ T cells (Figure 21C, 21G) alone did not elicit inflammatory infiltrates in lung, whereas, $CD4^+$ and $CD8^+$ T cells injected as combined population to the mice (Figure 21D, 21G) precipitated strong inflammatory infiltrates. No pathological findings were observed when PBS or culture supernatant was injected into normal mice (Figure 22E, 22F, 21G). This analysis shows that the cooperation between CD4 and CD8 T cell is important to elicit inflammatory infiltrates in lung. It also further excludes that simple foreign body reaction is the major pathogenic event.

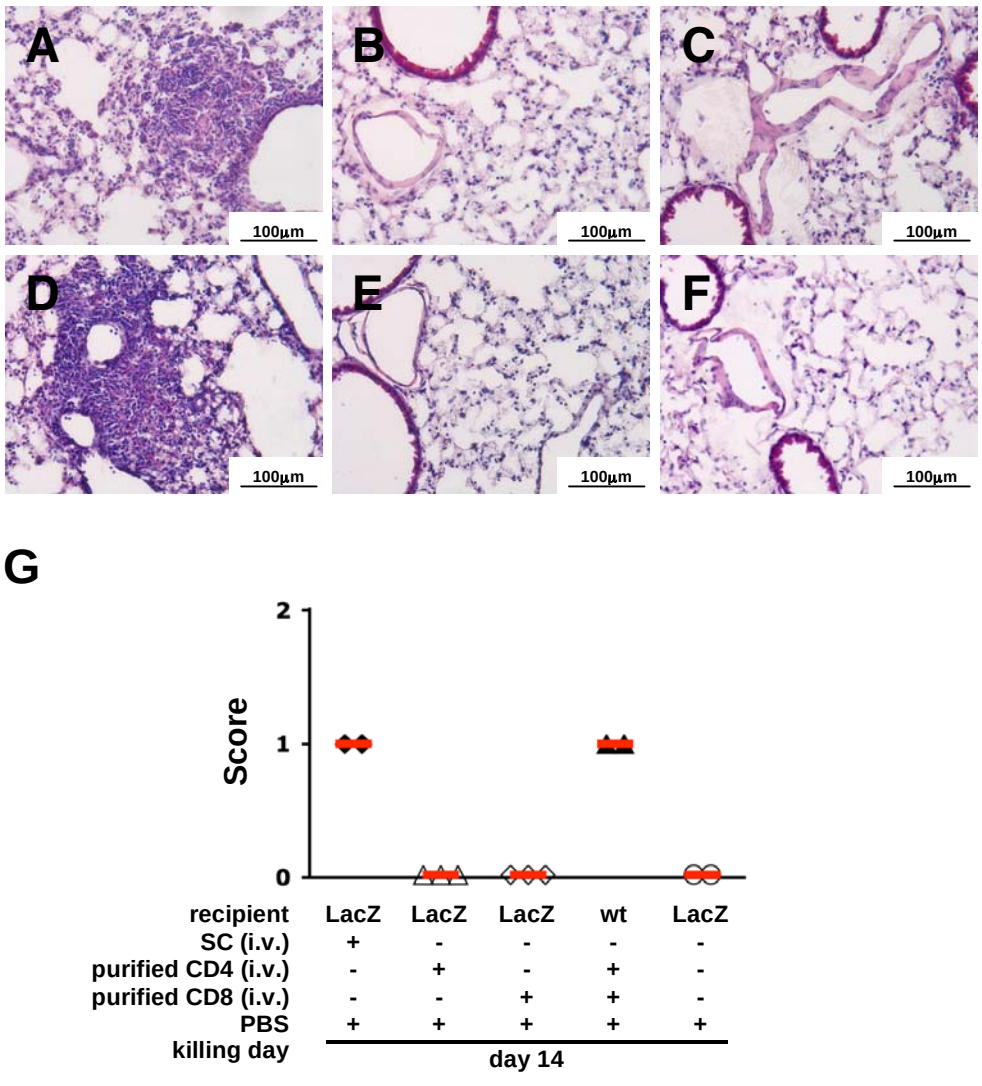


Figure 21. CD4 or CD8 alone dose not induce lung inflammatory infiltrates.

HE staining of lung after received different cell population (A) 25 × 10^6 splenocytes, (B) 4.5 × 10^6 purified CD4, (C) 3 × 10^6 purified CD8, (D) 2.5 × 10^6 purified CD4 and CD8, (E) PBS,

(F) without injection. (G) Inflammatory infiltrates severity scores in lung of all recipients. Red lines represent median values.

