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Development of DC-specific lentiviral vectors: novel DC-targeting strategies for immunomodulation

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1 SUMMARY

Dendritic cells (DCs) play an important role in orchestrating both innate and adaptive immune responses. They are specialized in presenting antigens to T cells, which can be derived from self or foreign origin. DCs express a large variety of pattern recognition receptors (PRRs), which allow sensing and capturing of pathogenic invaders. During pathogen encounter DCs undergo a maturation process, which makes them potent stimulators of various T cell responses. In contrast, presentation of self-antigen induces tolerance in naive T cells. In this study, we used a lentiviral gene transfer system with DC-specific promoters to address various questions regarding DC biology *in vivo*. Thereby, bone marrow chimeras were generated using lentivirally transduced hematopoietic stem cells. It was demonstrated that a 1.7kb fragment of the mouse DC-STAMP promoter drives DC-specific transgene expression, which induced both central and peripheral tolerance in CD4 and CD8 T cells. In the next step, we made use of this model to investigate the role of mature CD8 T cells, which re-enter the thymus. These mature re-entering CD8 T cells interfered with negative selection of developing T cells with the same specificity by elimination of antigen-presenting cells (APCs) in the thymus. This APC elimination was antigen-specific and affected both DCs and medullary thymic epithelial cells (mTECs). In addition, we investigated mechanisms of transcriptional regulation in DCs. Computational comparison of mouse CD11c and DC-STAMP promoter regions across species identified common regulatory elements in a defined orientation, the so-called CD11c/DC-STAMP promoter model. Mapping of CD11c promoter activity by *in vivo* promoter analysis confirmed the importance of the region, where the CD11c/DC-STAMP promoter model was predicted to be located. Screening a mouse promoter database for this model resulted in the identification of two novel candidate genes, so far not connected to DC biology. It was shown that these two genes are expressed in DCs and are partly coregulated with CD11c and DC-STAMP. In summary, transcriptional targeting of DCs by lentiviral vectors was successfully used to address questions regarding immunomodulatory capacity and transcriptional regulation of DCs.

2 ZUSAMMENFASSUNG

Dendritische Zellen (DZ) spielen eine wichtige Rolle in der Steuerung der angeborenen und adaptiven Immunantwort. Sie sind in spezieller Weise dazu geeignet Antigene für T-Zellen zu präsentieren, wobei dies entweder fremde oder Selbst-Antigene sein können. DZ exprimieren eine Vielzahl von Rezeptoren, die bestimmte Muster auf Pathogenen erkennen und dazu dienen diese in die Zelle aufzunehmen. Dabei machen die DZ einen Reifungsprozess durch, der dazu führt, dass sie auf potente Weise verschiedenste T-Zell-Antworten auslösen. Wenn die DZ hingegen Selbst-Antigen präsentieren, führt dies zu Toleranzentwicklung in naiven T-Zellen. In dieser Arbeit haben wir ein lentivirales Gentransfer-System mit DZ-spezifischen Promotoren verwendet um verschiedene Fragestellungen hinsichtlich der Biologie von DZ *in vivo* zu behandeln. Dabei wurden Knochenmarks-Chimären mit Hilfe von hämatopoetischen Stammzellen generiert, die zuvor lentiviral infiziert worden waren. Es wurde gezeigt, dass ein 1.7kb großes Fragment des Maus-DC-STAMP-Promotors DZ-spezifische Expression von Transgenen steuert, was sowohl zentrale als auch periphere Toleranz in CD4 und CD8 T-Zellen auslöste. Anschliessend machten wir uns dieses Modell zu Nutze um die Rolle von reifen CD8 T-Zellen zu untersuchen, die zurück in den Thymus wandern. Dort beeinträchtigten diese reifen CD8 T-Zellen durch die Eliminierung Antigen-präsentierender Zellen (APZ) die negative Selektion von sich entwickelnden T-Zellen, die dieselbe Spezifität hatten. Die Eliminierung von APZ war Antigen-spezifisch and betraf sowohl DZ als auch medulläre thymische Epithelzellen. Des Weiteren wurden Mechanismen der transkriptionellen Regulation in DZ untersucht. Dazu wurden CD11c und DC-STAMP Promotoren verschiedener Spezies mit Hilfe von Bioinformatik verglichen. Dies führte zur Identifikation von gemeinsamen regulatorischen Elementen in einer bestimmten Orientierung zueinander, dem sogenannten CD11c/DC-STAMP-Promotor-Modell. Die Aktivität des CD11c Promoters wurde anhand von *in vivo* Promotoranalyse auf diesen Bereich eingegrenzt, in dem das vorhergesagte CD11c/DC-STAMP-Promotor-Modell lag. Somit konnte die Wichtigkeit des auf bioinformatischen Analysen aufgebauten Modells bestätigt werden. Dann wurde eine Datenbank aus Maus-Promotoren nach dem CD11c/DC-STAMP-Promotor-Modell durchsucht. Dabei wurden zwei Gene identifiziert, die zuvor noch in keiner Verbindung zu DZ-Biologie standen. Es wurde gezeigt, dass die beiden

Gene in DZ exprimiert und teilweise mit CD11c und DC-STAMP koreguliert werden. Transkriptionelles Targeting von DZ durch lentivirale Vektoren wurde erfolgreich eingesetzt um Fragen hinsichtlich immunmodulatorischer Fähigkeiten und transkriptioneller Regulation von DZ zu behandeln.

3 INTRODUCTION

3.1 Dendritic cells

Dendritic cells (DCs) play a central role in orchestrating the adaptive immune system. They integrate distinct signals and direct the quality of the T cell response either towards immunity or tolerance. DCs are localized at body or internal surfaces like skin, lung and intestine to monitor for the presence of pathogenic invaders and in T cell areas of lymphoid tissues. DCs comprise a very heterogeneous cell population that has been characterized with many subsets, which differ in location, phenotype and function. In the steady state, DCs migrate from peripheral tissues to the draining lymph nodes and present self-antigen to naive T cells, inducing tolerance. Upon encounter of pathogen, DCs undergo a maturation process, which leads to more pronounced migratory behavior and an increase in stimulatory capacity. Depending on the type of pathogen and the microenvironmental circumstances, distinct T cell responses are induced resulting in protective immunity.

3.1.1 Antigen processing and presentation

DCs are specialized and very efficient in antigen processing and presentation. Inside the cell, protein antigens are processed into peptides and loaded onto MHC-molecules. These peptide-MHC (pMHC)-complexes are transported to the cell surface, where they are presented to naive T cells. There are two forms of antigen processing: the endogenous and the exogenous pathway. In the endogenous pathway, proteins that are produced inside the cell are degraded in the cytosol by the proteasome and transported via TAP into the ER, where the peptides are loaded onto MHC-I molecules. Such proteins include mainly self-proteins, but also tumor- or virus-associated proteins. MHC-I molecules present peptides to CD8 T cells, which differentiate into cytotoxic T lymphocytes (CTL) able to eliminate transformed or infected cells.

The exogenous pathway processes extracellular material, such as antigens derived from bacteria, parasites or toxins. One important characteristic of DCs is to react to changes in their environment. They are equipped with a variety of receptors, so called pattern recognition receptors (PRR) to sense and capture foreign antigen

(Ag). These include Toll-like receptors (TLR), Fc-receptors, mannose receptor, complement receptors and receptors to bind apoptotic cells. After uptake, these antigens enter the endosomal pathway and are subsequently degraded by lysosomal proteolysis. In special compartments with low pH, the peptides are loaded onto MHC-II molecules and subsequently presented to CD4 T cells.

In addition to these two pathways described, there is also the possibility that exogenous antigen is presented on MHC-I, a process called “cross-presentation”. This pathway is of particular importance for the priming of CTLs against tumors and viruses that do not infect DCs directly. The molecular mechanism of cross presentation is not completely understood, but there is evidence that a specialized compartment, the early endosome, favors cross-presentation (1). Moreover, this compartment can be specifically targeted, as the mannose receptor mediates routing of antigen to this location (2). There is a DC population specialized in cross-presentation, in mice characterized by expression of CD8 (see 3.1.5). One possible explanation for this unique ability is the fact that in these cells the milieu in the early endosomal compartment is specifically regulated to keep it at neutral pH (3). This inhibits antigen degradation and allows escape to the cytosol, where it is cleaved by the proteasome. Enhanced by TLR stimulation, TAP gets recruited to the early endosome and enables the re-import of the generated peptides (4). This process circumvents the problem that peptides derived from endocytosed material have to compete in the ER with a high number of endogenous peptides for MHC-I loading.

3.1.2 DC maturation

In the classical model, DCs can have two phenotypically distinct states. In the immature state, they have high endocytic capability and express low levels of MHC-II and costimulatory molecules like CD80, CD86 and CD70 on the surface. As a consequence they are inefficient in T cell stimulation, and rather act to induce tolerance. In contrast, pathogen encounter induces extensive upregulation of MHC-II, costimulatory molecules and chemokine receptors that favor migration to lymph nodes, where T cells are effectively primed. This is described as the mature state of DCs. Downregulation of endocytosis was previously described as a hallmark of DC maturation. Recently, this view has been revised and it is now

clear that while mature DCs do indeed show reduced levels of macropinocytosis, they retain the ability to capture antigen via receptor-mediated endocytosis (5). The strict concept that DC maturation forms the crucial switch between tolerance and immunity has also been modified, because there was evidence that DC maturation is a continuous process with various outcomes regarding T cell responses. The example of IL-12 production (Fig. 1) clearly shows that the duration, combination and timing of signals are important for determining the stimulatory capacity of the DC (6). Therefore a new terminology based on the effector function of the DC has been proposed (7): the classification in tolerogenic and immunogenic DCs.

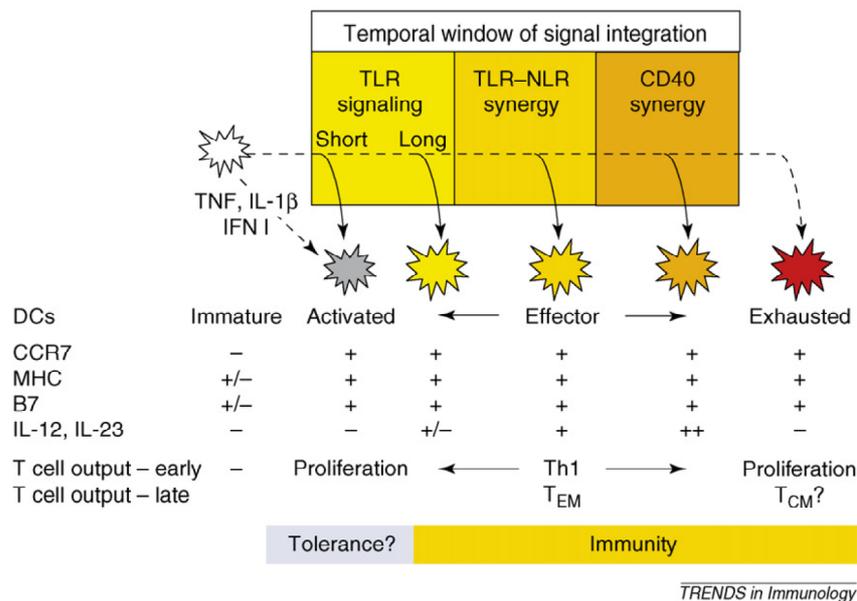


Figure 1 Signal integration model of DC activation.

The accumulation of activation signals in DCs results in an increasing capacity to produce IL-12. This has a strong impact on the generation of CD4 (Th1) and CD8 effector T cells. TLR Toll-like receptor, NLR Nod-like receptor, T_{EM} effector memory T cell, T_{CM} central memory T cell. Adapted from (6).

3.1.3 DCs induce immunity

DCs have the unique ability to prime naive T cells. They are able to upregulate costimulatory molecules to levels exceeding those expressed by other antigen presenting cells (APC), such as B cells or macrophages. As a consequence, T cells get a strong stimulus via CD28 (signal 2) in addition to the TCR signal (signal 1), which pushes them over the activation threshold (Fig. 2).

Activation of a CD4 T cell leads to upregulation of CD40L, allowing interaction with CD40 on the DC, which stimulates the DC to produce cytokines (signal 3). Their nature depends on the type of pathogen that the DC had encountered before and has an important impact on the further development of the CD4 T cell, known as T helper (T_H) polarization. Distinct T_H cell subsets are classified based on their cytokine expression profiles and associated roles for the immune response. T_H1 cells help to clear intracellular pathogens, whereas T_H2 cells combat extracellular pathogens like parasites and play a role in allergy. A third recently discovered subset, the T_H17 cell, is important for immune responses against extracellular bacteria and fungi and is involved in autoimmune reactions.

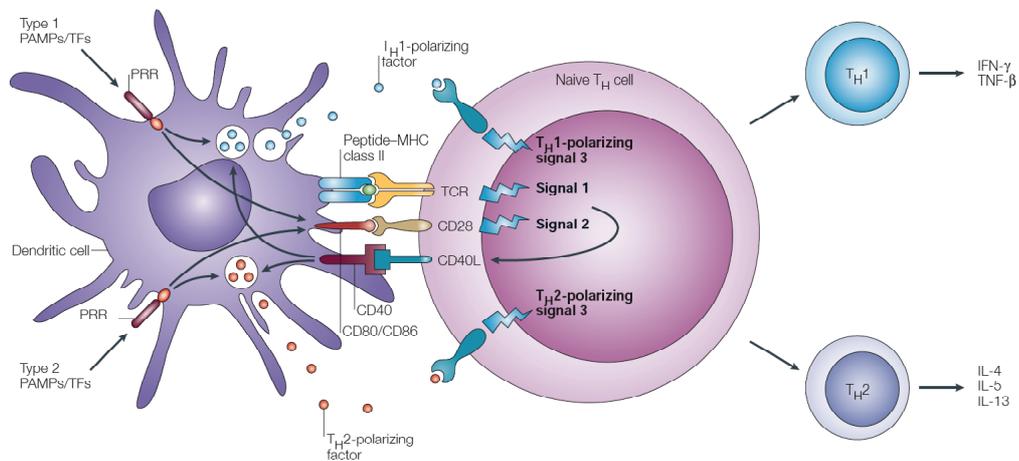


Figure 2 T cell stimulation and polarization requires three DC-derived signals.

The process in which the TCR recognizes a complex of MHC molecule and cognate peptide is defined as signal 1. Signal 2 comprises triggering of CD28 by costimulatory molecules like CD80 or CD86, which are upregulated after recognition of PAMPs by PRRs on the DC. Activation by these two signals leads to upregulation of CD40L on the CD4 T cell. On the DC, the binding of CD40 enhances the production of secreted factors (signal 3) that shape the T cell polarization. PAMP, pathogen associated molecular pattern; PRR, pattern recognition receptor. Adapted from (8).

The priming of CD8 T cells takes place in a different manner. As the generation of cytotoxic T lymphocytes (CTL) poses the risk of autoimmunity, it must be carefully regulated. Therefore an additional level of control is put in place: CD8 T cells do not express CD40L and cannot stimulate the DC. They rely on CD4 T_H cells that recognize the same cognate antigen on the DC and “license” it via CD40 to prime the CD8 T cells (9). As a consequence, CTL survival and memory formation are increased. However, in some cases a strong inflammatory stimulus can substitute T cell help. Licensed DCs upregulate costimulatory molecules,

downregulate inhibitory molecules like PD-L1 and are stimulated to produce CCR5 ligands. Recently, an alternative mechanism has been described, in which NKT cells provide help in response to glycolipid antigens and trigger DCs to produce CCR4 ligands (10). As a result, naive T cells are attracted and can more easily find the “right” DC, a concept called guided priming or signal 0 (11).

3.1.4 DCs in tolerance induction

The immune system has to deal with a tremendous variety of pathogens. To be prepared for any possible threat, it generates a large repertoire of T cells with many different specificities by TCR gene rearrangement. However, the random nature of this process is accompanied by the risk of producing autoreactive TCRs. To avoid autoimmune reactions, different mechanisms of tolerance induction are employed.

3.1.4.1 Central tolerance

In the thymus, T cells that develop and react strongly to self-peptide/self-MHC complexes are eliminated by a process called “negative selection”. This “quality control” should also include all potential self-antigens a T cell might potentially encounter in the periphery. However, the expression of some antigens is restricted to certain tissues, such as insulin in the pancreas. This problem is solved by promiscuous gene expression in medullary thymic epithelial cells (mTECs), a process regulated by the autoimmune regulator (AIRE). This transcriptional control element recognizes inactive chromatin and induces gene expression by recruitment of chromatin remodeling complexes and on the level of pre-mRNA processing (12). In addition, it has been described to enhance antigen presentation (13).

Negative selection takes place in the medulla and is mediated by two distinct players: DCs and mTECs. Both cell types are equally efficient in clonal deletion of CD8 T cells, but there are differences regarding CD4 T cells. Initially, mTECs were considered to function merely as antigen reservoirs for DCs, as some findings suggested that DCs are necessary for the deletion of CD4 T cells (14, 15). But a more recent report showed that mTECs also participate in negative selection (16).

Therefore it might depend on the amount or sub-cellular localization of the antigen, whether Ag-transfer to a DC is necessary for deletion of CD4 T cells.

In addition to this mechanism there is a second process to ensure central tolerance: CD4 T cells with intermediate TCR affinity are directed to develop into regulatory T cells (Treg). For this function, mTECs are essential (17).

However, central deletion is not complete and the exit of some mature autoreactive T cells to the periphery still occurs (18).

3.1.4.2 Peripheral tolerance

The process of inactivating autoreactive T cells that have escaped thymic deletion is known as peripheral tolerance. This can occur through deletion, anergy or induction of Tregs. Anergy is defined as an unresponsive, nonproliferative state. In contrast, T cells undergoing clonal deletion initially proliferate, but are unable to develop effector functions and ultimately die by apoptosis.