3.2.3. Route of administration affects the lung inflammatory infiltrates

Since inflammatory infiltrates were found exclusively in the lung, we next tested the role of the administration route. When we looked for LII two weeks after i.v., s.c., or i.p. injection of DC, only i.v. injected DC induced lung inflammatory infiltrates in mice, whereas no inflammatory infiltrates occurred in s.c. and i.p. injected recipients (Figure 22). Both TM-LacZ mice, that express high amount of β-galactosidase in the lung (Figure 2C, 2D), and wt mice developed similar inflammatory foci (Figure 22, 17B). These results demonstrated that intravenous injection and not antigen expression was essential for the development of lung inflammatory infiltrates in mice. Particularly, antigen presentation by endothelial cells was not involved in pathogenesis.

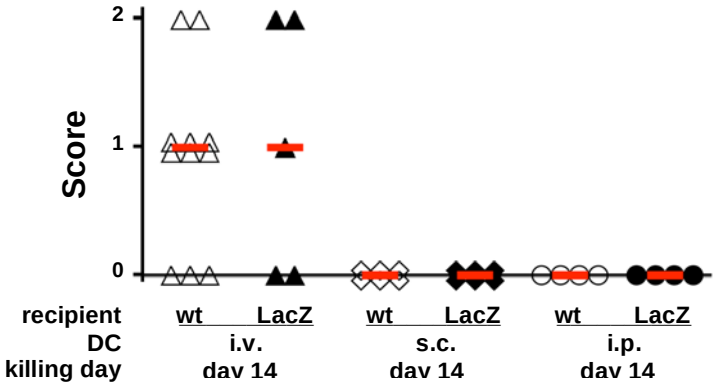


Figure 22. Route of administration induce lung inflammatory infiltrates in mice.
0.5×10^6 β-gal$_{(500\text{-}507)}$-pulsed DC were adoptively transferred i.v., s.c. or i.p. into the mice and lungs were analyzed on day 14. Inflammatory infiltrates severity scores of wt (open symbols) and TM-LacZ (close symbols) mice after adoptive transfer. Red lines represent median values.

3.2.4. Inflammatory cells showed high proliferation rate in lesion area

Lung inflammatory infiltrates built up in lung over time (Figure 23). There were very few infiltrates observed two days after splenocytes injection i.v.. The peak of infiltrates occurred two weeks after injection, and persisted for at least five weeks. This observation suggests that LII developed by proliferation rather than by simple trapping. This hypothesis was confirmed by *in vivo* BrdU labeling! One week before sacrifice, the recipients were fed with BrdU in the drinking water. The newly synthesized DNA was detected in the inflammatory infiltrates by immunohistochemistry (65 ± 2.5% and 64 ± 1.4% proliferated cells in splenocytes recipients and DC recipients, respectively) (Figure 24A, 24C). In normal lung tissue, as expected, had a low proliferation index and only few lung resident cells (4 ± 0.3% and 3.5 ± 0.5% proliferated cells in splenocytes recipients and DC recipients, respectively) (Figure 24C) were positive for BrdU. This experiment shows that LII developing over time contained a large number of proliferating cells.

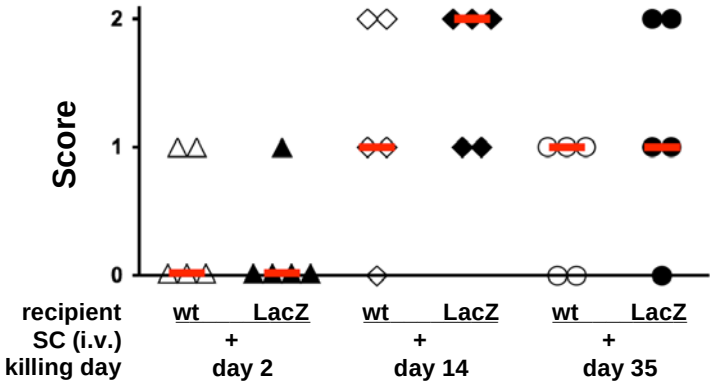


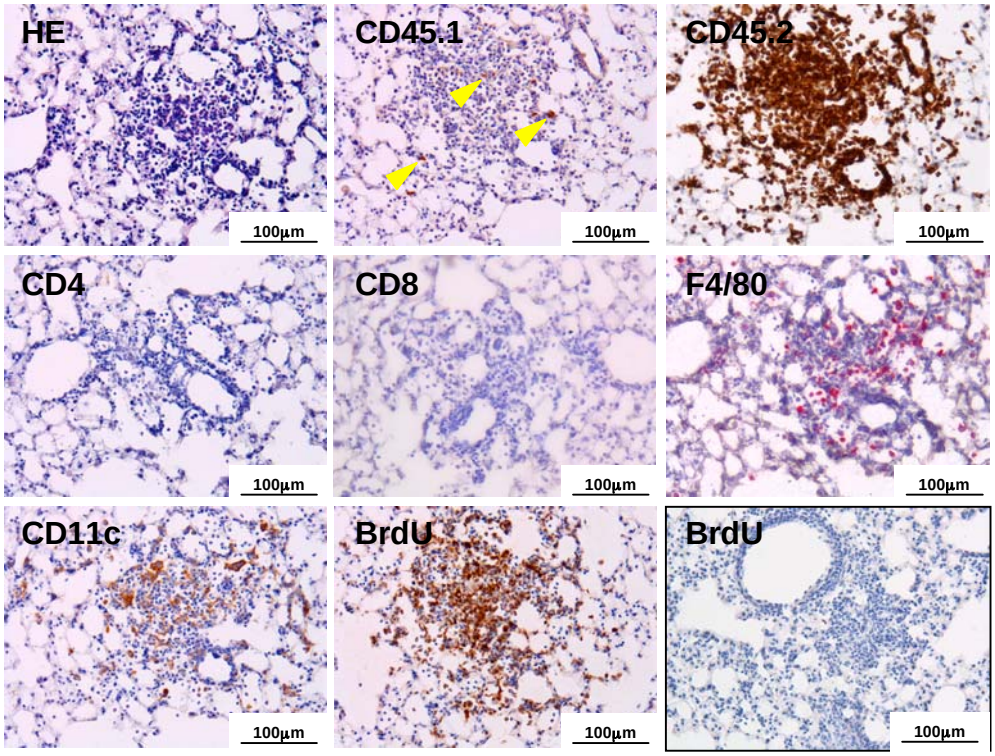
Figure 23. Intravenous injection of splenocytes induced persistent lung inflammatory infiltrates.

25×10^6 β-gal$_{(500\text{-}507)}$-primed splenocytes were adoptively transferred i.v. into the mice, and lungs were analyzed on day 2, 14 and 35. Inflammatory infiltrates severity scores of wt (open symbols) and TM-LacZ (close symbols) mice after adoptive transfer. Red lines represent median values.

3.2.5. Inflammatory cells are the recipient origin

We next wished to determine whether LII were of donor or of recipient origin. To check the origin of inflammatory cells in lung inflammatory infiltrates area, C57BL/6-Ly5.1 splenocytes or DC were injected intravenously into C57B/L6-Ly5.2 recipients. After two weeks, only 1% of CD45.1^{+} donor cells (Figure 24A, 24D) were detectable in the lung of the recipient after the injection of splenocytes. These cells were scattered throughout the organ. About 3% of CD45.1 positive cells were found in the spleen and 0.6% in peripheral blood (Figure 24E). After the injection of DC, 0.7% of CD45.1 positive cells were found in lung (Figure 24D), only very few in the spleen and in peripheral blood (Figure 24E). Again, the majority of cells in the LII were CD45.2 positive, i.e. of recipient origin (Figure 24A). This observation suggests that donor splenocytes or / and DC injection stimulated focal lung inflammatory cell proliferation of recipient cells.