Their potent Ag-presenting properties make DCs an important player in the induction of peripheral tolerance. Recent experimental evidence has shown that constitutive ablation of DCs indeed results in the generation of autoimmunity (15). In the steady state, DCs sample self-antigen and present it in the absence of costimulation and proinflammatory cytokines, which results in T cell anergy or deletion. When the capacity of DCs to take up antigen is inhibited experimentally, autoreactive T cells accumulate in peripheral organs and can cause autoimmune disease (19). The amount of antigen has been suggested to be a critical factor deciding between the two T cell fates: high levels of antigen mediating a chronic high TCR signal favor generation of anergy (20). How the process of tolerance induction operates on a molecular level has been addressed by Probst et al (21): resting DCs mediate negative signals by triggering inhibitory receptors like PD-1 and CTLA-4 on CD8 T cells. However, on CD4 T cells, expression of ICOS (inducible costimulator) has been described to be necessary for the induction of anergy (22). The molecular basis for clonal deletion of CD8 T cells is characterized by Bim-upregulation and decreased levels of Bcl-2 and IL-7R α chain (23). Notably, there were overlaps observed in the molecular signatures of T cells undergoing deletional apoptosis or anergy.

Another mechanism by which DCs promote tolerogenic responses involves the production of IDO (indoleamine 2,3- dioxygenase), which is induced via

interaction with CTLA-4. On the one hand, this enzyme catalyzes the degradation of the essential amino acid tryptophan, which results in the inhibition of T cell proliferation. As a second effect, metabolic products of this pathway promote the induction of T cell apoptosis (24).

The generation or expansion of Tregs presents an indirect way to induce peripheral tolerance, as this cell type is specialized in the suppression of T cell responses, e.g. through secretion of the immunoregulatory cytokines IL-10 and TGF- β . One distinguishes between natural Tregs, which are generated in the thymus, and induced Tregs (iTregs) that develop from naive CD4 T cells in the periphery. For the generation of iTregs, DCs produce factors like TGF- β , IL-10 and retinoic acid. Tregs in turn constitutively express CTLA-4, which induces down modulation of costimulatory molecules on DCs (25). This interferes with subsequent activation of other T cells, promoting the tolerogenic phenotype of the DC.

3.1.5 DC subsets

There are several types of DCs, each characterized by different cellular markers and functions. A first distinction can be made between plasmacytoid and conventional DCs. Plasmacytoid DCs (pDC) express the B cell marker B220 and are present in primary and secondary lymphoid organs as well as in blood. They are specialized in the response to viruses, as they express a set of intracellular PRRs recognizing viral nucleic acids and can produce high levels of type I interferon.

Conventional DCs can be further divided into migratory and lymphoid resident DCs. Migratory DCs are originally located in peripheral tissues such as skin, lung and intestine, where they act as sentinels and a first-line of defense against pathogenic invaders. Via the lymphatics, they enter the draining lymph nodes, where they directly present antigen to T cells or transfer it to lymph node resident DC populations (26). The best-studied example for migratory DCs are Langerhans cells from the epidermis.

In the mouse, lymphoid resident DCs are classified as either CD8 positive or negative DCs. The latter express CD11b and are mainly involved in the priming of CD4 T cells (27). The CD8 positive DC subset is the main producer of IL-12, thereby driving T_H1 development and promoting CD8 T cell responses (28). The

most outstanding feature of the CD8 positive DCs is their ability to cross-present antigen, which is necessary to exert two opposing functions. On the one hand, it allows priming of CTLs against viruses that do not directly infect DCs, while on the other hand, it induces CD8 T cell tolerance against self-Ag that is only expressed in certain tissues and not by DCs themselves (19). Supporting these functions, Clec9A, a receptor for necrotic cells, is preferentially expressed by the CD8 positive DC subset (29). In addition, only the CD8 positive DC subset is able to produce high amounts of TGF- β and thereby mediate the generation of iTregs (30). In contrast, CD8 negative DCs have a higher capability to support the expansion of natural Tregs.

As the CD8 positive DC subset has these special functions, the definition of a human equivalent was an important task, but unfortunately no CD8 expressing DC subset has been found in humans. However, the XC chemokine receptor 1 (XCR1) was recently identified as a common marker for the DC population specialized in cross-presentation (31). Expression of XCR1 could help to amplify CD8 T cell responses. After antigen recognition CD8 T cells start to produce XCL1, the ligand for XCR1, potentially recruiting CD8 positive DCs to the site of first antigen encounter (32).

The “division of labor” regarding cross-presentation between DC subsets has important consequences: activated CTLs are able to kill DCs in an Ag-dependent fashion in peripheral tissues (33) and lymph nodes (34). This inhibits the generation of new CTLs specific for the same antigen, providing negative feedback regulation, but does not compromise the activity of the CTL itself. In contrast to CD4 T cells, activated CTLs function on “autopilot”(35) and do not need continuous presence of antigen or stimulation by DCs. If all DCs were able to cross-present, they would all be eliminated by antigen-specific CTLs during a systemic viral infection. However, the existence of multiple DC subsets means that there are still other DCs available that can maintain T_H cell responses important for antibody production and macrophage activation (36).

Another example of division of labor between different DC subsets within the same organ can be observed in the gut. CD103 positive DCs have tolerogenic capacity, in that they induce Treg cells, whereas the CD103 negative subset is the

main producer of proinflammatory cytokines and generates protective immunity to pathogens (37).

Finally, another population of DCs has been described to emerge during systemic inflammation: monocytes can develop into inflammatory DCs, termed TIP-DCs, as they mainly produce TNF and inducible nitric oxide synthase (38).

3.1.6 DCs in immunotherapy

The ability to orchestrate both arms of immunology, protective immunity and self-tolerance, makes DCs attractive targets for immunotherapy. DCs can be used to boost immune responses against pathogens or tumors as well as to dampen undesired immune activity such as autoimmunity, allergy or transplant rejection. Currently, two DC-based immune therapy approaches are followed in preclinical and clinical studies:

In vivo targeting of DCs

This method involves the direct injection of DC-specific monoclonal antibodies coupled to Ag. It offers the possibility to specifically target the DC subset of choice, as they differ in their surface receptors (e.g. DEC-205 on CD8 positive DCs and the 33D1 Ag on CD8 negative DCs in mice). There is preclinical evidence that Ag-targeting to DEC-205 in the absence of a maturation stimulus results in tolerance induction (39), whereas using a similar antibody with an adjuvant has been found to improve cellular and humoral immune responses against a HIV-Ag (40).

Ex vivo manipulation of DCs

Cells from donor blood are used *ex vivo* to generate DCs, which are manipulated by Ag-loading and subsequently injected into the patient. In culture, the process of Ag-loading and maturation can be tightly controlled, but it is necessary to generate high cell numbers, which is very cost intensive. In clinical trials using DCs loaded with tumor Ag, durable complete clinical responses were elicited in a small number of patients with metastatic melanoma (41, 42).

Another therapeutic possibility is gene transfer, which can be carried out by transfection of nucleic acids or by viral transduction (see 3.2). In mouse models,

tolerogenic effects were achieved by adenoviral transfer of soluble TNF receptor (43) or IL-10 (44) into DCs, which resulted in prolonged allograft survival. Lentiviral transfer of a neuropeptide into DCs also induced a tolerogenic phenotype, which was demonstrated to be beneficial in autoimmunity models (45).

Different cell types can be infected and genetically modified: *ex vivo* DCs or hematopoietic stem cells (HSCs) that can also give rise to DCs in the recipient. The latter method has the advantages that the DCs are not directly exposed to manipulation, which could change their phenotype, and that natural DC subsets are involved instead of the artificially generated *in vitro* DC type.

3.2 Gene therapy

Gene transfer can be used to treat diseases, an approach known as gene therapy. As some diseases are caused by mutation of a single gene, this offers the possibility to cure the patient by transfer of the original gene sequence to restore its function. For this purpose viral vectors are particularly suitable, because it is part of their life cycle to enter a cell. These vectors can be used to transduce HSCs or other cell types, which are subsequently transferred back into the patient. To ensure long-term benefits for patients, stable gene delivery is necessary and desirable. Retroviruses meet this requirement with their unique ability to integrate into the host cell genome. As a result, the introduced genetic information is constantly passed on to daughter cells during cell division. In addition, retroviral vectors are characterized by having a very low immunogenicity in comparison to adenoviral vectors.

Gene therapy using retroviral vectors has been efficiently applied for various human diseases. These include the lymphocyte linked severe combined immunodeficiency (SCID) (46, 47), the phagocyte linked immunodeficiency chronic granulomatous disease (CGD) (48), the metabolic disorder adrenoleukodystrophy (49) and the blood disorder β -thalassemia (50). Also in cancer treatment a gene therapy approach can be used to engineer T cells to express tumor-specific receptors, which has been successfully used in cases of neuroblastoma (51).

For gene therapy approaches, two members of the retroviral family have mainly been used: γ -retroviruses and lentiviruses. They differ in the mode of integration

site selection. Both favor transcriptionally active regions, but γ -retroviruses have a particular preference for transcriptional start sites. This process bears the risk that downstream proto-oncogenes are activated, which can lead to tumorigenesis. Unfortunately, cancer development has indeed been observed in some patients after gene therapy treatment. However, there is evidence that lentiviral vectors display a less harmful distribution of chromosomal integrations (52) and have another major advantage over γ -retroviruses: the unique ability to infect non-dividing target cells.

The risk of tumorigenesis is the major drawback of gene therapy. The development of novel improved vectors is focused on attempting to avoid this: e.g. chromatin insulators, which flank the integrating sequence are used to make integration safer (53) and some progress is currently being made in the development of non-integrating lentiviral vectors (54).

3.2.1 Gene transfer by lentiviral vectors

In this study, we applied lentiviral gene transfer to address questions of DC biology *in vivo*. The lentiviral vectors we used for experimentation are derived from the human immunodeficiency virus (HIV)-1. This virus belongs to the family of retroviruses, which contain a single-stranded RNA genome that is reverse transcribed into double-stranded DNA before integration into the host cell genome. The HIV-1 genome is composed of regulatory, accessory and structural genes with different functions (Table 1).

Before the HIV-1 vector was first used for experimental gene transfer into cells, several modifications were introduced in order to increase biosafety (55). To this end, the genetic information was separated into two plasmids (Fig. 3), which are transiently cotransfected into a producer cell line. The transfer vector contains an expression cassette with the transgene of interest under the control of an internal promoter, which is flanked by long terminal repeats (LTRs). The LTRs are composed of an U3, R and U5 region. The U3 region contains the viral promoter elements, whereas the R and U5 regions are required for reverse transcription. All relevant HIV-1 proteins are encoded by the packaging plasmid regulated by a CMV promoter. The fact that only the transfer vector contains the packaging signal (Ψ), which ensures transfer into the viral capsid, renders the virus

replication-deficient: after infection the genetic information for all viral proteins is missing in the host cell.

Table 1 HIV-1 genes and gene products with their respective functions.

Some genes encode for polyproteins that are subsequently cleaved. Modified from (55).

Gene	Encoded protein (s)	Function
Regulatory genes		
<i>tat</i>	Tat	trans-activation of gene expression
<i>rev</i>	Rev	nuclear export of late mRNAs, promotion of polysomal binding to RRE-containing RNAs
Accessory genes		
<i>vif</i>	Vif	enhancement of virus transmission
<i>vpr</i>	Vpr	nuclear transport of proviral DNA, induction of G ₂ arrest in dividing cells
<i>vpu</i>	Vpu	CD4 degradation, virus maturation and release
<i>nef</i>	Nef	CD4 and MHC-I down regulation, enhancement of virus replication
Structural genes		
<i>Gag</i>	matrix, capsid, nucleocapsid, p6	formation of viral particles, packaging of viral genomic RNA
<i>pol</i>	reverse transcriptase, integrase, protease	reverse transcription, integration and virion maturation
<i>env</i>	gp120, gp41	binding and entry into the host cell

The *env* gene encoding the envelope proteins has been deleted from the viral genome, as these proteins bind preferentially CD4 on T cells and this is impractical for an experimental approach, in which all cells should be targeted. Therefore the lentivirus is associated with a different surface glycoprotein: it is “pseudotyped” with the glycoprotein of vesicular stomatitis virus (VSV-G), which mediates a broad cell tropism, potentially through binding of phosphatidylserine (PS) in cell membranes. However, a recent report showed that PS is not the cell surface receptor for VSV-G, but is rather involved in a step after viral entry (56). The genetic information for VSV-G is supplied on a third plasmid under the control of a CMV (cytomegalovirus) promoter (Fig. 3).

Besides the *env* gene, the *vpu* gene was also deleted in the first generation vector system (Fig. 3a). As it has been shown that none of the accessory genes of HIV-1 were necessary for the lentiviral life cycle, they were deleted as well (second generation vector system, Fig. 3b). In the next step, the *tat* gene was also deleted

and only *gag*, *pol* and *rev* remained, whereby for biosafety reasons the latter was separated on another plasmid (Fig. 3c). Furthermore, the LTRs in the transfer vector were modified in the third generation vector system: a deletion was introduced into the U3 region (Δ U3) of the 3' LTR, which serves as template for the U3 in the 5' LTR during reverse transcription. As a result, the promoter activity of the 5' LTR is abolished, a process defined as self-inactivation. Moreover, the U3 in the 5' LTR was replaced by a CMV promoter, which prevents replacement of the Δ U3 in the 3' LTR by homologous recombination. Finally, the woodchuck post-transcriptional response element (WPRE) was included in the transfer vector, which improves transgene expression levels by enhancement of 3'-end processing and polyadenylation. In this study, the following three plasmids were transiently co-transfected into the producer cell line for generation of lentivirus: the envelope plasmid together with the packaging plasmid of a second generation system and the SIN transfer vector (red boxes in Fig. 3).

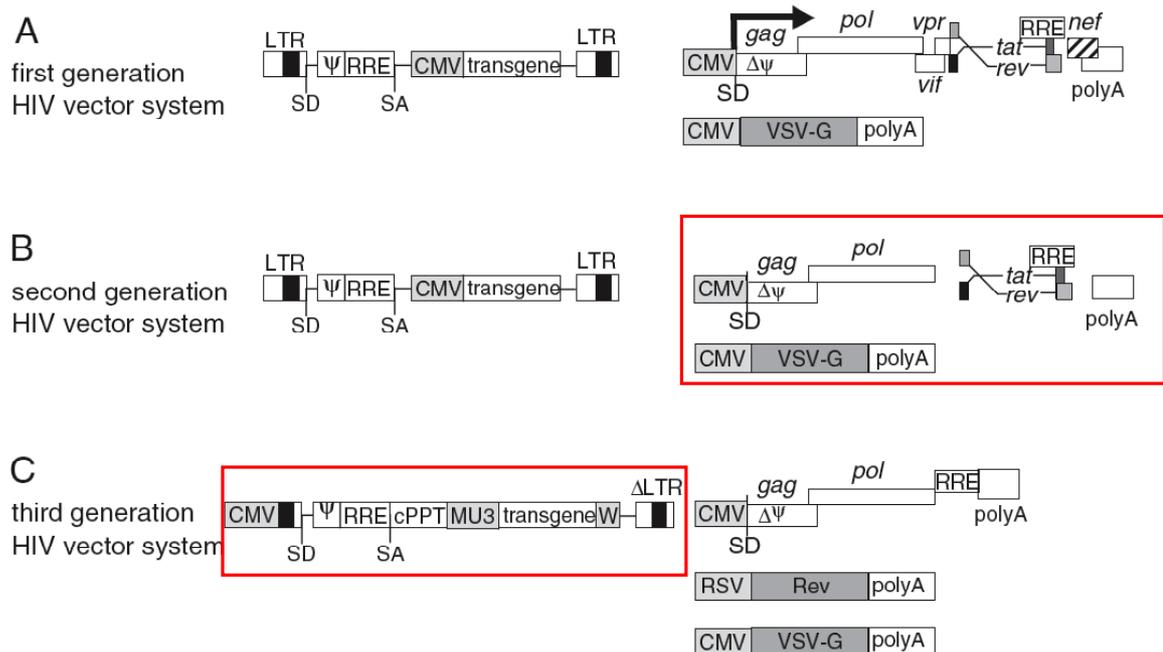


Figure 3 Schematic representation of various lentiviral vector systems.

All systems use the VSV-G envelope glycoprotein for pseudotyping. The packaging constructs differ in the amount of attenuation, HIV-1 genes have been consecutively deleted. CMV, cytomegalovirus; Ψ , packaging signal; RRE, Rev response element; LTR, long terminal repeat; cPPT, central polypurine tract; W, WPRE woodchuck hepatitis virus post-transcriptional regulatory element; SD, splice donor site; SA, splice acceptor site. Modified from (55).

In particular, internal promoters with the potential to drive gene expression in DCs were used and analyzed for tissue specificity.

Lentiviral supernatants were used to infect hematopoietic stem cells (HSCs), which had been enriched prior to use by 5-FU (5-Fluoro-uracil) treatment of bone marrow donor mice (Fig. 4).

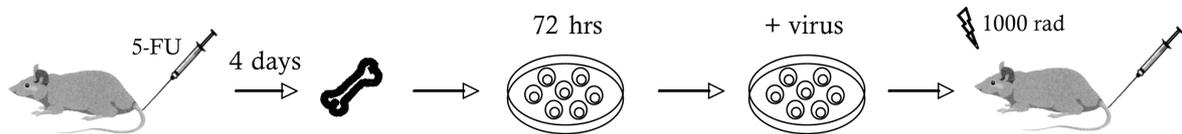


Figure 4 Generation of bone marrow chimeras.

Donor mice were injected i.v. with 5-FU (5-Fluoro-uracil). Four days later bones were taken and bone marrow containing mainly HSCs was isolated. After incubation for 72 h in the presence of a stimulating cytokine cocktail, cells were transduced with lentivirus and subsequently injected into lethally irradiated recipient mice.

Upon generation of a viral DNA genome through reverse transcription the 5' LTR is replaced by the non-functional 3' LTR ($\Delta U3$). As a consequence, the viral promoter is inactivated and only the internal promoter region is active in infected cells. After transduction, the virus is removed and the infected HSCs are subsequently injected into lethally irradiated mice, which are then analyzed after 8 weeks to allow full reconstitution of the immune system.

3.3 Aims of the project

3.3.1 Development of a DC-specific lentiviral vector for long-term induction of antigen-specific tolerance

In humans it has been demonstrated that Ag-specific tolerance can be induced by injection of immature peptide-loaded DCs (57), but that effect only lasted for 6 months (58). Disadvantages of this procedure are not only the short duration but also undesired changes in the DC phenotype by *ex vivo* manipulation. To solve these problems we decided to use a model, in which HSCs are transduced by a lentiviral vector before injection into the recipient. The ability of lentiviruses to integrate into the genome ensures long-term modification and HSCs provide a constant source for genetically modified DCs, which can develop without direct

manipulation. In order to restrict antigen expression to DCs, a lentiviral vector with a DC-specific promoter should be used.

3.3.2 Investigating the role of peripheral T cells in negative selection

Primarily, the thymus is considered to be a generative organ, where progenitor cells enter and leave after several stages of development as mature CD4 or CD8 single positive (SP) T cells. More recently, several reports showed that 'back-migration' can also occur and mature peripheral T cells may re-enter the thymus (59-62). Apparently around 10^5 mature T cells from the periphery can be accommodated in a specific thymic niche (60) and mainly localize to the medulla (63). Multiple possible functions have been assigned to these cells, such as maintenance of medullary thymic epithelial cells (mTEC) or direct mediation of thymocyte selection in certain experimental settings (64-67). None of these observed effects are "classical" functions for T cells and were therefore discussed as "epiphenomena" (59). Peripheral T cells, which re-enter the thymus of normal mice, are not naive but rather activated and cycling (68). Upon activation, T cells change their homing receptors in order to mediate effector functions in all tissues of the body, including the thymus (63).

In lymph nodes (34) and tissues (33) cytotoxic effector T cells can eliminate dendritic cells (DCs) in an antigen-specific manner. We wanted to investigate, whether peripheral T cells can also remove APCs upon return into the thymus and thereby potentially alter negative selection.

3.3.3 *In vivo* promoter analysis

Dendritic cells (DCs) have a variety of biological features. We wanted to know how these are regulated on a transcriptional level and define relevant DC-specific regulatory transcriptional networks. One important characteristic of DCs is to sense and react to changes in their environment. This makes conventional promoter analysis studies difficult to perform, as DCs quickly alter their phenotype in response to *in vitro* manipulation. We used a novel method for promoter analyses, in which HSCs are genetically manipulated by lentiviral vectors and expression of the reporter gene is monitored in various primary cell

types, which develop without further manipulation in irradiated recipient mice. In addition, comparative promoter analysis by computational means was employed to define evolutionary conserved regions within promoter sequences. Furthermore, the characterization of regulatory elements within the mouse CD11c promoter could help to identify a smaller and still functional promoter fragment. As lentiviral vectors have size limitations, this could be of use for future cases, in which transgenes with greater length are inserted behind the promoter.

4 MATERIALS AND METHODS

Both materials and methods are listed alphabetically. Company headquarters are only indicated at first mention.

4.1 Materials

4.1.1 Antibodies

Table 2 Antibodies used in flow cytometry.

Specificity (anti-mouse)	Conjugate	Clone	Supplier
B220	PerCP	RA3-6B2	Becton, Dickinson & Co. (BD), Franklin Lakes, NJ, USA
CD3	PerCP	145-2C11	BD
CD4	PE PerCP PE-Cy7	GK1.5	BD
CD8	PE PerCP APC-Cy7	53-6.7	BD
CD11b	PE	M1/70	BD
CD11c	APC	HL3	BD
CD19	PerCP PE-Cy7	1D3	BD
CD24	PE	M1/69	BD
CD25	FITC	PC61	BD
CD44	PE	Pgp-1, Ly-24	BD
	Alexa Fluor 405	IM7.8.1	life technologies, Carlsbad, CA, USA
CD45.1	APC eFluor 450	A20	eBioscience, San Diego, CA, USA
CD62L	PE	Mel-14	BD
CD69	PE	H1.2F3	BD

CD90.1	FITC PerCP	OX-7	BD
F4/80	PE	BM8	BD
Gr-1	PE	RB6-8C5	BD
I-A ^b	PE	AF6-120.1	BD
IFN γ	PE APC	XMG1.2	BD
κ chain	PE	polyclonal	Southern Biotec (Birmingham, AL, USA)
λ chain	PE	polyclonal	Southern Biotec (Birmingham, AL, USA)
Ly6C	FITC	AL-21	BD
Neutrophils	Biotin	7/4	AbD Serotec (Oxford, UK)
NK1.1	PE PE-Cy7	PK136	BD
PD-1	FITC	J43	BD
Qa-2	Biotin	1-1-2	BD
TNF α	FITC PE	MP6-XT3	BD
V α 2 TCR	FITC APC	B20.1	BD
V β 5.1/5.2 TCR	FITC PE	MR9-4	BD

Streptavidin-APC was purchased from life technologies and Streptavidin-PE from Southern Biotec. The MHC tetramers H-2K^b/SIINFEKL (OVA₂₅₇₋₂₆₄) and H-2K^b/SSIEFARL (HSVgB₄₉₈₋₅₀₅) were either directly PE-labeled or used in combination with a PE-Fluorotag and were purchased from ProImmune (Oxford, UK).

4.1.2 Chemicals

If not stated differently, chemicals were purchased from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma-Aldrich (St. Louis, MO, USA). All buffers and solutions were prepared using double distilled water.

4.1.3 Consumables

Centricon filter	Millipore Corporation, Billerica, MA, USA
disposable syringe filter (0.2 + 0.45 µm)	Nalgene Nunc Int., Rochester, NJ, USA
bottle filter	Nalgene Nunc Int. Rochester, NJ, USA
disposable injection needle Japan (26 G x 1/2")	Terumo Medical Corporation, Tokyo,
disposable syringe (1+5 ml)	Braun, Melsungen, Germany
reaction container 0.2 ml	Nunc, Wiesbaden, Germany
reaction container 1.5 ml und 2 ml	Eppendorf, Hamburg, Germany
reaction tube 5 ml	BD, Franklin Lakes, NJ, USA
reaction tube 15 ml und 50 ml	Greiner, Frickenhausen, Germany

Other materials and plastic wares were purchased from BD, Nunc (Wiesbaden, Germany) and Greiner (Frickenhausen, Germany).

4.1.4 Devices

Analytic scale (Adventurer, Ohaus Corp., Pine Brooks, NJ, USA), automatic pipettors (Integra Biosciences, Baar, Switzerland), bench centrifuge (Centrifuge 5415 D, Eppendorf, Hamburg, Germany), cell counter (Coulter Counter Z2, Beckman Coulter, Krefeld, Germany), centrifuge (Rotixa RP, Hettich, Tuttlingen, Germany), chemical scale (Kern, Albstadt, Germany), flow cytometer (FACSCalibur, FACSCantoII and FACS Aria, BD), incubator (Hera cell, Heraeus Kendro Laboratory Products, Hanau, Germany), laminar airflow cabinet (Heraeus), magnetic stirrer (Ika Labortechnik, Staufen, Germany), PCR-machine (Biometra, Goettingen, Germany), pH-meter (Inolab, Weilheim, Germany), pipettes (Gilson, Middleton, WI, USA), power supply (Amersham Pharmacia, Piscataway, NJ, USA), real-time PCR machine (Lightcycler, Roche, Basel, Switzerland or CFX96 Real Time System, BIO-RAD, Hercules, CA, USA), vacuum pump (KNF Neuberger, Munzingen, Germany), vortex-Genie2 (Scientific Industries, Bohemia, NY, USA), water bath (Grant Instruments Ltd., Barrington Cambridge, UK). All other devices are mentioned in the methods section.

4.1.5 Media and solutions

ACK buffer	8.29 g NH ₄ Cl 1 g KHCO ₃ 37.2 mg Na ₂ EDTA H ₂ O ad 1 l pH 7.2-7.4 adjusted with 1 N HCl sterilized by 0.2 μm filtration
PBS	150 mM NaCl 10 mM Na ₂ HPO ₄ 2 mM KH ₂ PO ₄ pH 7.4 adjusted with 5 N NaOH
PBS-FBS	Dulbecco's PBS without Ca ²⁺ / Mg ²⁺ 2% FBS (v/v)
FACS buffer	PBS 2% FBS (v/v) 0.01% NaN ₃ (v/v)
5-Fluoro-uracil (5-FU)	20 mg/ml in Dulbecco's PBS pH 10-11 adjusted with NaOH vortex until complete dilution pH 7.5 adjusted with HCl Sterilized by 0.2 μm filtration Stored at -20°C
MACS buffer	Dulbecco's PBS without Ca ²⁺ / Mg ²⁺ 0.5% FBS (v/v) 2 mM EDTA
RFI	15% Glycerin (v/v) 100 mM KCl 50 mM MnCl ₂

	<p>30 mM C₂H₃KO₂ 10 mM CaCl₂ pH 5.8 adjusted with 0.2 mM acetic acid Sterilized by 0.2 μm filtration Stored at 4°C</p>
RFII	<p>15% Glycerin (v/v) 10 mM MOPS 10 mM KCl 75 mM CaCl₂ pH 6.8 adjusted with 1 N NaOH Sterilized by 0.2 μm filtration Stored at 4°C</p>
50x TAE buffer	<p>242 g Tris 57.1 ml 100% acetic acid (v/v) 100 ml 0,5 M EDTA (pH 8.0) H₂O ad 1 l</p>
<u>Solutions used for transfection</u>	
2xHBS	<p>50 mM HEPES 280 mM NaCl 1.5 mM Na₂HPO₄-Dihydrate pH 7.05 adjusted with NaOH Sterilized by 0.2 μm filtration Stored at -20°C (≤ 6 months)</p>
CaCl ₂	<p>2.5 M CaCl₂ Sterilized by 0.2 μm filtration Stored at -20°C</p>

Cell culture media

All culture media and solutions were purchased from Gibco (Invitrogen, Carlsbad, CA, USA) unless otherwise stated.

DC medium	RPMI 1640 + glutamine (PAA, Pasching, Austria) 5% FBS (inactivated, v/v) 500 mM β -mercaptoethanol 100 U/ml penicillin 100 μ g/ml streptomycin 25 ng/ml GM-CSF
HSC medium	Stemline II hematopoietic stem cell expansion medium (Sigma-Aldrich) 100 U/ml penicillin 100 μ g/ml streptomycin 50 ng/ml hIL-6 10 ng/ml mIL-3 50 ng/ml mSCF
293FT medium	Dulbecco's Modified Eagle Medium (DMEM) with glutamax-I 10% FBS (inactivated, v/v) 100 U/ml penicillin 100 μ g/ml streptomycin 0.1 mM non-essential amino acids (MEM) 10 mM HEPES 500 μ g/ml geneticin
293FT transfection medium	same as 293FT medium, without geneticin
NIH3T3 medium	DMEM 10 % FBS (non-inactivated, v/v) 100 U/ml penicillin 100 μ g/ml streptomycin

Freezing medium

90% FBS

10% DMSO

4.1.6 Mouse strains

All mice were bred and maintained in the mouse facility of the Institute of Immunology (LMU, Munich, Germany).

C57BL/6 (B6)

This inbred strain has the MHC-haplotype H-2^b. Mice from the C57BL/6 strain express the allele Ly5.2 (CD45.2) on all leukocytes and Thy1.2 (CD90.2) on all T cells. Two congenic strains were also used that either express Ly5.1 (CD45.1) or Thy1.1 (CD90.1) on a B6 background.

OT-I

CD8 T cells from OT-I mice express the transgenic V α 2/V β 5 TCR specific for OVA (ovalbumin)₂₅₇₋₂₆₄ in the context of MHC-I H-2K^b (69). These mice were kept on a B6 background either expressing the Ly5.1 or the Thy1.1 allele.

OT-II

CD4 T cells from OT-II mice (70) express the transgenic V α 2/V β 5 TCR specific for OVA₃₂₃₋₃₃₉ that is recognized in the context of MHC-II I-A^b (71). These mice expressed the congenic marker Thy1.1 on a B6 background.

RIP-mOVA

RIP-mOVA mice express a membrane-bound form of OVA under control of the rat insulin promoter (RIP) (72). In the pancreas and testis of these mice OVA is expressed as a model auto-antigen. When RIP-mOVA mice receive OT-I T cells by adoptive transfer and are immunized, they develop diabetes (73). The progress of diabetes is monitored by measuring the glucose concentration in the urine (Diabur 5000, Roche).

4.1.7 Peptides, protein and oligonucleotides

The peptides OVA₂₅₇₋₂₆₄ and HSVgB₄₉₈₋₅₀₅ were purchased from PolyPeptide Group (Strasbourg, France). Ovalbumin (albumin from chicken egg white, Grade V) was purchased from Sigma-Aldrich.

Oligonucleotides were purchased from MWG-Biotech AG (Ebersberg, Germany) and are indicated in the relevant context in the methods section. Sequencing reactions were carried out by Sequiserve (Vaterstetten, Germany) or MWG-Biotech AG.

4.1.8 Vectors

Cloning vector

For subcloning, the plasmid pBluescript-II-KS+ (pBS, Agilent Technologies, Santa Clara, CA, USA) was used.

Herpes Simplex vector

The recombinant, replication deficient HSV-1 vector HSV-OVA was produced by P. Marconi (University of Ferrara, Italy). It is based on T0ZGFP, a low toxicity HSV vector, in which three immediate early genes are deleted. The hCMV- β -Globin-OVA expression cassette has been inserted into the UL41 locus (74).

Lentiviral vectors

All lentiviral vectors used in this work are based on FUGW (75). In this vector, as described above, the promoter/enhancer-containing region localized in the 3'LTR is deleted. As a result, the virus is inactivated after integration and replication is blocked. For all cloning reactions the FUGW vector was digested with Pac I and Age I to remove the original Ubiquitin C promoter. The mouse CD11c promoter sizes 2000bp and 1500bp correspond to the restriction sites Hind III and SexA I within the 5kb promoter, respectively. Initially, the 1000bp fragment was amplified by PCR with 5'-ATTTGCGGCCGCTAGCACCCCAGTTCTTTGCT-3' and 5'-TCGCGACTGCAGCCCACTGGAGAA-3' primers and cloned into a different vector with Not I and Nru I. From there it was isolated with Not I, the sticky end was filled up by Klenow enzyme, and with Age I. This fragment was ligated into the FUGW, which has been treated with Pac I, Klenow enzyme and

Age I. To generate the smaller mouse CD11c promoter-GFP constructs, various promoter fragments were amplified using standard PCR. The following primers were used: 900 5'-CCTTAATTAACAATGCTTACCCACCCCCTC-3', 750 5'-CCTTAATTAACAGTTTTTAGTATTCTCTTGACCTTGG-3', 500 5'-GCTATTAATTAATATGTTGAGCAAATGACTAAT-3', 400 5'-GCTATTAATTAATGTGCTTACTTCTTAGTCTACTTCCA-3' with the same reverse primer 5'-GCATACCGGTGCGACTGGAGAACAGAAGCA-3' for all of the constructs. The minimal SV40 promoter was amplified from the pGL3-Promoter vector (Promega, Madison, WI, USA) using the primers 5'-CCTTAATTAAGCGATCTGCATCTCAATT-3' and 5'-GCATACCGGTGCCAAGCTTTTTGCAAAGC-3'. To combine the CD11c promoter fragments with the minimal SV40 promoter, the fragments were amplified by PCR (CD11c^{500-400bp} 5'-CCGCTCGAGCGGTATGTTGAGCAAATGAC-3' and 5'-GGAAGATCTTCCTGATCCATGTAGGGAGC-3', CD11c^{750-574bp} 5'-CTAGCTAGCATTGCTTCTGAAATTCAG-3' and 5'-GAAGATCTGAGTAAAAGCAGATGG-3', CD11c^{750-400bp} 5'-CTAGCTAGCATTGCTTCTGAAATTCAG-3' and 5'-GGAAGATCTTCCTGATCCATGTAGGGAGC-3') and cloned first into the pGL3-Promoter vector using the following restriction enzymes: Xho I and Bgl II (CD11c^{500-400bp}) or Nhe I and Bgl II (CD11c^{750-574bp}, CD11c^{750-400bp}). In the next step the CD11c fragments were isolated together with the minimal SV40 promoter: Sma I and Hind III for CD11c^{500-400bp} or Nhe I and Hind III for CD11c^{750-574bp} and CD11c^{750-400bp}. All these fragments were completely blunt-ended and ligated into a blunt-ended FUGW. All CD11c-GFP constructs were validated by sequencing.

For this work also other lentiviral constructs were used, which have been previously described (76):

The DC-STAMP-OVA virus contains a membrane bound form of ovalbumin (fused to the membrane part of the transferrin receptor) under the control of a 1704bp promoter fragment of mouse DC-STAMP. Specificity of the relevant promoter fragment was first analyzed with a lentiviral vector containing GFP as a reporter gene (DC-STAMP-GFP). As control virus a modified form of FUGW was used, which contains the Ubiquitin C promoter without any transgene.

4.2 Methods

4.2.1 Cellular and immunological methods

4.2.1.1 Adoptive cell transfer

This method involves transfer of T cells from a donor mouse into a recipient mouse. T cells were isolated from spleens and/or lymph nodes of donor mice using negative selection (MACS, see 4.2.1.10). The purity of T cells was determined by flow cytometry (see 4.2.1.5.) before i.v. transfer into syngenic recipients of the same sex. Congenic markers (Ly5.1 or Thy1.1) allowed subsequent detection of transferred T cells in the recipient.

4.2.1.2 Cell culture

Culture and lentiviral transduction of hematopoietic stem cells (HSC)

Donor mice were injected i.v. with 5-Fluorouracil (5-FU, 150 mg/kg body weight, Invivogen, San Diego, CA, USA), after four days mice were sacrificed and bone marrow was isolated. 5-FU is a pyrimidin analog, which inhibits DNA synthesis and therefore leads to apoptosis of proliferating cells. Thereby 5-FU treatment enriches the non-dividing stem cell population in the bone marrow. The cell suspension was depleted of erythrocytes (Mouse Erythrocyte Lysing Kit, R&D Systems, Minneapolis, MN, USA), resuspended in HSC medium (1×10^6 cells/ml) and cultured in 100 mm plates at 37°C and 5% CO₂. The cells were stimulated with a cytokine mixture (Miltenyi Biotec, Bergisch Gladbach, Germany) containing murine IL-3 (10 ng/ml), murine SCF (50 ng/ml) and human IL-6 (50 ng/ml). At day 3 of culture cells were spin-infected (300 g, 2 h at 32°C) with cell-free stocks of lentivirus (MOI between 0.2 and 5) in the presence of protamine sulfate (4 µg/ml, Sigma-Aldrich). After four more hours incubation at 37°C the virus was removed, cells were incubated in HSC medium and $1-3 \times 10^6$ cells per recipient mouse were injected i.v. in PBS the next day.