A



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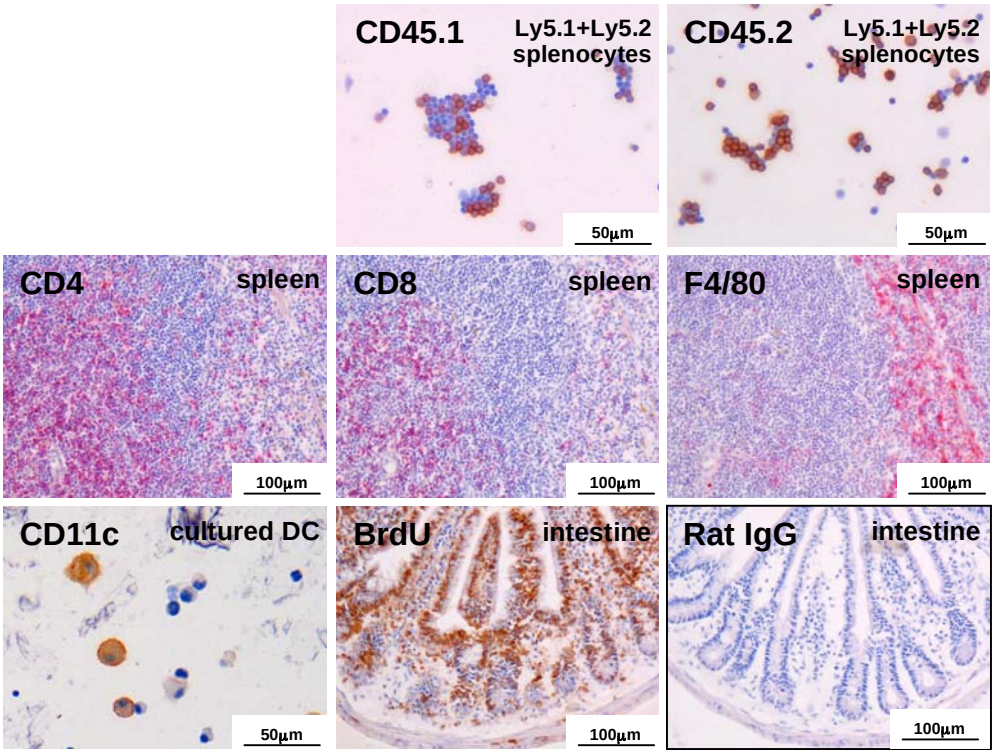
CD45.1
Ly5.1+Ly5.2 splenocytes
50μm
CD45.2
Ly5.1+Ly5.2 splenocytes
50μm
CD4
spleen
100μm
CD8
spleen
100μm
F4/80
spleen
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CD11c
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BrdU
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Rat IgG
intestine
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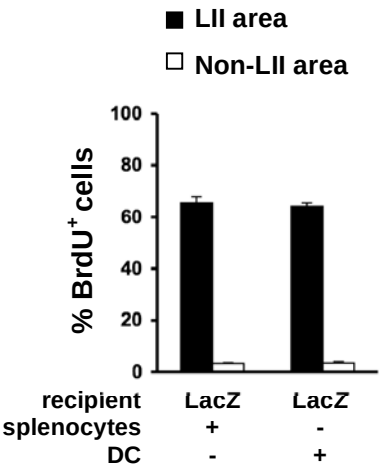
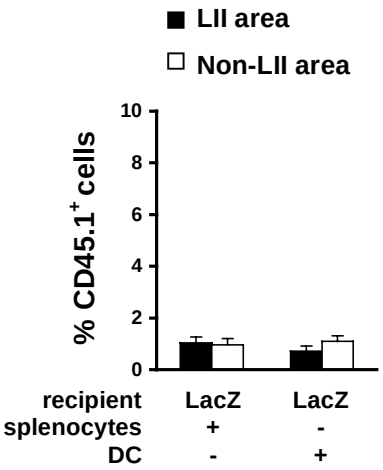
C


LII area
Non-LII area
% BrdU+ cells
100
80
60
40
20
0
recipient splenocytes DC
LacZ + -
LacZ - +

D


LII area
Non-LII area
% CD45.1+ cells
10
8
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4
2
0
recipient splenocytes DC
LacZ + -
LacZ - +

E

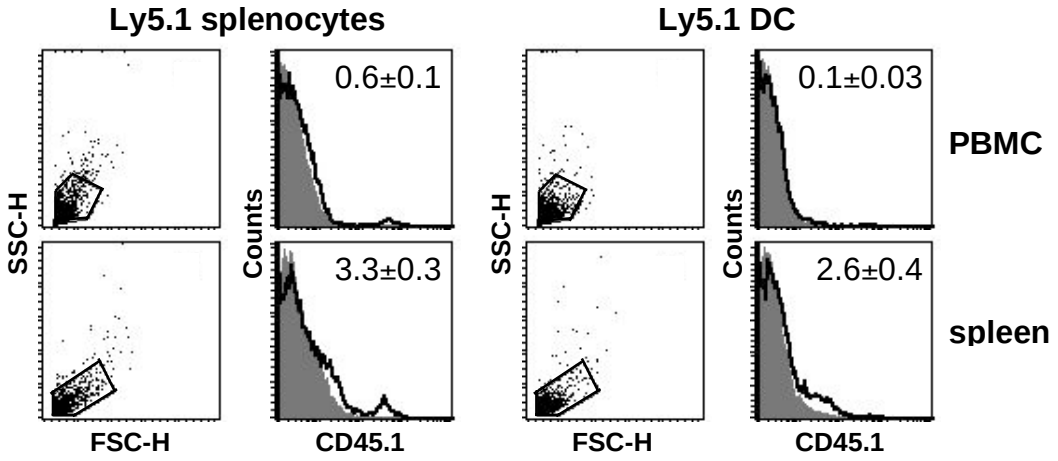

Figure 24. Origin and proliferation state of lung inflammatory infiltrates.