Culture of dendritic cells

Addition of the cytokine GM-CSF leads to *in vitro* differentiation of DCs from bone marrow in the course of several days. A modified version of Inaba's protocol (77)

was used. Bone marrow cells were depleted of erythrocytes (Mouse Erythrocyte Lysing Kit, R&D Systems) and 1×10^6 cells/ml were cultured in DC-medium in a total amount of 10 ml per 100 mm plate at 37°C and 5% CO₂. Each 2-3 days fresh medium was added. If desired, lentiviral transduction was performed at day 1 of culture with 1×10^6 cells/ml, in a total of 2 ml per well in a 6 well plate. The transduction protocol was the same as for NIH3T3 cells (see below).

Culture of 293FT and NIH3T3 cells

293FT (human embryonic kidney cell line) and NIH3T3 (mouse embryonic fibroblast cell line) cells were cultured in their respective media at 37°C and 10% CO₂. They were kept in 175 cm² and 75 cm² tissue culture flasks, respectively. Both cell lines were split every other day taking care that less than 75% confluence was reached.

Transduction of NIH3T3 cells for lentivirus titration

NIH3T3 cells were plated with 4×10^4 cells/well in 24-well plates 18 h before transduction. A total of 6 wells were prepared per virus to be titrated. Two additional wells were plated to determine the number of cells per well at the time of transduction. Using NIH3T3 medium, different dilutions were made depending on the virus stock: for concentrated virus dilutions between 1/100 and 1/10000 were adequate, whereas for unconcentrated virus dilutions between 1/5 and 1/100 were used. NIH3T3 cells were transduced with 1 ml of various virus dilutions using spin infection (300 g, 2h at 32°C) in the presence of 8 µg/ml polybrene (Hexadimethrine-Bromide, Sigma-Aldrich). After 4 h at 37°C the virus dilution was replaced with NIH3T3 medium and cells were incubated for two more days. Then cells were harvested with Trypsin/EDTA and total genomic DNA was isolated for qPCR analysis (see 4.2.2.8).

4.2.1.3 CFSE staining

Labeling of cells with CFSE (carboxyfluorescein-diacetate-succinimidylester, life technologies) allows tracking of cell division both *in vitro* and *in vivo*. CFSE diffuses into the cell, where it binds to amino groups of proteins. By cleavage with intracellular esterases, CFSE becomes a fluorescent dye. After each cell division the amount of dye is divided equally between the daughter cells. As a

consequence, fluorescence intensity is reduced by 50%, leading to a characteristic peak pattern, in which the number of cell divisions can easily be identified. For CFSE labeling, a single cell suspension was resuspended in prewarmed PBS containing 0.1% FBS and 5 μ M CFSE is added while vortexing to ensure homogeneous staining. Cells are incubated for 10 min at 37°C and protected from light. The reaction is stopped by adding an equal volume of pure FBS. The cells are washed two times with PBS and resuspended in the desired amount of PBS or culture medium.

4.2.1.4 Determination of cell numbers

To determine the cell number of primary cells a Coulter counter Z2 instrument (Beckman Coulter) was used. Cell count and size is measured by the change of electrical resistance that a cell causes by passing through a small hole in an electrode. For analysis 10 μ l of cell suspension was diluted in 10 ml conductive solution (Isoton II, Beckman Coulter) and 2 drops of a lytic reagent (ZAP-OGLOBIN II, Beckman Coulter) were added to remove residual erythrocytes.

For cell counting of cell lines like 293FT and NIH3T3 a Neubauer counting chamber was used. 10 μ l cell suspension were mixed with 90 μ l Trypan blue, which stains dead cells. Only live cells were counted and the actual cell number (unit 10^6 /ml) was calculated by division of the resulting number by 10. Total cell numbers of a certain population within an organ were calculated by multiplying the percentage of the population of interest with the cell number of the respective organ and dividing it by 100.

4.2.1.5 Flow cytometry - Fluorescence-Activated Cell Sorting (FACS)

In flow cytometry various characteristics of single cells such as size, granularity and molecular marker expression can be detected. Cells are stained with fluorochrome-coupled antibodies against surface or intracellular antigens. In a fluid stream cells pass a laser beam and several detectors. The resulting information is collected and can be used for identification of distinct cell populations within a heterogeneous mixture of cells.

An advanced development of classical flow cytometry is cell sorting. On a specialized instrument (FACSAria, BD) the population of interest can be defined by the user and is then collected by electrostatic droplet deflection.

In a 5 ml reaction tube 50 μ l of a single cell suspension ($1-5 \times 10^6$ cells) were mixed with 50 μ l of a 2x concentrated antibody solution at an appropriate dilution (antibodies were titrated before use). The tubes were incubated in the dark at 4°C for 20 min. The cells were then washed with 2-3 ml FACS buffer to remove excess of unbound antibodies (300g, 4°C). If biotinylated antibodies were used, a second staining step with fluorochrome-conjugated streptavidin followed. For intracellular cytokine staining splenocytes (10×10^6) were restimulated for 4 h in 1 ml with 2 μ g SIINFEKL or SSIEFARL peptide in the presence of 2 μ l GolgiStop (BD), which blocks protein secretion. Intracellular staining was performed using the Cytofix/Cytoperm kit (BD) according to the manufacturer's protocol. MHC tetramers were used according to the manufacturer's protocol (ProImmune).

Prior to acquisition all samples were filtered (41 μ m mesh; Reichelt Chemietechnik, Heidelberg, Germany) to remove cell aggregates. Data were acquired on a FACSCalibur with two lasers (488 and 633 nm) or on a FACSCanto II instrument with three lasers (488, 633 and 405 nm) and analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

4.2.1.6 Generation of bone marrow chimeras

Recipient mice were lethally irradiated with two separate doses (2x 550 rad) using a Cesium source (Gammacell 40, AECL, Mississauga, Canada) and supplied with neomycin (1.2 g/l, Sigma-Aldrich) containing drinking water for five weeks. Chimeras were analyzed 8-10 weeks after bone marrow transfer. For most of the experiments HSCs that had been lentivirally transduced were transferred intravenously. In some cases untreated bone marrow was used directly after isolation.

4.2.1.7 Harvesting of blood and organs from mice

Harvesting peripheral blood

Before blood extraction, mice were placed under an infrared lamp for few minutes to generate vasodilatation. Mice were put in a trap and a small cut was made in the tail vein with a scalpel blade. In an eppendorf tube 3-5 drops (100-150 μ l) of blood were collected and mixed with 50 μ l heparin-sodium (25000 I.E./5 ml, Ratiopharm, Ulm, Germany).

Harvesting organs and preparation of single cell suspensions

Mice were sacrificed by cervical dislocation, fixed with needles on a styrofoam pad, disinfected with 70% ethanol and cut open. Thymus, lymph nodes and spleen were harvested with fine tweezers and kept on ice in RPMI medium. For generation of single cell suspensions organs were placed in a petri dish (\varnothing 5 cm) between two 150 μ m meshes (Reichelt Chemietechnik) and mashed with a 1 ml syringe plunger (Omnifix, Braun, Melsungen, Germany). For optimal recovery of dendritic cells (DC) organs were treated by enzymatic digestion: injection with a solution containing Liberase CI (0.42 mg/ml) and DNase I (0.2 mg/ml, both from Roche) and incubation for 25 min at 37°C, followed by mechanical dispersion using a cell strainer (100 μ m, BD).

For preparation of bone marrow the hind legs were removed. The bones were cleaned from muscles, separated into tibia and femur and quickly disinfected with 70% ethanol. The terminal parts of the bones were cut open and the bone marrow was flushed out with needle and syringe. For large-scale isolation bones were placed in medium and carefully fragmented with a mortar and pestle. Bone marrow was harvested from the supernatant and filtered through a cell strainer.

Erythrocyte lysis

Erythrocytes from peripheral blood were lysed using Pharm Lyse reagent (BD) according to the manufacturer's instructions.

Cell pellets from organs were resuspended in 4 ml ACK buffer and left for 5 min at RT. Afterwards 10 ml FACS buffer was added, the cells were centrifuged (5 min at 4°C, 300 g) and resuspended in culture medium or FACS buffer. A more gentle treatment (Mouse Erythrocyte Lysing Kit, R&D Systems) was used according to

the manufacturer's instructions for bone marrow preparations or if cells were used for intracellular cytokine staining.

After erythrocyte lysis cell pellets were resuspended in the desired amount of medium or FACS buffer.

4.2.1.8 Histology

Organs were embedded in O.C.T. compound (Sakura Finetek, Zoeterwoude, The Netherlands), snap frozen and cut in 5 μm sections on a cryostat instrument (Jung Frigocut 2800 E, Leica Microsystems, Wetzlar, Germany). Sections were air-dried for at least 1 h, fixed with acetone (-20°C for 10 min) and stained by standard protocol with anti-CD8-PE (clone 53-6.7, eBioscience). Analysis was performed on a BX41 microscope equipped with a F-view II camera and cell^F software (all from Olympus, Hamburg, Germany).

4.2.1.9 Immunizations

Virus stocks were thawed on ice and treated with ultrasound for 10 seconds (Ultrason E, Greiner, Frickenhausen, Germany). Virus concentration was adjusted with PBS and 4×10^6 pfu of rHSV-1 expressing OVA (HSV-OVA) was injected i.v. per mouse.

4.2.1.10 Magnetic cell sorting (MACS)

Magnetic cell sorting (MACS, Miltenyi Biotec) is a technique that allows isolation of various cell subpopulations based on their expression of different antigens on the cell surface. In general there are two possible methods for cell sorting: labeling the population of interest (positive selection) or labeling all other cells (negative selection). The MACS principle is based on the use of monoclonal antibodies that are conjugated to superparamagnetic microbeads. After labeling, the cells are applied to a column that is placed in a magnetic field of a MACS separator. There are different columns for different purposes and for different numbers of cells. Labeled cells (the positive fraction) are retained inside the column by the magnetic field, while the unlabeled ones (the negative fraction) pass through. The column is washed three times with MACS buffer to remove excess unlabeled cells. After

removal of the column from the magnetic field, the cells retained in column can be eluted. MACS separation was applied to purify dendritic cells (CD11c microbeads, positive selection) and CD8⁺ T cells (CD8⁺ T cell Isolation Kit, negative selection) from cells isolated from spleen, lymph nodes and thymus. For some experiments, bone marrow preparations were depleted of CD8 T cells using CD8 microbeads. All procedures were performed according to the manufacturer's instructions.

4.2.1.11 Production of lentiviral vectors

For virus production 293FT cells (Invitrogen) were transiently transfected using the standard calcium phosphate precipitation method. 293FT cells were plated 14-18 hours before transfection (5.5×10^6 cell per 100 mm cell culture plate) and kept at 37°C and 10% CO₂. Throughout the whole virus production 293FT medium without geneticin was used to avoid additional cellular stress. For the transfection procedure 20 µg lentiviral vector, 15 µg pCMVΔR8.2 and 10 µg pMD2G (coding for VSV-G) were mixed with 100 µl CaCl₂ and sufficient water for a total volume of 1 ml. Under vortexing 1 ml 2xHBS was added. This solution was added dropwise to the plates, which were incubated for 3-5 hours. Afterwards cells were washed with pre-warmed PBS and fresh 293FT medium (10 ml) was added. The handling of the plates was performed very carefully to avoid detachment of the cell monolayer. Supernatants were harvested and filtered (0.45 µm filter, Nalgene Nunc) on three consecutive days starting one day after transfection. All supernatants were pooled and stored at 4°C until the last harvest, when they were either directly partitioned into aliquots or concentrated using Centricon filter devices (Plus-80, Millipore, Billerica, MA, USA) according to the manufacturer's instructions. This method of ultrafiltration is based on the following principle: centrifugal force drives a solution against a membrane with a particular cutoff (100kDa). The membrane traps high-molecular substances like viruses, whereas smaller molecules pass through. Thereby the lentiviral supernatant could be highly concentrated (up to 100-fold) without loss of virus. Aliquots were snap-frozen in liquid nitrogen and stored at -80°C until use.

4.2.2 Molecular biology methods

4.2.2.1 Agarose gel electrophoresis

This technique was used to separate DNA fragments according to their length. By comparison to a 100bp or 1kb ladder (New England Biolabs (NEB), Ipswich, MA, USA) the actual size of the fragments was estimated. Before gel loading the DNA samples were mixed with gel loading dye (10% glycerol, xylene cyanol FF). Separation was carried out by application of constant voltage (80V) to an electrophoresis chamber containing conductive buffer (TAE). Depending on the size of the DNA fragment of interest different amounts of agarose were used (0.5-2% w/v). DNA was visualized by addition of ethidium bromide to the gel (0.5 µg/ml) and subsequent examination under UV light (312 nm, Intas, Goettingen, Germany). For cloning, DNA fragments were excised with a clean scalpel and then isolated from the gel (see 4.2.2.4).

4.2.2.2 Cleavage of DNA with restriction enzymes

Restrictions enzymes were used to characterize and identify DNA fragments, as well as to prepare DNA fragments for cloning. All restrictions enzymes were purchased from NEB and were used according to the manufacturer's instructions.

4.2.2.3 Culture of bacteria

Transformed bacteria were cultured in reaction tubes with LB medium (Roth, Karlsruhe, Germany) in a shaker (Infors, Bottmingen, Switzerland) at 37°C overnight. Since all plasmids and vectors contained an ampicillin resistance gene, 100 µg/ml of the antibiotic was added to the LB medium for selection of transformed bacteria. For generation of single colonies LB-agar plates (7.5 g agar/500 ml LB medium, containing 100 µg/ml ampicillin) were placed in an incubator at 37°C overnight.

4.2.2.4 DNA and RNA isolation and purification

The following kits were used according to the manufacturer's protocols. During RNA isolation residual amounts of DNA were removed by on-column DNase I treatment. All kits were purchased from Qiagen (Hilden, Germany):

Purification of DNA fragments from agarose gel	QIAquick Gel Extraction Kit
Isolation of plasmid DNA (small-scale)	QIAprep Spin Miniprep Kit
Isolation of plasmid DNA (large-scale)	QIAfilter Plasmid Maxi Kit
Isolation of genomic DNA	DNeasy Blood & Tissue Kit
Isolation of total RNA	RNeasy Mini Kit
	RNeasy Micro Kit (for small cell numbers)

4.2.2.5 Ligation of DNA fragments

Ligation reactions were composed of 50 ng vector DNA and a threefold molar excess of insert DNA in ligase buffer and 400U T4 DNALigase (NEB) in a final volume of 20 μ l. The reaction was performed at RT for 1 h or at 4°C overnight. For transformation into competent bacteria 5 μ l of the ligation reaction was used.

4.2.2.6 Measurement of nucleic acid concentration

Nucleic acid concentrations were determined by UV absorbance measurement at 260 nm. For this purpose samples were either used undiluted and measured directly with a NanoDrop instrument (Thermo Fisher Scientific, Waltham, MA, USA) or diluted in plastic cuvettes (Brand, Wertheim, Germany) and measured with a Biophotometer (Eppendorf, Hamburg, Germany). The 260/280 ratio is an indicator for nucleic acid purity: values between 1.8-2 are desirable, as this means a low amount of protein contamination.

Titration of lentiviral vectors was performed by qPCR (see 4.2.2.8) using absolute quantification. Therefore a standard curve was prepared with serial dilutions of plasmid DNA, where concentrations were very low and high accuracy was necessary. Therefore a Qubit fluorometer and the dsDNA HS Assay Kit (both from Invitrogen) were used according to the manufacturer's protocols. This method

involves the addition of a DNA binding fluorescent dye, which increases sensitivity.

4.2.2.7 Polymerase chain reaction (PCR)

Using this method, a particular DNA sequence is amplified from a small amount of template DNA. For this process specific primers that flank the region of interest are used. For subsequent molecular cloning of the PCR product restriction sites can be incorporated terminally in the primer sequence. Thermal cycles alternate that allow DNA denaturation, primer annealing and DNA synthesis. For PCR the Platinum Taq High Fidelity DNA polymerase (Invitrogen) was used to avoid undesired mutations during the reaction.

Reaction composition:

5-50 ng plasmid DNA

0.4 μ M primer for

0.4 μ M primer rev

1 x High Fidelity PCR buffer

200 μ M dNTP mix

2 mM MgSO₄

1 U Platinum Taq High Fidelity DNA polymerase

H₂O ad 50 μ l

The melting temperature (T_M) of primers was calculated with the following formula: $T_M = [(G+C) \times 4^\circ\text{C}] + [(A+T) \times 2^\circ\text{C}]$

Depending on the T_M the annealing temperature (T_A) of primers in the PCR reaction was calculated:

$$T_A = T_M - 5^\circ\text{C}$$

PCR reactions were performed with a T3 Thermocycler (Biometra) using the following program:

Step 1:	94°C	5 min	
Step 2:	94°C	30 sec	
Step 3:	55°C	30 sec	(T _A can vary dependent on T _M)
Step 4:	68°C	1 min per kb	back to step 2 (30 cycles)
Step 5:	68°C	10 min	
Step 6:	4°C	∞	

PCR products were analyzed on an agarose gel and excised, if used for cloning.

4.2.2.8 Quantitative PCR (qPCR)

Quantitative PCR is used to determine the exact amount of a particular DNA sequence within a sample. There are two different methods to detect the amount of PCR product during the PCR reaction, namely in “real time”: SYBR green is a fluorescent dye that intercalates with any double stranded DNA, whereas TaqMan probes bind specific sequences. These probes are oligonucleotides that are labeled with fluorescent dyes, which only give a signal when the probe is bound to DNA. The cycle number (crossing point, CP) when fluorescence intensity exceeds a certain threshold is correlated with the initial amount of the relevant template DNA.

Detection via SYBR green

Two days after lentiviral transduction genomic DNA from NIH3T3 cells was isolated and eluted in 150 µl water. DNA was analyzed in duplicates by quantitative PCR (Lightcycler FastStart DNA Master^{PLUS} SYBR Green I Kit, Roche) on a Lightcycler Carousel-based system (Roche). Viral integration (forward 5'-TGAAAGCGAAAGGGAAACCA-3' and reverse 5'-CCGTGCGCGCTTCAG-3') was investigated per cell (BDNF forward 5'-ACGACATCACTGGCTGACAC-3' and BDNF reverse 5'-CATAGACATGTTTGCGGCATC-3'). Standard curves were generated with 100-fold serial dilutions of plasmids containing the relevant template DNA (FUGW or pBS-BDNF) and absolute quantification was used to calculate the viral titers.

Reaction composition:

2 µl DNA

0.75 mM primer for (1 mM for Bdnf)

0.2 mM primer rev (1 mM for Bdnf)

1x Master SYBR Green I mix

H₂O ad 20 µl

The following PCR program was used:

Step 1: 95°C 10 min

Step 2: 95°C 10 sec

Step 3: 60°C 5 sec

Step 4: 72°C 10 sec back to step 2 (40 cycles)

Melting curve acquisition was performed as suggested by the manufacturer's protocol.

The final calculation of the virus titer was done using the following formulas:

$$\text{Virus titer (TU/ml)} = \frac{\text{number of NIH3T3 cells} \times \text{number of virus copies per genome}}{\text{volume of virus (ml)}}$$

$$\text{MOI} = \frac{\text{volume of virus (ml)} \times \text{virus titer (TU/ml)}}{\text{number of cells}}$$

Detection with TaqMan probes

Equal amounts of RNA were used for cDNA synthesis with the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). The TaqMan assay was performed with the LightCycler TaqMan Master Kit (Roche) and the Universal ProbeLibrary Set mouse (Roche) according to the manufacturer's instructions on a CFX96 Real Time System (BIO-RAD) using the primers and probes listed in Table 3. Expression levels were normalized to Ubiquitin C or CD11c and relative quantification was calculated using the $\Delta\Delta\text{CT}$ -method (78).

Table 3 Primers and probes for quantitative TaqMan PCR.

Gene	Forward Primer	Reverse Primer	Probe #
UBC	5'-GAGTTCCGTCTGCTGTGTGA-3'	5'-TCACAAAGATCTGCATCGTCA-3'	77
CD11c	5'-CCAGTTGGAGCTTCCAGTAAA-3'	5'-CCTTTTCTGAGGTTGA GAAGTTAAG-3'	46
DC-STAMP	5'-GTATCGGCTCATCTCC TCCA-3'	5'-ACTCCTTGGGTTTCCTTGCTT-3'	11
PPEF2	5'-TTCTGT CACAACCGCA AGG-3'	5'-TCTGTTGCTGCCAACTTCAT-3'	16
PFTK1	5'-ATGGACAAGCACCCCTGGA-3'	5'-CGCAGCAGCTGAAATAAAA-3'	58
OVA	5'-GCTATGGGCATTACTGACGTG-3'	5'-TGCTGAGGAGATGCCAGAC-3'	41

4.2.2.9 Production of chemo-competent bacteria

A single colony of *Escherichia coli* (*E. coli*) Stbl3 (Invitrogen) was inoculated in LB medium without ampicillin and incubated overnight. The next day, 1 ml of this culture was diluted in 99 ml of LB medium containing 10 mM MgCl₂ and incubated at 37°C in a shaker until an OD₆₀₀ of 0.4-0.6 was reached. The bacterial culture was immediately cooled on ice for 10 min, followed by centrifugation at 3000g, 4°C for 25 min. The pellet was resuspended in 40 ml pre-cooled RFI medium and left on ice for 15 min. After centrifugation, the pellet was resuspended in 4 ml RFII medium and incubated on ice for further 15 min. Aliquots of 100 µl were prepared on dry ice and stored at – 80°C.

The *E. coli* Stbl3 strain is specifically designed for cloning of lentiviral vectors. Presence of long terminal repeats (LTRs) makes these vectors unstable, because it results in undesired homologous recombination. The *E. coli* Stbl3 strain is deficient for the relevant recombinase.

4.2.2.10 Transformation of chemo-competent bacteria

Competent bacteria (100 µl) were thawed on ice for 5 min. Plasmid DNA (1 µl intact plasmid, 5 µl ligated plasmid) was added to the cells, carefully mixed and incubated on ice for 30 min. A heat shock was performed at 42 °C for 45 seconds and the vial was placed directly back on ice. LB medium (500 µl) was added and the cells were incubated for 1 h at 37 °C with shaking. The cells were then plated

on pre-warmed LB agar plates containing ampicillin and incubated overnight at 37 °C. For transformation of an intact highly concentrated plasmid 100 µl of bacterial suspension was plated, whereas for a ligated plasmid all cells were used.

4.2.3 Computational promoter analysis

Sequence information of promoter regions was retrieved from *EIDorado* software (Genomatix, Munich, Germany). In this program, a promoter is generally assumed (without further experimental information to integrate) to be located 500bp upstream and 100bp downstream of the transcriptional start site (TSS), but for some analyzes we enlarged the regions further upstream to -1400 or -1900bp. In the figures, the location of TSSs is depicted according to *EIDorado*, which uses information derived from CAGE tags and individual cDNAs. For definition of TFBSs the Matrix family library (Version 8.2, Genomatix) was used. To avoid redundancy, various information about binding sequences is used to calculate a weight matrix. Based on similarities in binding pattern and functionality the matrices are clustered into families (79). The version of the vertebrate library we used contains 727 matrices grouped in 170 families. Using *Frameworker* software (Genomatix) different promoter regions were compared and screened for a set of TFBSs with a defined order and orientation, a so-called “framework”. Default settings were used except for the maximum distance variance between two elements, which was increased from 10 to 20bp (CD11c mouse and human with DC-STAMP mouse) or 30bp (CD11c rat). In addition, the search was sometimes limited to the respective TF families (CD11c rat and DC-STAMP rat). The CD11c/DC-STAMP promoter model was refined by adjusting the distance ranges to the values from the CD11c rat search result. Subsequently, the model was used to perform a mouse promoter database search with default settings (*ModellInspector*, Genomatix).

We also employed another platform, the “immunological genome project” (www.immgen.org, (80)), where microarray data of various primary cell types are compared and gene constellations are calculated.

4.2.4 Statistical analysis

P-values were calculated with Student's t test using PRISM software (GraphPad software, La Jolla, CA, USA) and are defined as: ***: $p < 0.001$, **: $p = 0.001$ to 0.01 , *: $p = 0.01$ to 0.05 . Error bars represent standard deviations (SD). All experiments had a sample number of at least three mice per group, unless otherwise stated.

5 RESULTS

5.1 Transcriptional targeting of antigen to DCs by the DC-STAMP promoter induces tolerance *in vivo*

A fragment of the mouse DC-STAMP promoter (1704bp, -1565bp to +131bp) was analyzed for tissue specificity in order to transcriptionally target DCs. Therefore a lentiviral vector was used, in which the promoter region drives eGFP as reporter gene (Fig. 5a). Using this vector, bone marrow chimeras were generated by transduction of hematopoietic stem cells (HSC) and subsequent injection into lethally irradiated recipients. Analysis of GFP expression by flow cytometry in various cell types demonstrated that the DC-STAMP promoter (pDC-STAMP) fragment was only active in DCs but not in other cell types from spleen, thymus and lymph nodes (Fig. 5b and (76)).

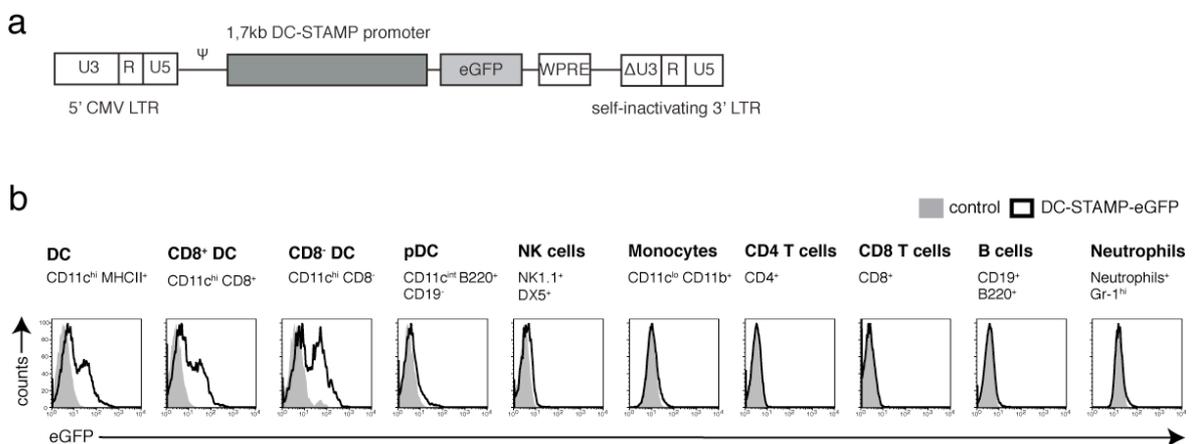


Figure 5 The pDC-STAMP^{1.7kb} drives expression selectively in DCs.

(a) Schematic representation of the lentiviral-based SIN-vector containing a fragment of the mouse DC-STAMP promoter (1704bp) to control expression of eGFP (enhanced Green Fluorescent Protein). CMV, cytomegalovirus; Ψ, packaging signal; SIN, self-inactivating; LTR, long terminal repeat; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; ΔU3, deletion in the U3 region. (b) Promoter activity of the mouse DC-STAMP promoter *in vivo* as measured by flow cytometry. Spleen cells from bone-marrow chimeras generated with HSCs transduced with the DC-STAMP-GFP lentivirus were analyzed for GFP expression. The different cell types were identified based on the expression of the indicated markers. Overlays were generated after gating on the relevant population using cells from C57BL/6 mice as negative controls. Data shown are representative of two independently performed experiments with 4-5 mice per group.

As this strategy represents a novel method to deliver transgenes specifically to DCs *in vivo* without changing their phenotype, it allowed us to address the question, if DCs could induce tolerance in this system. We chose a membrane-bound form of ovalbumin (OVA) as a model antigen, which was expressed under the control of pDC-STAMP (Fig. 6a).

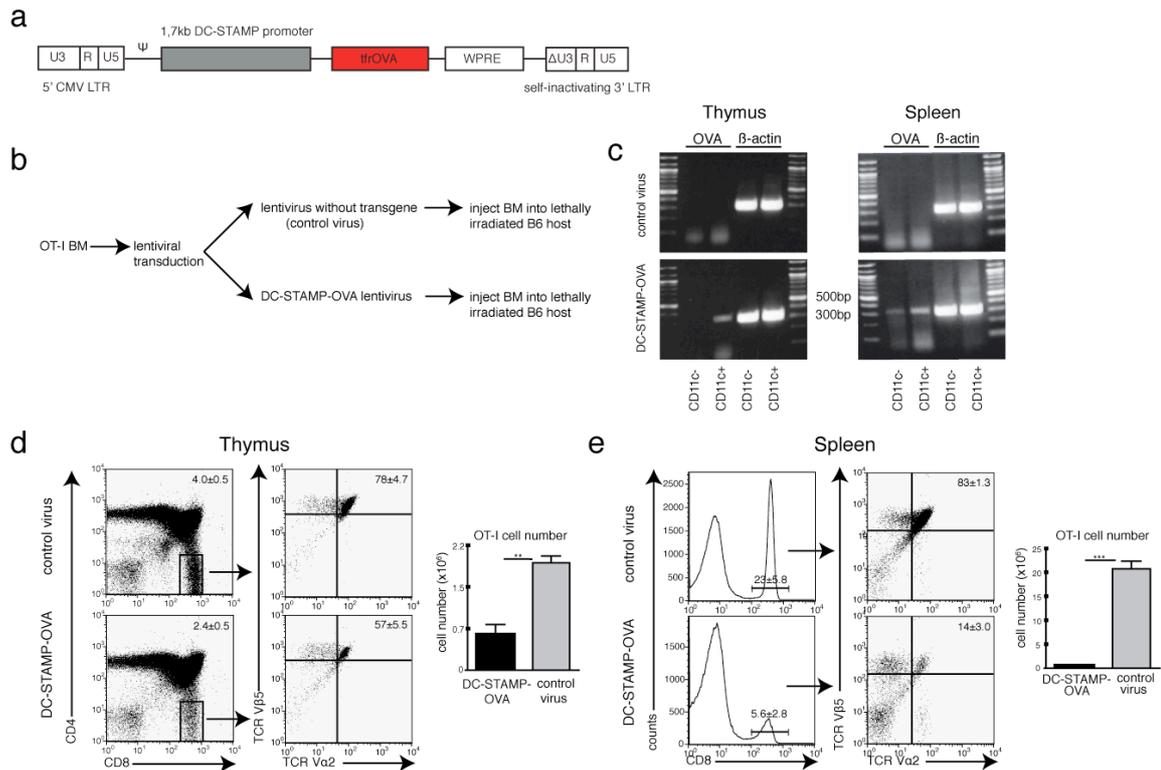


Figure 6 OVA expression in DCs induces central and peripheral tolerance of OT-I T cells.

(a) Schematic representation of the DC-STAMP-OVA lentiviral vector. A fusion of ovalbumin to the transmembrane part of the transferrin receptor (trfOVA) is controlled by the 1.7kb fragment of the mouse DC-STAMP promoter. From here on this vector is called DC-STAMP-OVA. (b) Experimental setup for generation of BM-chimeras. Bone marrow HSCs from OT-I mice were depleted of CD8 T cells and transduced with DC-STAMP-OVA or control lentivirus. Lethally irradiated B6 hosts were reconstituted with the respective HSCs and analyzed after 8 weeks by flow cytometry. (c) Analysis of OVA expression in CD11c-positive and -negative cells from DC-STAMP-OVA and control virus chimeras. From thymus and spleen, cell fractions were isolated by magnetic bead sorting and RNA was prepared. RT-PCR analysis identified a 317bp fragment for trfOVA and a 302bp fragment for β -actin as control. (d) Flow cytometric analysis of thymocytes. OT-I T cells were identified according to expression of CD8, TCRV α 2 and TCRV β 5 as indicated by gates and quadrants. Total numbers of OT-I T cells in the respective chimeras were determined (**, p=0.004). (e) Frequencies of splenic OT-I T cells were determined by flow cytometry. Total numbers of OT-I T cells from spleens of both types of chimeras were compared (***, p=0.0002).

Chimeras were generated using HSCs from OT-I transgenic mice, which express an OVA-specific TCR on CD8 T cells (Fig. 6b). Before transfer, those HSCs were either transduced with DC-STAMP-OVA or control lentivirus. As a result, OT-I T

cell development could be monitored in the absence or presence of OVA-expressing DCs, which were found both in thymus and spleen (Fig. 6c). DC-specific expression of OVA induced central tolerance of OT-I T cells, as seen by reduced frequencies and total numbers of thymic transgenic T cells in comparison to control chimeras (Fig. 6d). In spleen, OT-I T cell numbers were even more drastically reduced, indicating peripheral tolerance had been induced (Fig. 6e). It is important to note that transgenic OVA expression was detected in BM-chimeras even after ten months (data not shown).

Taken together, the DC-STAMP lentivirus allows transcriptional targeting of Ag specifically to DCs. In this system, DC-specific expression of Ag induces central and peripheral tolerance, which is Ag-specific and long lasting. The results described in this section have been recently published (76).

In the course of these experiments we made an interesting observation: a low number of mature OT-I T cells, which is usually present in the bone marrow HSCs of OT-I donor mice, had an inhibiting effect on central deletion of developing OT-I T cells, which was typically induced in DC-STAMP-OVA chimeras. As a consequence, we depleted the HSCs of OT-I donor mice of CD8 T cells before use and decided to further investigate the potential role of mature OT-I T cells in the thymus (see 5.2).

5.2 Peripheral T cells re-enter the thymus and interfere with negative selection *in vivo*

5.2.1 Homeostatically expanding activated CD8 T cells re-enter thymus and pancreas

To analyze the potential functions of thymus homing mature T cells we employed one of the best-studied autoimmune mouse models, the RIP-mOVA mouse, which expresses the membrane bound form of chicken OVA in pancreatic β -cells as well as in thymic mTECs (14). These mice were lethally irradiated and reconstituted with bone marrow (BM) from syngeneic OT-I mice, which either contained or was depleted of mature CD8 OT-I T cells (Fig. 7a). When OT-I T cells were depleted from OT-I-BM (Δ CD8) before reconstitution (Fig. 7a), recipient chimeras showed no signs of diabetes (Fig. 7b). In contrast, chimeras receiving non-depleted BM developed lethal diabetes within 7 days (Fig. 7b), which was caused by OT-I T cells massively infiltrating the pancreas (Fig. 7c, d). In the same mice, we also found substantial numbers of OT-I T cells in the spleen (Fig. 7d, e). Notably, there were more OT-I T cells in the pancreas (Fig. 7h) and those cells displayed a much higher activation status ($CD44^{\text{high}}Ly6C^{\text{high}}$, Fig. 7e) compared to the splenic counterparts. This shows that these highly activated effector cells tend to accumulate in their target organ, the pancreas, where they encounter their cognate antigen OVA.

At day 8 after irradiation the thymus is only starting to be reconstituted and contains very few cells, mainly CD4 CD8 double negative T cells. As expected there were nearly no CD4 or CD8 single positive (SP) T cells in the thymus of Δ CD8 chimeras (Fig. 7f). In the undepleted chimeras, however, half of the cells were CD8 SP T cells and of those the vast majority expressed the OVA-specific transgenic TCR (Fig. 7f). The fact that those thymic OT-I cells express markers of mature thymocytes ($CD24^{\text{lo}}Qa-2^{\text{high}}$, Fig. 7g), which could not have developed in this short period, argues that these cells have re-entered the thymus from the periphery.

To test if this re-migration is Ag-dependent and to characterize those cells in more detail, we transferred CFSE-labeled OT-I T cells together with normal B6 BM into

lethally irradiated B6 mice. The migratory behavior of adoptively transferred T cells was investigated in the absence of antigen 5 or 11 days after transfer (Fig. 8a). Already at day 5 after transfer re-migrated OT-I T cells were found in the thymus, although numbers were relatively low (Fig. 8b).