C57BL/6-Ly5.1 splenocytes or DC were adoptively transferred intravenously to the C57BL/6-Ly5.2 recipients, and the recipient mice were fed with BrdU drinking water for one week before sacrificed. Two weeks after adoptive transfer, (A) Immunohistochemistry analysis of lung inflammatory infiltrates for CD45.1 (donor leucocytes), CD45.2 (recipient leucocytes), CD4, CD8, F4/80, CD11c and BrdU (new synthesized DNA). Non-BrdU fed mouse lung was stained as control showed in black frame. Yellow arrowheads indicate CD45.1 cells. (B) Mixed Ly5.1 and Ly5.2 splenocytes (upper row for CD45.1 and CD45.2), spleen (middle row for CD4, CD8 and F4/80), cultured DC (lower left for CD11c) and BrdU fed mouse intestine (lower middle) were stained as control. In black frame shows Rat IgG stained BrdU fed mouse intestine. (C) The percentage of BrdU positive stained the cells in LII and in health area. (■) indicates in LII area, (□) indicates in health area. (D) The percentage of CD45.1 positive stained the cells in LII and in health area. (E) PBMC and spleen cells of recipient were detected by FACS stained with FITC labelled anti-CD45.1. (F) The percentage of CD4, CD8, F4/80 and CD11c positive cells in LII area after various leucocytes i.v. injection. Percentage of positive cells / total cells in area was calculated as: positive stained cells in lesion area / total cells in lesion area × 100%.

3.2.6. $CD11c^+$ cells were the dominant population within LII

To further analyze the cellular composition of cells in the area of LII, 10μm thick lung cryosections were stained with antibodies specific for helper T cells (CD4), cytotoxic T cells (CD8), macrophages (F4/80) and dendritic cells (CD11c). CD11c positive cells, macrophages, a few $CD4^+$ cells, but no $CD8^+$ cells were found in the infiltrates after splenocytes or / and DC i.v. adoptive transfer (Figure 18B, 19C, 24F). Interestingly, the cellular composition in LII was similar after DC and splenocytes adoptive transfer, respectively. Few resident F4/80 and CD11c positive cells were detectable by immunohistochemical staining, but they are scattered in a different pattern of distribution in the normal looking lung (Figure 19B, 24F).

3.2.7. Endothelial cells were preserved in inflammatory infiltrates area

TM-LacZ mice express β-galactosidase on endothelial cells and LII formed exquisitely around pulmonary blood vessels. To analyze the integrity of blood vessel in the centre of LII, the X-gal substrate staining was used to identify the EC on TM-LacZ lung section. In 6/62 normal TM-LacZ mice, the X-gal substrate staining for examining β-galactosidase activity was complete negative, 7/67 mice was negative in 10-20 % of lung area, due to the technical limitation, e.g. 4% PFA perfusion fixation did not completely reach every capillary of entire lung resulting the loss of β-galactosidase activity. We observed this in 4 / 31 experimental TM-LacZ mice, as well, the X-gal substrate staining for enzymatic β-galactosidase activity was complete negative, even in the unaffected lung area. After intravenous injection of various leucocytes into the mice, most inflammatory infiltrates were found around endothelial cells of vein (Figure 25B) and artery (Figure 25C) in TM-LacZ mouse, but the EC themselves were preserved (Figure 25D). Taken together, these results showed that the lung inflammatory cells are of recipient

origin, mostly CD11c^{+} and were formed by local proliferation. No substantial tissue injury did occur and even vascular EC were mostly preserved.