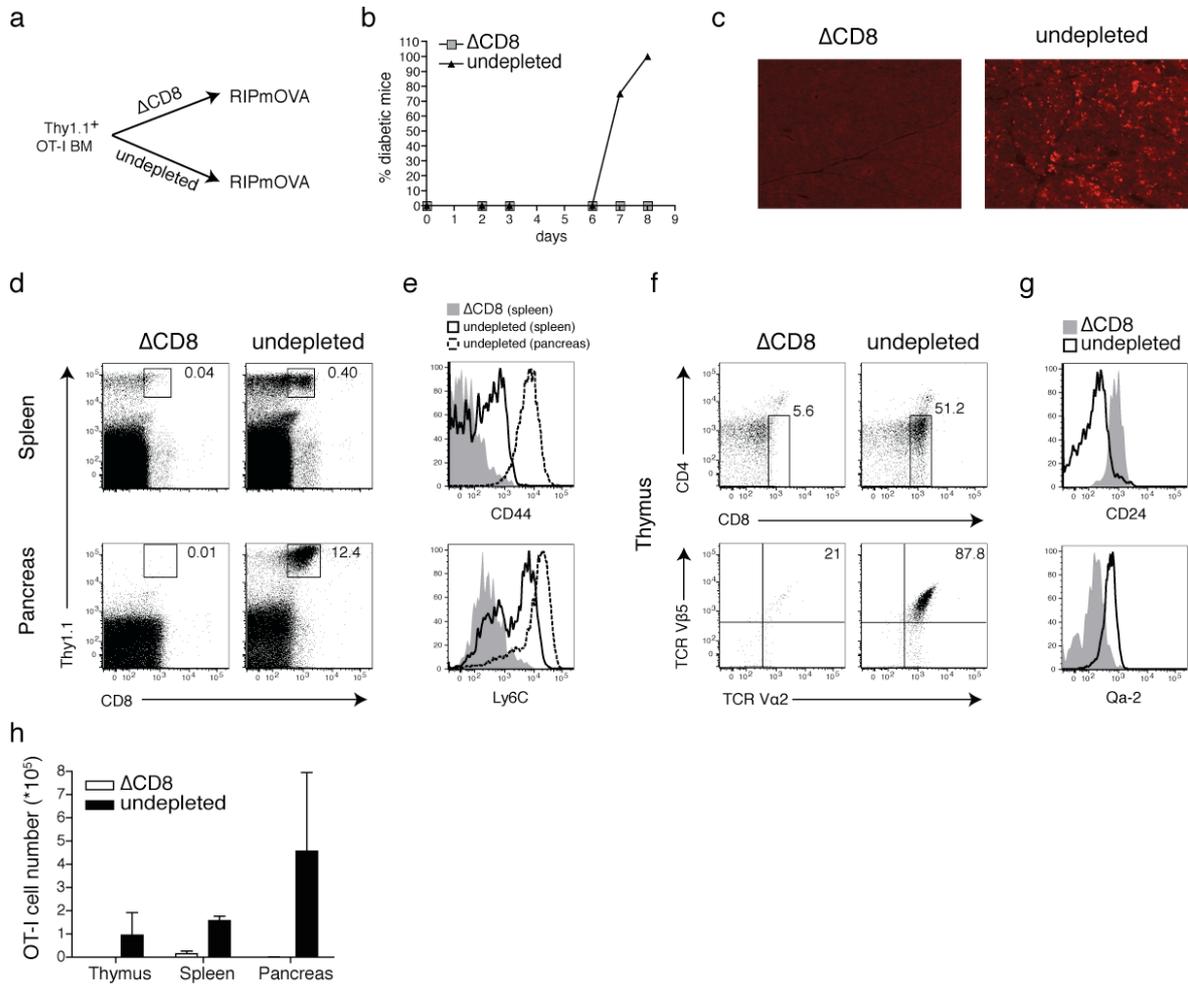


Figure 7 OT-I T cells induce diabetes in lymphopenic RIPmOVA mice.

(a) Thy1.1⁺ OT-I bone marrow (BM) was transferred either undepleted or after depletion of CD8 T cells (ΔCD8) into lethally irradiated RIPmOVA mice. (b) Mice were monitored for onset of diabetes and sacrificed 8 days after BM-transfer. (c) Immunofluorescence staining of pancreatic sections for CD8 (magnification 10x). Frequency (d) and surface phenotype (e) of donor-derived Thy1.1⁺ CD8 T cells in spleen and pancreas. (f) Flow cytometric analysis of Thy1.1⁺ donor-derived cells in the thymus. The frequency of CD8⁺ SP T cells and the percentage of TCR Vα2⁺ and Vβ5.1/5.2⁺ cells among them is indicated. (g) Developmental status of OT-I T cells in the thymus. (h) Total cell numbers of Thy1.1⁺ OT-I T cells in the indicated organs. Data are representative of three independent experiments with similar results (n=3-4 mice per group).

The number of cells re-entering the thymus increased over time, approximately 200-fold from day 5 to 11. The mature OT-I T cells also repopulated other organs, including the spleen, lymph nodes and pancreas. However, there was an obvious

difference in cell quality at day 11: re-migrated cells in the thymus had undergone the most divisions (CFSE^{lo}) and were highly activated (CD62L^{lo}) as compared to cells in the spleen and lymph nodes (Fig. 8c). Pancreatic cells showed an intermediate phenotype. In keeping with a previous report from allogeneic settings (81), our data show that homeostatic proliferation generates T cells that are able to enter the thymus also independently of cognate antigen.

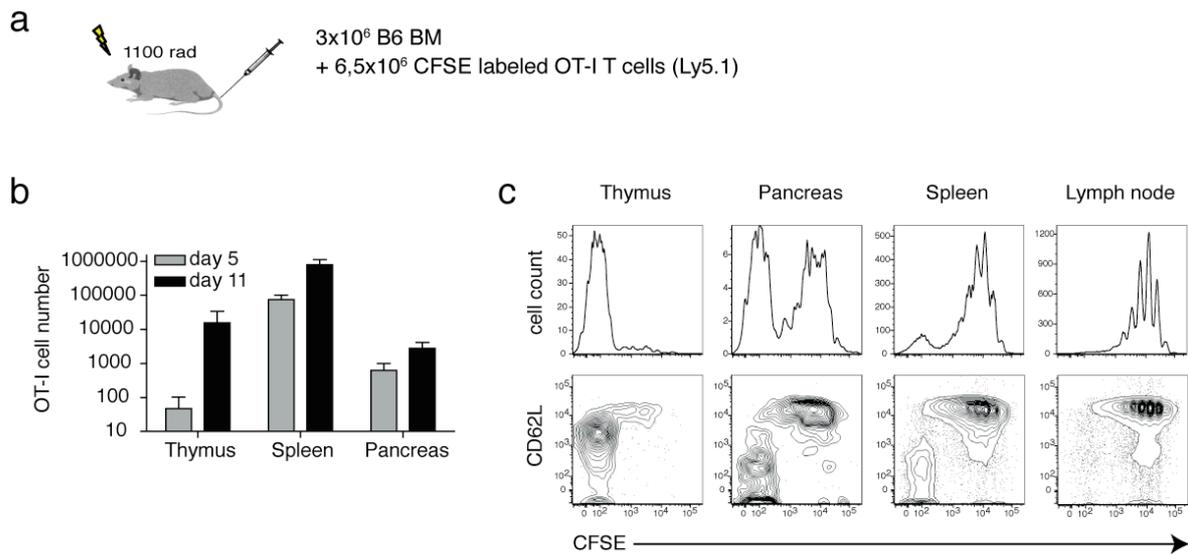


Figure 8 Most activated OT-I T cells can enter the thymus.

(a) Lethally irradiated B6 mice were reconstituted with syngeneic BM (3×10^6) together with CFSE labelled Ly5.1⁺ OT-I T cells (6.5×10^6) and analyzed at day 5 and 11 post transfer. (b) Total cell number (log scale) of adoptively transferred OT-I T cells in thymus, spleen and pancreas at day 5 (grey bar) and day 11 (black bar). (c) CFSE profile and CD62L expression of OT-I T cells in the indicated organs at day 11. OT-I T cells were identified based on the expression of CD8 and the congenic marker (Ly5.1). Data are representative of two independent experiments with similar outcomes (n=3 per group).

5.2.2 Thymus re-entry of peripheral T cells leads to Ag-specific removal of thymic APCs and deficient negative selection

We next wondered if these highly activated re-entered T cells display effector functions within the thymic microenvironment. To explore this we employed the lentiviral transduction system described above (see 5.1). Lentiviral transduction of BM HSCs with virus carrying the DC-STAMP-promoter allows specific transcriptional targeting of transgenes to DCs for induction of central and peripheral tolerance (76). This model offered the possibility to directly address the

question, if mature CD8 T cells from the periphery are able to interfere with thymic deletion of T cells, which recognize a defined antigen (OVA) specifically expressed by DCs.

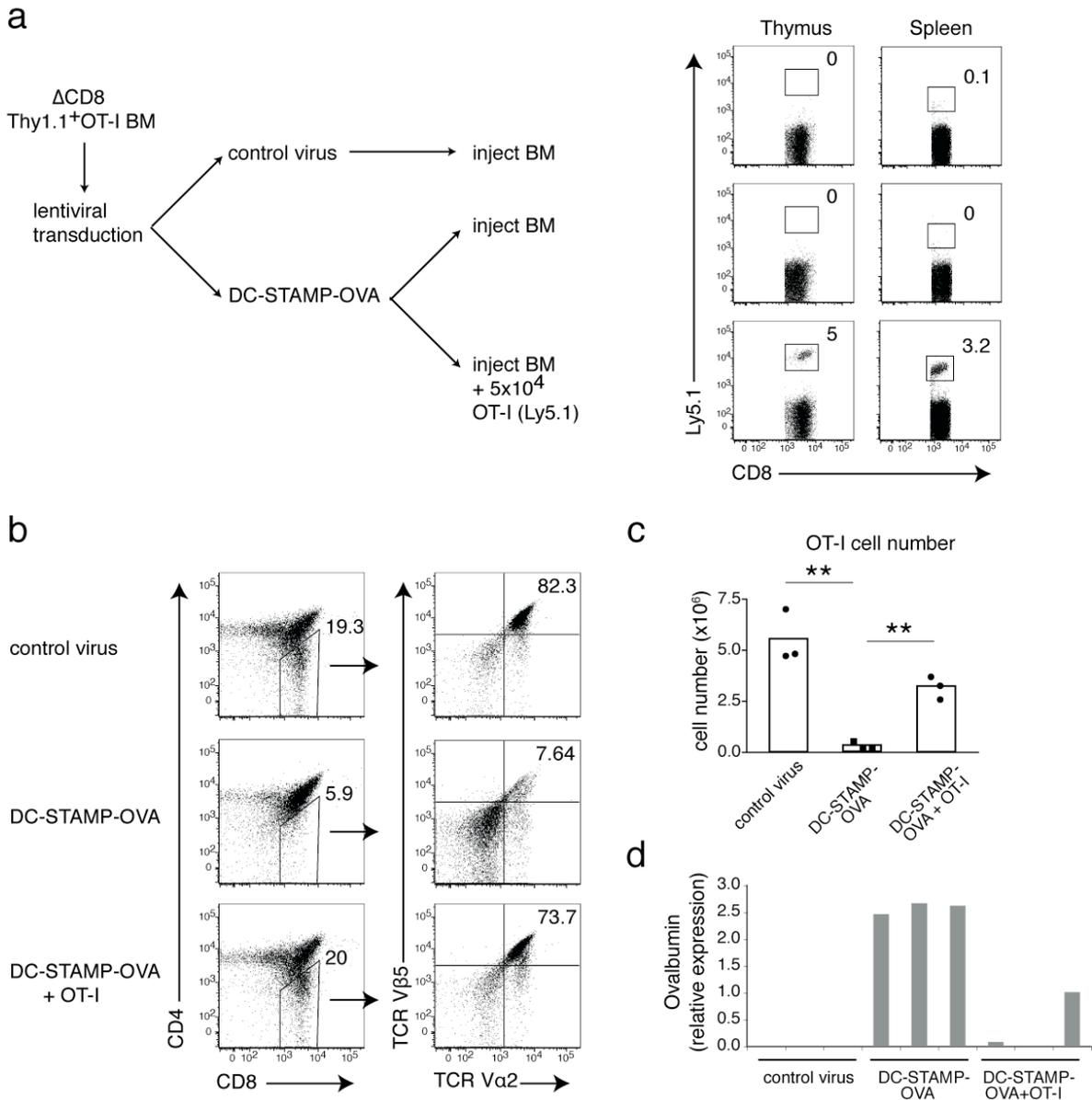


Figure 9 Adoptively transferred OT-I T cells disturb DC-mediated negative selection of BM-derived T cells.

(a) Thy1.1⁺ OT-I BM HSCs were CD8 T cell depleted (Δ CD8) and transduced with control lentivirus or lentivirus encoding ovalbumin (OVA) under control of the DC-STAMP promoter (DC-STAMP-OVA). The DC-STAMP-OVA lentivirus treated BM was injected into irradiated B6 mice either alone or together with 5×10^4 Ly5.1⁺ OT-I T cells. The presence of these cells is demonstrated in representative flow cytometric analyses of thymus and spleen (right) after gating on CD8 T cells. (b) Flow cytometric analysis of thymocytes after gating on donor specific (Thy1.1) cells (not shown). Expression of OT-I TCR V α 2 and V β 5.1/5.2 on CD8 SP cells identifies them as BM-derived OT-I T cells. (c) Total cell number of BM-derived OT-I T cells in the thymus. (d) RNA from thymic cells was analyzed by quantitative PCR. In this bar graph, one bar represents one individual mouse and expression of OVA is shown relative to CD11c. Results are representative of three independent experiments with 3-4 mice per group.

We generated bone marrow chimeras using CD8 T cell depleted BM from Thy1.1⁺ OT-I donors, which was transduced either with DC-STAMP-OVA or control lentivirus and injected into lethally irradiated hosts. The DC-STAMP-OVA treated BM was used either alone or mixed with low numbers of Ly5.1⁺ OT-I T cells isolated from lymph nodes, representing mature CD8 T cells (5×10^4 , Fig. 9a). As such, their influence on negative selection could be investigated. The use of different congenic markers allowed tracking of the origin of OT-I T cells. We monitored the development of Thy1.1⁺ BM-derived “endogenous” OT-I T cells in thymi without OVA (control virus) or in the presence of their cognate antigen OVA on thymic DCs (DC-STAMP-OVA lentivirus, Fig. 9a). In the third group (DC-STAMP-OVA + Ly5.1⁺OT-I), thymic DCs expressing OVA could be potential targets for activated re-entering Ly5.1⁺ OT-I T cells. If back-migrated T cells indeed function to kill thymic OVA⁺ DCs, negative selection should be obstructed, thereby allowing endogenous Thy1.1⁺ OT-I T cells to develop. As expected, adoptively transferred Ly5.1⁺ OT-I T cells homeostatically expanded and were found in the thymus and spleen of chimeric mice (Fig. 9a, right). While the endogenous BM-derived Thy1.1⁺ OT-I T cells developed normally in chimeras generated with control lentivirus treated BM (i.e. in the absence of OVA-expressing DCs), their frequencies (Fig. 9b) and total numbers (Fig. 9c) were diminished to background levels in DC-STAMP-OVA chimeras due to negative selection, as previously published (76).

In contrast, the presence of re-migrated OT-I T cells in DC-STAMP-OVA chimeras strongly interfered with negative selection, as observed by the development of the endogenous Thy1.1⁺ donor-derived OT-I T cell population at near normal frequencies (Fig. 9b, c). When OT-I T cells were transferred into control chimeras (Fig. 10a), they re-migrated to the thymus, but did not influence the development of endogenous OT-I thymocytes (Fig. 10b). This control experiment excludes the possibility that the mere presence of mature OT-I T cells boosts the development of endogenous OT-I T cells.

In order to investigate, if OT-I T cell development was due to a lack of OVA expression in the thymus, we performed qPCR on thymic cells from the different groups. This analysis revealed that re-migrating Ly5.1⁺ OT-I T cells caused disappearance of OVA mRNA-expression, while OVA remained detectable in thymi of DC-STAMP-OVA treated chimeras (Fig. 9d). As we have previously

shown in DC-STAMP-OVA chimeras that thymic expression of OVA is confined to DCs (76), our data suggest that mature T cells expand homeostatically in lethally irradiated hosts and enter the thymus, where they eliminate thymic DCs. This allows endogenous thymocytes to develop in the absence of negative selection.

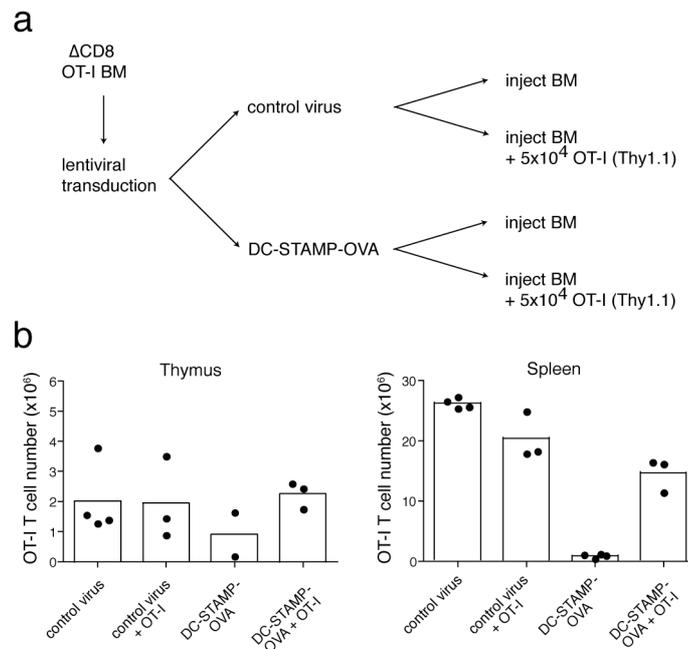


Figure 10 Adoptively transferred OT-I T cells have no influence on the development of BM-derived OT-I cells in control chimeras

(a) OT-I BM HSCs were CD8 T cell depleted and transduced with DC-STAMP-OVA or control lentivirus. In both groups BM was either injected alone or together with 5×10^4 mature OT-I T cells (Thy1.1) into lethally irradiated B6 recipients. (b) Total cell number of BM-derived OT-I T cells in thymus (left) and spleen (right) is displayed.