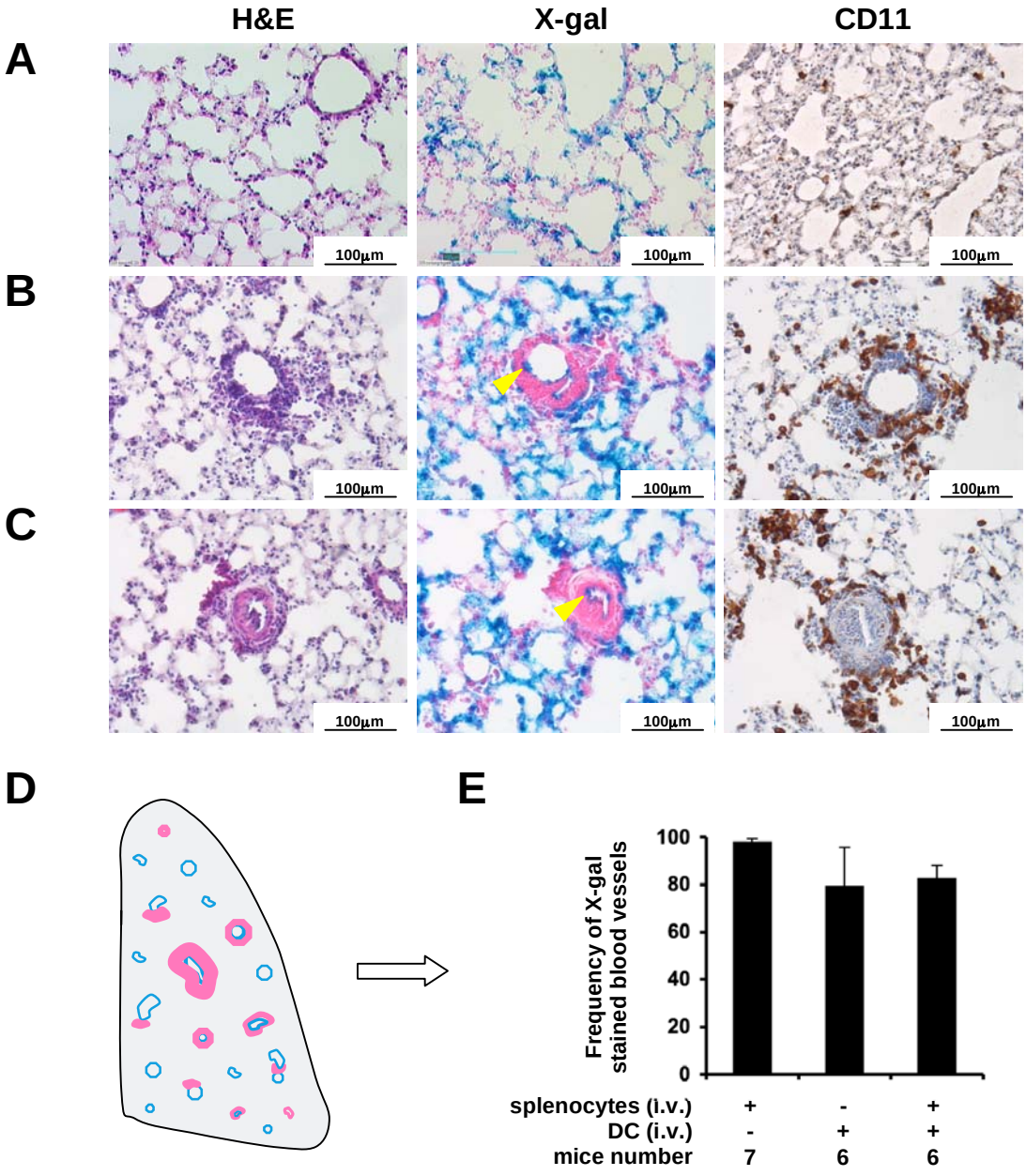

Figure 25. Endothelial cells were preserved in inflammatory infiltrates.

25 × 10^{6} β-gal$_{(500\text{-}507)}$-primed splenocytes or 0.5 × 10^{6} β-gal$_{(500\text{-}507)}$-pulsed DC or combined cells were adoptively transferred i.v. into the mice, and lungs were analyzed on day 14. 10μm thick lung cryosections after received 25 × 10^{6} β-gal$_{(500\text{-}507)}$-primed splenocytes were stained with H&E (A), X-gal (B) and CD11c (C). Upper row is normal lung, middle and lower row shows inflammatory infiltrates around vein and artery. Yellow arrowheads indicate endothelial cells stained with X-gal. (D) Schematic shows the X-gal staining of lung. Blue circles indicate

X-gal stained blood vessels, pink indicate inflammatory infiltrates. (E) Summary of endothelium integrity of TM-LacZ mice using X-gal staining. Frequency of X-gal stained blood vessels = number of positive stained blood vessels in inflammatory infiltrates of whole lung area / all vessels in inflammatory infiltrates of whole lung area × 100% = 10 /12 * 100 = 83.3%.

4. Discussion

In this thesis project, I made two important observations: first, despite of successful induction of an antigen-specific CTL-response, CTL-mediated endothelial injury in a mouse model of endothelial cell-selective antigen presentation could not be observed in vivo. Second, intravenous injection of splenocytes, dendritic cells, or both, induced reproducibly focal, non-specific perivascular lung inflammatory infiltrates containing abundant $CD11c^+$ cells.
In the following paragraphs these two findings are discussed separately.