As we have demonstrated before (Fig. 6e and (76)) that OVA expression in DCs leads to peripheral tolerance, we also analyzed splenocytes from the same mice as in Figure 9. In control chimeras, OT-I T cells were present at normal frequency (Fig. 11a) and numbers (Fig. 11b), but in DC-STAMP-OVA chimeras those few OT-I T cells that left the thymus were even more reduced in the periphery.

This residual cell population was characterized by surface markers associated with chronic infection or constant presence of antigen (82): upregulation of PD-1 and downregulation of Ly6C (Fig. 11c). However, in the presence of adoptively transferred OT-I T cells (Ly5.1), endogenous OT-I T cells remained in the periphery, although not reaching normal levels (Fig. 11a, b), and displayed marker expression of naive T cells (Fig. 11c). QPCR analysis of splenocytes showed

reduced OVA expression if mature OT-I T cells had been adoptively transferred (Fig. 11d). These findings demonstrate that CD8 T cells can eliminate DCs in an Ag-dependent manner not only in lymph nodes (34) and tissues (33) but also in the spleen.

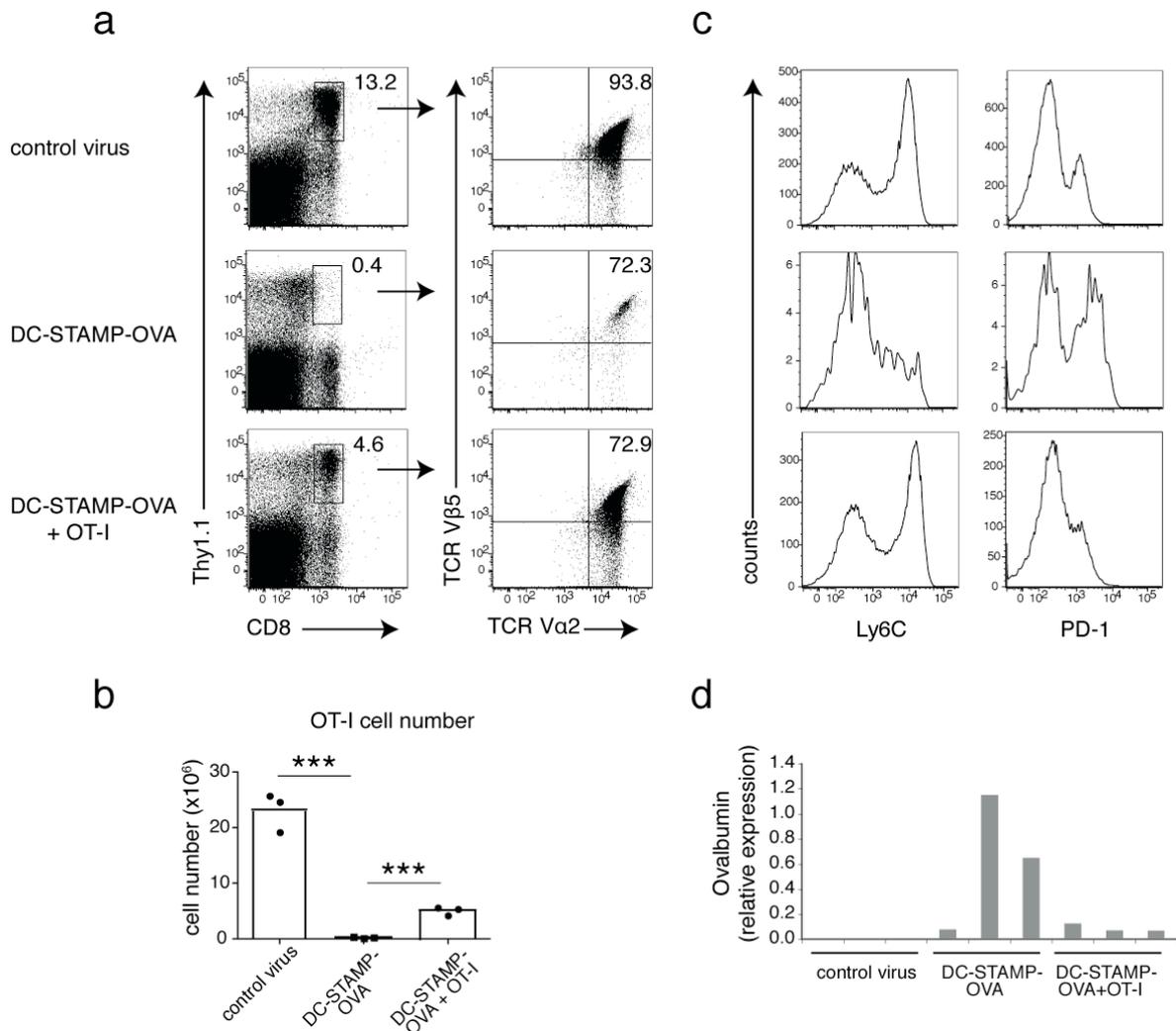


Figure 11 Adoptively transferred OT-I T cells disturb DC-mediated peripheral tolerance of BM-derived T cells.

Flow cytometric analysis of splenocytes from the same experiment as in Figure 9 (for experimental setup see Fig. 9a). Frequency (a) and total cell number (b) of BM-derived (Thy1.1) OT-I T cells in spleen. (c) Analysis of activation markers on BM-derived OT-I T cells from spleen. (d) Expression of OVA relative to CD11c in splenocytes. In this bar graph, one bar represents one individual mouse. Results are representative of one out of three independent experiments with 3-4 mice per group.

As we have shown (76) that antigen expression in DCs also results in thymic depletion of CD4 T cells, we wanted to know if peripheral OT-I T cells would similarly interfere with negative selection of OT-II cells. OT-II BM was transduced

with control virus or DC-STAMP-OVA virus, which was either injected alone or together with mature OT-I T cells (Fig. 12a). In addition, we wanted to test whether the number of OT-I -“contaminants” could be decreased. Therefore, we transferred instead of 5×10^4 either 1×10^4 or 1×10^3 cells (Fig. 12a). OT-I T cells were indeed able to interfere with negative selection of OT-II cells. As few as 1×10^3 OT-I contaminants were sufficient to inhibit negative selection, allowing normal percentages of OT-II cells to develop (Fig. 12b, left). Moreover, also in the spleen equal percentages of OT-II cells were present in comparison to control chimeras (Fig. 12b, right). This indicates that also in this setting, regarding CD4 T cells, removal of Ag-expressing DCs in thymus and spleen by adoptively transferred OT-I T cells interferes with central and peripheral tolerance.

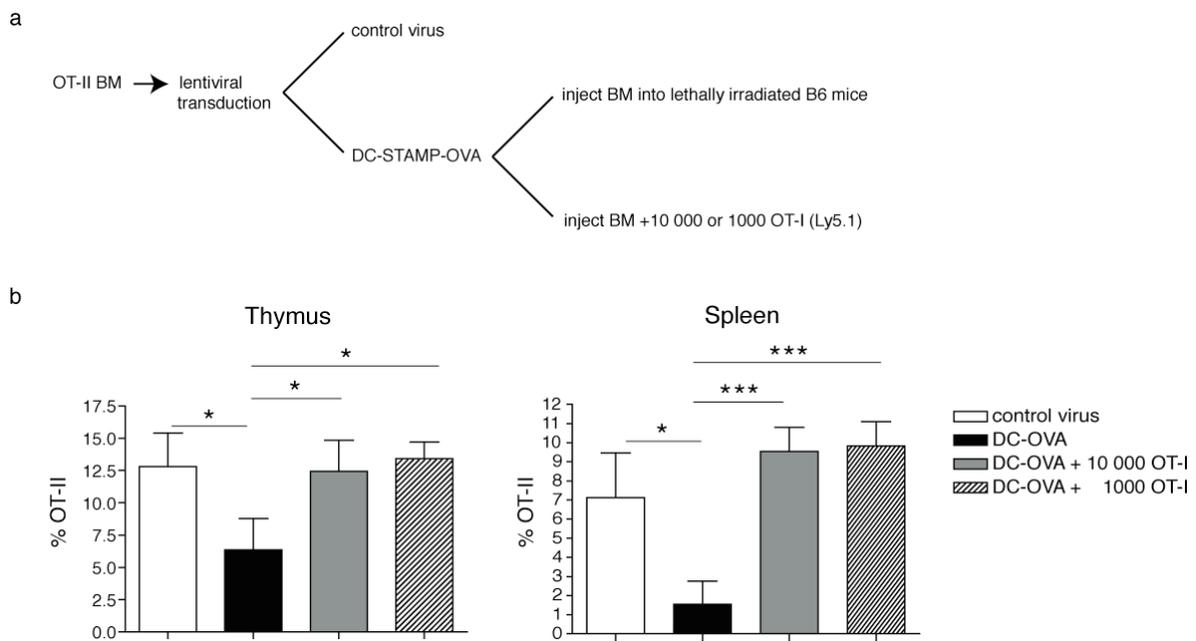


Figure 12 Adoptively transferred OT-I T cells disturb DC-mediated negative selection of OT-II T cells.

(a) OT-II BM HSCs were transduced with control lentivirus or DC-STAMP-OVA lentivirus. The DC-STAMP-OVA lentivirus treated BM was injected into irradiated B6 mice either alone or together with 1×10^4 or 1×10^3 Ly5.1⁺ OT-I T cells. (b) Percentage of OT-II T cells in thymus (left) and spleen (right). Data represent two independent experiments with 2-4 mice per group (*: $p < 0.05$ and ***: $p < 0.001$).

In addition to thymic DCs, mTECs are another key mediator of central tolerance induction. These cells efficiently express and present self-Ags, and might serve as an Ag-source for thymic DCs (83). In order to study whether mTECs could also be targets for mature re-entered thymic CD8 T cells, we employed RIP-mOVA mice,

in which the self-Ag OVA is expressed selectively in thymic mTECs (14). Following lethal irradiation of RIP-mOVA hosts, we adoptively transferred Thy1.1⁺ΔCD8-OT-I BM either alone or together with Ly5.1⁺OT-I T cells (Fig. 13a).

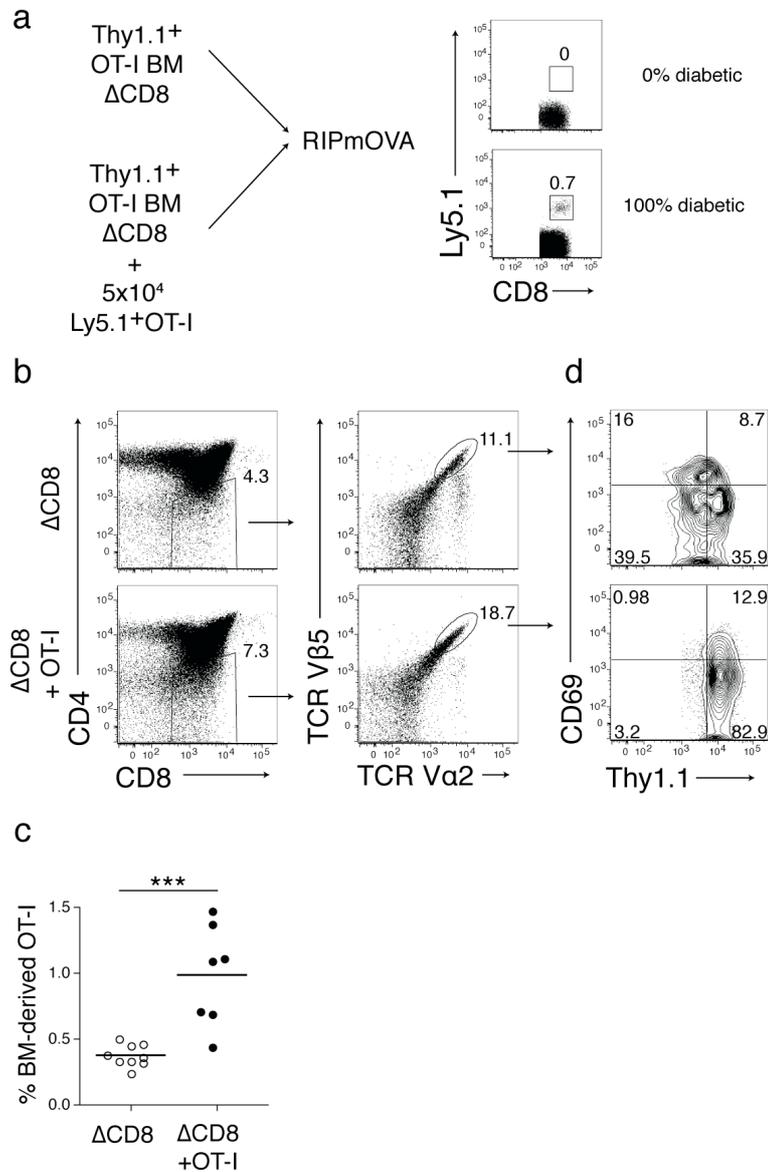


Figure 13 Adoptively transferred OT-I T cells disturb mTEC-mediated negative selection of BM-derived T cells.

(a) Thy1.1⁺ OT-I BM was depleted of CD8 T cells (ΔCD8) and injected into lethally irradiated RIPmOVA mice either alone or together with 5x10⁴ Ly5.1⁺ OT-I T cells (ΔCD8+OT-I). Chimeras were analyzed at day 22 after transfer. Representative flow cytometric analysis of thymocytes after gating on CD8 SP thymocytes is shown (right). (b) Thymocytes were analyzed for CD4 and CD8 expression by flow cytometry after gating on donor cells (Thy1.1). The frequency of OT-I TCR Vα2 and Vβ5.1/5.2 positive cells was determined. (c) Percentage of BM-derived OT-I T cells in the thymus of ΔCD8→RIPmOVA (n=9) and ΔCD8+OT-I→RIPmOVA chimeras (n=7) were compared (***, p=0.0003). (D) Frequency of CD69^{hi} and Thy1.1^{lo} cells in the BM-derived OT-I population. Data are representative of two independent experiments with 4-9 mice per group.

This experiment is analogous to Figure 7, but in this case the OT-I contaminants were isolated separately and carried a congenic marker. As described before (Fig. 7), the presence of mature OT-I cells in irradiated RIPmOVA mice resulted in the development of severe diabetes within one week. As this is too early to investigate thymic T cell development, we had to treat the diabetic mice with insulin, which enabled us to look at later time points (day 22).

As previously shown, mTECs of RIP-mOVA mice express transgenic OVA (14) and therefore induce efficient negative selection. Consistently, only low numbers of BM-derived Thy1.1⁺CD8⁺ SP T cells expressing the transgenic OT-I TCR V α 2V β 5 combination could be found in mice reconstituted with Δ CD8-BM (Fig. 13b, c). These cells were CD69^{high}Thy1.1^{lo} (Fig. 13d), showing a phenotype of recent activation, probably by Ag-recognition (84, 85). In contrast, development of endogenous Thy1.1⁺OT-I T cells was much more efficient in RIP-mOVA hosts that had also received mature peripheral Ly5.1⁺OT-I T cells (Fig. 13b, c). These BM-derived OT-I T cells had a naive surface phenotype (CD69^{lo}Thy1.1^{high}) (Fig. 13d), suggesting development in absence of Ag. These results demonstrate that not only DCs, but also mTECs, are targets for re-entering CD8 T cells.

5.2.3 Re-entered mature T cells induce development of self-reactive endogenous T cells

We next wanted to monitor if the observed inhibition of thymic negative selection is restricted to the development of transgenic T cells or would also occur in normal mice with polyclonal T cell repertoires. To this end we generated C57BL/6 chimeras using C57BL/6-BM transduced with control or DC-STAMP-OVA lentivirus. The DC-STAMP-OVA treated BM was injected either alone or together with low numbers of Thy1.1⁺ OT-I T cells (Fig. 14a). As in this system the frequencies of Ag-specific T cells are too low to detect, thymic development could not be monitored. Therefore, we had to use an indirect approach, in which peripheral Ag-specific T cells were expanded by immunization and subsequently analyzed for functionality. Before immunization we removed the adoptively transferred Thy1.1⁺ OT-I T cells by injection of a Thy1.1-specific depleting antibody to avoid interference with the expansion of polyclonal T cells (Fig. 14b).