Part 1. Endothelial cells as targets of antigen-specific CTL

The major goal of this thesis project was to investigate antigen-specific and antigen-dependent endothelial injury mediated by antigen-specific CTL *in vivo*. In order to reach this goal, a mouse model was chosen in which E.coli β-galactosidase (LacZ) was expressed under the control of the thrombomodulin promoter, i.e. selectively in vascular endothelial cells. In these TM-LacZ mice, LacZ expression in the vascular EC was patchy, not uniform. Pulmonary blood vessels were uniformly and strongly LacZ positive whereas liver EC were virtually LacZ negative. This inherent variability in antigen-expression representing an internal control should guarantee the identification of truly antigen-specific endothelial injury. Adoptive transfer of β-$gal_{(500-507)}$-primed wild type splenocytes followed by repetitive subcutaneous immunization of TM-LacZ mice with β-$gal_{(500-507)}$-pulsed DC was able to break tolerance against the autoantigen LacZ in these TM-LacZ mice. In the spleen, on average 1 (0.54-1.2)% of total $CD8^+$ T cells were shown to be β-$gal_{(500-507)}$ specific effector cells. This was similar to the result of this immunization procedure in wt mice. However, despite of the successful immunization, the mice did not develop disease. Body weight was stable over time and at the tissue level, microscopic analysis did not reveal antigen specific endothelial injury or tissue infiltration by $CD8^+$ T cells. This is surprising since in a similar

model, in which the same antigen (LacZ) is expressed under the control of the smooth muscle cell actin promoter [69], DC immunization using the same peptide was also able to break self-tolerance but lead to $CD8^+$ T cell-mediated vasculitis. Furthermore, in various models of antigen-specific, peptide-dependent, MHC class I restricted immune responses, the number of peptide-specific CTL in the spleen was accurately reflecting an efficient generation of systemically active effector T cells [71,80-82]. Our negative result could have several explanations. The peptide-specific CTL that were detected after β-gal immunization could be self-reactive low affinity T cells [83] and therefore, the amount of β-gal peptide presented by EC might be too low for efficient target cell injury *in vivo*. However, the cytotoxic potency of the cell lines that emerged from these splenocytes after *ex vivo* expansion was remarkable since at an E:T ratio of <10:1 and with peptide concentrations of 10^{-5} M for target cell pulsing still more than 60% specific killing was observed (Figure 5B). Furthermore, upregulation of antigen-expression in target tissues by poly(I:C) treatment did not precipitate endothelial injury (Figure 15B). Finally, endothelial cells could present a distinct peptide repertoire and lack the immunodominant $\beta\text{-gal}_{(500\text{-}507)}$ peptide among their MHC class I ligands. In fact we have previously shown [84] for human EC that $SMCY_{(311\text{-}319)}$ derived peptide FIDSYICQV was presented at a negligible amount on the cell surface. Therefore, SMCY-specific CTL with high potency were unable to recognize and eliminate male target EC but were successfully eliminating male B-lymphoblastoid cells. A recent publication described ignorance of endothelial cells by peptide-specific CTL as a mechanism of antigen-specific tolerance in another mouse model of endothelial β-galactosidase expression [57]. The Tie2-LacZ mice express the E.coli protein uniformly in all endothelial cells. In addition to the antigen-specific ignorance of endothelial cells, these mice also developed antigen-specific tolerance. This peripheral tolerance was shown to depend on crosspresentation of LacZ by $CD11c^+$ bone marrow derived cells, putatively dendritic cells. We also observed antigen-specific peripheral tolerance against LacZ in TM-LacZ mice, but only for intravenous immunization. As mentioned in the beginning, subcutaneous immunization could easily break tolerance in the TM-LacZ mice and resulted in large

numbers of antigen-specific, potent, $CD8^+$ splenocytes. If endothelial cells could specifically escape CTL-mediated injury by presenting insufficient amounts of antigen, mice which express β-galactosidase ubiquitously should develop GVHD-like disease after the same immunization procedure. In fact, Rosa26-LacZ mice, which express E.coli β-galactosidase in all nucleated cells, developed antigen-specific disease with relative weight loss immediately after immunization with β-$gal_{(500-507)}$-pulsed DC (Figure 16E). Furthermore, two of the immunized Rosa mice developed GVHD-like disease 10 weeks after immunization. Both Rosa26-LacZ and wt mice had signs of chronic tissue inflammation, but the injury score was more severe in the Rosa26-LacZ mice. This single, long term experiment demonstrates that antigen-specific, chronic GVHD like disease can be induced in mice in which one protein, LacZ, serves as a source for minor histocompatibility antigens when this protein is expressed ubiquitously in all tissues of the body and when its expression is not restricted to the endothelium.

We conclude that in this model of focal, endothelial cell restricted antigen expression, vascular injury cannot be induced despite of successful induction of an antigen-specific CTL response. This finding may have implications for non-cytopathic endothelial infections (e.g. by cytomegalovirus human immunodeficiency virus), immune-defense against endothelial neoplasias or to understand the phenotype of alloimmune diseases, particularly graft versus host disease after MHC matched stem cell transplantation.

Part 2. Lung inflammatory infiltrates after intravenous leukocyte injection

As an unexpected, novel finding we observed reproducibly perivascular inflammatory infiltrates after intravenous injection of various leukocyte populations over a broad range of cell counts. 25 x 10^6 splenocytes, 0.5 x 10^6 bone marrow derived dendritic cells, or both together induced focal, perivascular proliferation and expansion of mostly $CD11c^+$ cells. Interestingly, neither $CD4^+$ cells nor $CD8^+$ cells alone, but the combination of the two, were able to precipitate LII. Although some of the most advanced LII also contained giant cells resembling foreign body reaction, we had no evidence that this was the major pathogenic mechanism. 1) foreign body material was not observed in histological preparations, 2) neither $CD4^+$ nor $CD8^+$ cell suspensions induced the LII, but the combination of the two, 3) bone marrow cells were not sufficient to induce LII and 4) none of the cell culture media or washing buffers alone induced LII.

The obvious etiology of this phenomenon is intravenous injection of leukocyte suspensions of variable origin and composition. This suggests that it might represent a model for a variety of well known, well described lung diseases associated with stem cell transplantation (e.g. interstitial pneumonia syndrome [85]), blood transfusions (e.g. transfusion associated lung injury [86]) or adoptive lymphocyte transfer for fighting leukemia relapses or transplant associated viral infections [87].

On the other hand, the predominance of proliferating $CD11c^+$ cells regardless of the original cell suspension that was injected is puzzling. This suggests that the procedure elicited lung vascular injury and precipitated a common final pathway resulting in a uniform repair process eventually leading to granuloma formation. The granuloma were of variable severity but vascular endothelium within the LII was mostly preserved. Endothelial integrity and absence of vessel wall necrosis or erythrocyte extravasation argues against fully

developed vasculitis. This is also in accordance with the absence of any obvious sign of disease in these mice. Perivascular $CD11c^+$ dendritic cells are also an important and predominant leukocyte population in other vascular disorders like giant cell arteritis [88].

A strikingly similar finding has recently been reported in mice after intravenous injection of FasL-transfected dendritic cells [89]. We analyzed FasL expression by our bone marrow derived DC and found it to be negative. The common ground for the observation by Buoncore and us may not be the injected DC but the type of lung endothelial injury induced by the injected cells.

We may have established a novel animal model for an early stage of pauci-immune or ANCA-associated pulmonary vasculitis. It is highly reproducible and due to the persistent nature of the granuloma formation it is suitable for further interventions aiming at precipitating bona fide pulmonary vasculitis or aiming at resolution of the non-caseating granuloma.

5. References

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6. List of Abbreviations

APC	Antigen presenting cell
BM	Bone marrow
BMT	Bone marrow transplantation
BrdU	5-Bromo-2'-deoxyuridine
CTL	Cytotoxic T lymphocytes
DAB	3,3'-Diaminobenzidine
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
EC	Endothelial cell
ELISA	Enzyme-Linked ImmunoSorbent Assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDG	Fluorescein di-D-galactopyranoside
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gPBS	Glucose containing phosphate-buffered saline
GVHD	Graft versus host disease
HBSS	Hank's Buffered Salt Solution
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
IFNα	Interferon alpha
IFNγ	Interferon gamma
IHC	Immunohistochemistry
IL-2	Interleukin 2
IL-4	Interleukin 4
IPS	Idiopathic pneumonia syndrome
LII	Lung inflammatory infiltrates
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II

MPA	Microscopic polyangiitis
OVA	Chicken ovalbumin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PI	Propidium iodide
PMA	Phorphol myristate acetate
poly(I:C)	Polyinosinic–polycytidylic acid potassium salt
RT	room temperature
TCR	T cell receptor
TM	Thrombomodulin
TNFα	Tumor necrosis factor alpha
X-gal	5-Bromo-4-Chloro-3-Indoyl-D-Galactopyranoside
WG	Wegener's granulomatosis
wt	wild type

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