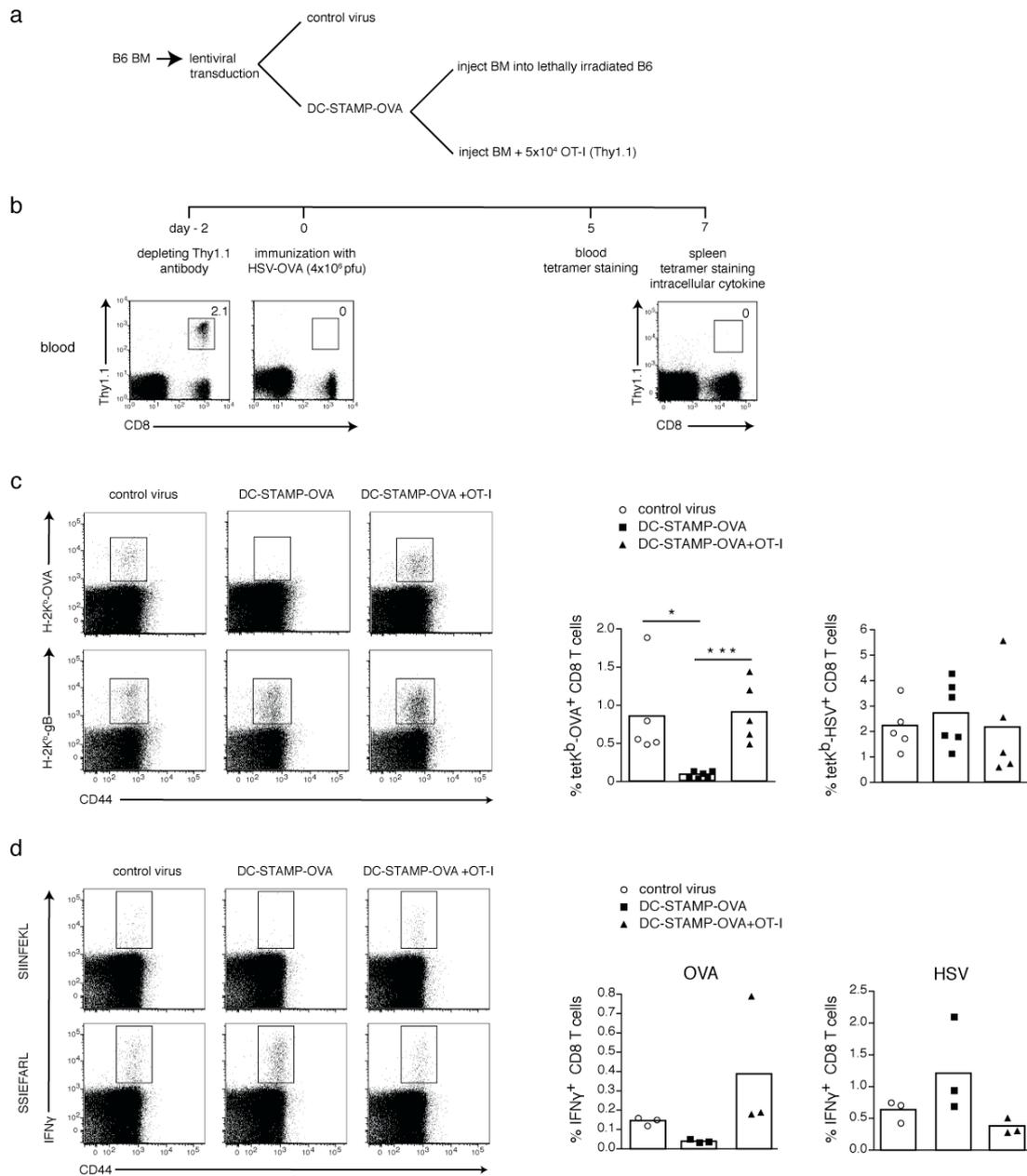


Figure 14 **Adoptively transferred OT-I T cells disturb negative selection of a polyclonal CD8 T cell repertoire.**

(a) B6 BM HSCs were transduced with control lentivirus or DC-STAMP-OVA lentivirus. The DC-STAMP-OVA lentivirus treated BM was injected into lethally irradiated B6 recipients either alone or together with 5×10^4 Thy1.1⁺ OT-I T cells. (b) After at least 8 weeks, the adoptively transferred OT-I T cells were removed by treatment with a depleting Thy1.1 antibody two days before immunization and remained undetectable throughout the course of the experiment. Shown are representative stainings of blood samples at the indicated time points. (c) Percentage of tetramer positive cells (H-2K^b-OVA and H-2K^b-gB) in the splenic CD8 T cell population seven days after immunization. Representative dot blots are shown and data from two independently performed experiments ($n=2-3$ per group) are pooled and statistically analyzed. (d) Spleen suspensions were stimulated *in vitro* with the OVA-peptide SIINFEKL or the gB-peptide SSIEFARL and analyzed after 5 h for surface expression of CD44 and production of IFN γ by intracellular staining. Representative flow cytometric data are shown after gating on the CD8 positive population. From one experiment, percentages of IFN γ producing cells among CD8 T cells are displayed ($n=3$ per group).

We then immunized mice with replication-deficient Herpes simplex virus (HSV) expressing recombinant OVA and monitored the induced CD8 T cell responses (Fig. 14c).

As expected, all chimeras mounted efficient polyclonal CD8 T cell responses against the unrelated MHC class I K^b-restricted HSV-gB epitope SSIEFARL (Fig. 14c, lower row). In contrast, while control chimeras also showed CD8 T cell responses to the K^b-restricted OVA-epitope SIINFEKL, DC-STAMP-OVA chimeras did not, as their T cell repertoire was devoid of OVA-specific cells due to lentivirus-mediated OVA-expression in thymic DCs leading to central tolerance (Fig. 14c, upper row; (76)). However, when adoptively transferred OT-I T cells were present and acted to remove OVA-expressing thymic DCs in DC-STAMP-OVA chimeras, OVA-specific peripheral CD8 T cells were again detectable in peripheral organs by MHC-tetramers (Fig. 14c), which also showed effector functions by production of IFN γ (Fig. 14d).

Taken together, these data indicate that the presence of mature CD8 T cells in a lymphopenic host disturbed common tolerance mechanisms and thereby induced the development of functional self-reactive CD8 T cells. As such, we could add another important function for re-entering T cells to the already proposed ones (61).

5.3 *In vivo* analysis of the mouse CD11c promoter: Identification of a Dendritic Cell-specific enhancer

5.3.1 Comparative promoter analysis of CD11c and DC-STAMP promoters across species identified conserved promoter structures

The 5.5kb region upstream of the transcriptional start site of the mouse CD11c gene (86) and the 1.7kb fragment of the mouse DC-STAMP promoter (Fig. 5 and (76)) were successfully used to drive DC-specific gene expression. In addition, it has been shown that DC-STAMP parallels the expression of CD11c (87). To investigate if these two promoters share common features regarding transcriptional regulation, we decided to compare the two promoters across species using computational analysis.

CD11c is an integrin αX , which forms a heterodimer with the integrin $\beta 2$ -chain (CD18). This complex is known to bind LPS, fibrinogen and the complement component iC3b and is therefore also called complement receptor 4 (CR4). It mediates cell adhesion by binding several adhesion molecules (ICAM-1, ICAM-2, VCAM-1). Considering these binding capacities, two roles have been proposed that can be functionally linked to DCs: antigen uptake and T cell activation (88, 89).

The dendritic cell-specific transmembrane protein (DC-STAMP) is preferentially expressed in DCs and localizes to the ER (87). It has been implicated in the differentiation of myeloid cells (90), the negative regulation of DC function (91) and also plays a role in osteoclast fusion (92). The currently known interaction partners for DC-STAMP are the ER-resident proteins OS9 (93) and LUMAN (94), a transcription factor. In mature DCs, DC-STAMP is involved in the translocation of LUMAN to the Golgi and thereby enables its activation in a process called regulated intramembrane proteolysis (RIP).

Comparative promoter analysis was used to identify transcription factor binding sites (TFBSs) conserved in order and distance, referred to as a promoter “framework”. In the first step, 1500bp (-1400/+100bp referring to the transcriptional start site) of the mouse and human CD11c promoters and 2000bp (-1900/+100bp) of the mouse DC-STAMP promoter were compared using the analysis tool *FrameWorker* (Genomatix GmbH).

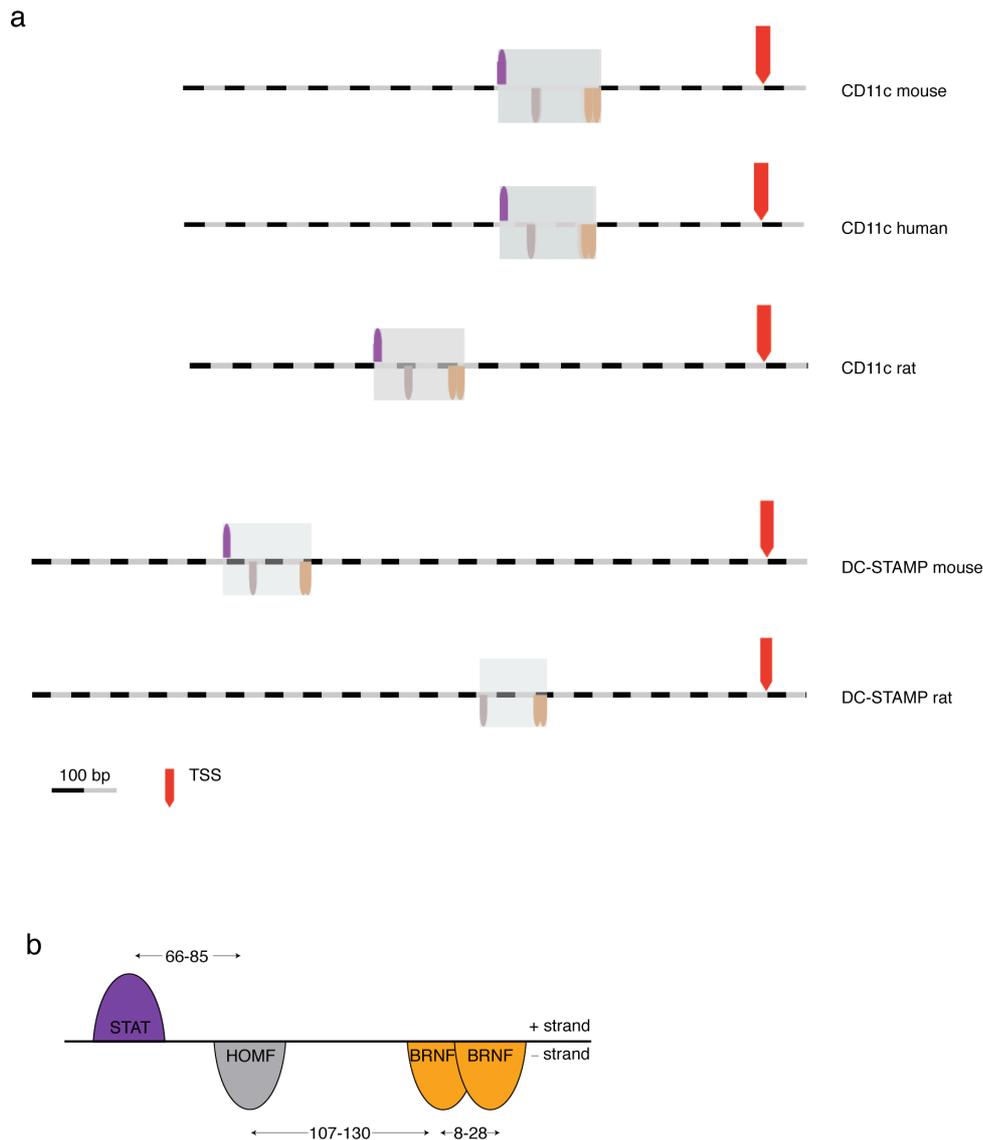


Figure 15 CD11c and DC-STAMP share a common promoter framework.

(a) Identification of a regulatory framework by computational comparison of the CD11c promoter with the DC-STAMP promoter across species. TSS, transcription start site. (b) Detailed representation of the CD11c/DC-STAMP promoter framework, which contains four elements over a length of 250bp. The transcription factor families and their respective orientation on the + and - strand are displayed. The indicated distance ranges result from the refinement of the framework in the rat CD11c promoter. STAT (signal transducer and activator of transcription), HOMF (Homeodomain transcription factors), BRNF (Brn POU domain factors).

As a result, two similar frameworks comprising four TFBSs were identified. The two frameworks differed only on position three and the remaining elements were identical. In the next step, we screened the orthologous promoters in the rat. As only one of the two frameworks was partly found in the rat DC-STAMP promoter (Fig. 15), we decided to focus on this framework, shown in Figure 15a, for verification studies (alternative framework see Fig. 25, APPENDIX).

The framework comprises 250bp and contains four TFBSs for the following transcription factor families: STAT (signal transducer and activator of transcription) binds the + strand, whereas HDMF (Homeodomain transcription factors) and two members of the BRNF (Brn POU domain factors) family bind the – strand (Fig. 15b).

Importantly, the regulatory region identified corresponds to one of the few regions conserved between mouse and human CD11c (data not shown, *DiAlignTF*, Genomatix) and comprises those nucleotides, which have been proposed to be a transcriptional enhancer in the human CD11c promoter (95). In addition, the fragment of the mouse DC-STAMP promoter (-1565/+131bp) that we previously used to drive gene expression specifically in DCs (Fig. 5 and (76)) also contains the transcriptional framework described.

5.3.2 Method for *in vivo* promoter analysis

In order to experimentally validate this hypothetical promoter framework we decided to analyze whether this region plays a role in the transcriptional activity of the mouse CD11c promoter. In contrast to the usual approach for promoter analysis, which applies cell lines as an artificial system, we decided to analyze the promoter in a more physiological setting and tested it directly *in vivo*. To this end, we cloned different fragments of the CD11c promoter (pCD11c) region into self-inactivating lentivirus upstream of GFP as a reporter gene (Fig. 16a). The resulting viruses were used to genetically modify HSCs of C57BL/6 mice, which were subsequently injected into irradiated recipient mice. In these chimeras, promoter activity was examined in various organs and primary cell types upon reconstitution of the hematopoietic system. This method offers an important advantage: promoter activity can be measured in steady state cells, as they are not manipulated directly- a problematic issue, if working with DCs.

In a first attempt to generate a smaller but still functional promoter fragment, we examined the activity of a pCD11c^{1500bp} fragment. Different lymphoid organs were analyzed by flow cytometry for transgene expression. GFP expression was detected preferentially in DC populations from the spleen, thymus and lymph nodes (Fig. 16b).

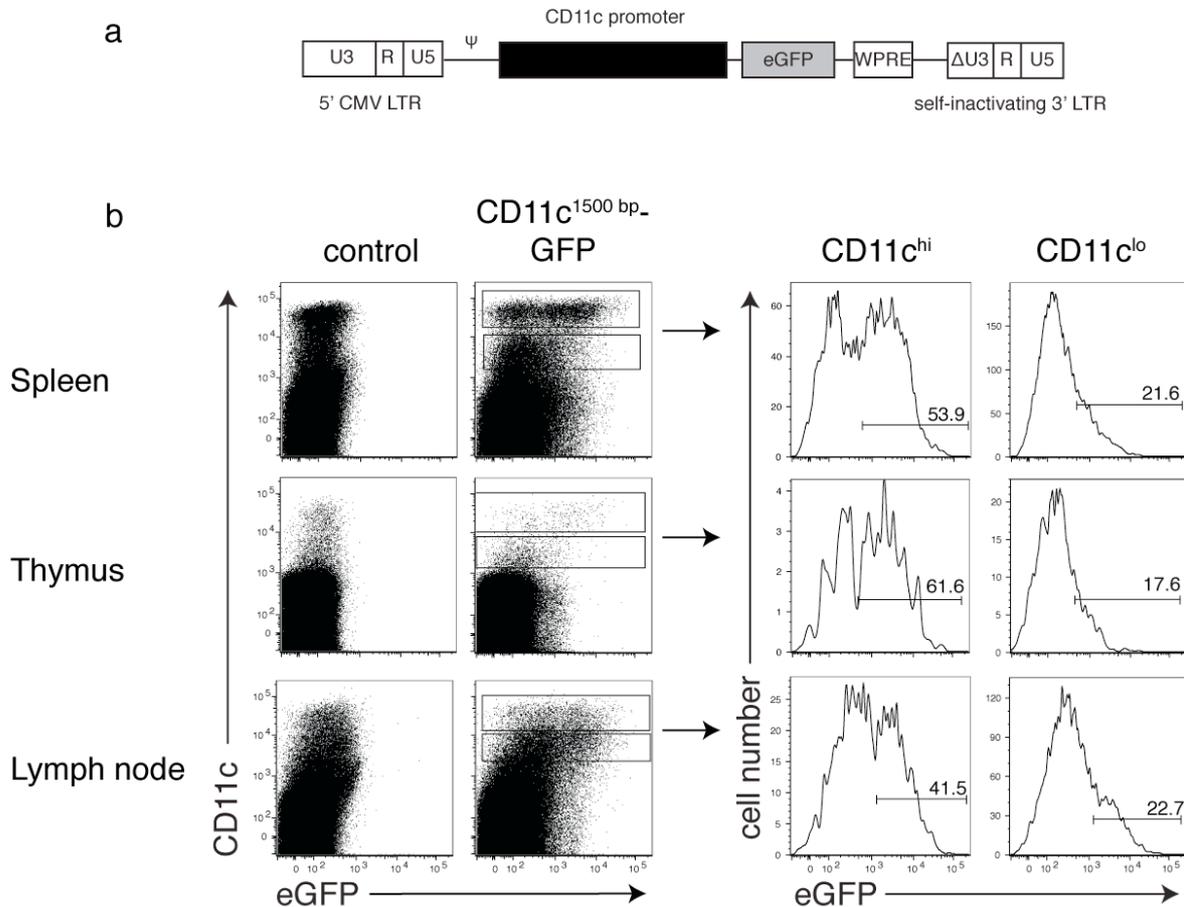


Figure 16 The pCD11c^{1500bp} drives expression selectively in DCs.

(a) Schematic representation of the lentiviral-based SIN-vector containing a fragment of the mouse CD11c promoter to control expression of eGFP (enhanced Green Fluorescent Protein). CMV, cytomegalovirus; Ψ, packaging signal; LTR, long terminal repeat; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; ΔU3, deletion in the U3 region. (b) Analysis of the CD11c^{1500bp} promoter activity in different organs. HSCs from C57BL/6 mice were modified by transduction with a lentiviral vector presented in (a) and injected into lethally irradiated C57BL/6 recipient mice. After 8 weeks, single cell suspensions of spleen, thymus and lymph nodes (mandibular, axillary and subiliac) from these chimeras were stained for CD11c and MHC-II and GFP expression was analyzed by flow cytometry. For detailed quantification, we gated on CD11c^{hi} and CD11c^{lo} cells and histograms were displayed. The data shown are representative of two independently performed experiments with 4-5 mice per group.

Low viral titers (MOI between 0.2 and 0.5) were used for transduction of HSCs in order to avoid multiple insertions per cell. This explains the occurrence of CD11c

positive, but GFP negative cells. The levels of GFP corresponded to the expression of CD11c, as CD11c^{lo} cells also showed less GFP expression than CD11c^{hi} cells (21.6% compared to 53.9% in spleen, respectively). This suggests that the artificial CD11c promoter fragment used contains important regulatory regions allowing similar expression like the CD11c promoter in its normal genomic context.

5.3.3 Sequential deletion analysis of the mouse CD11c promoter and evaluation *in vivo*

To further characterize the CD11c promoter region and verify the CD11c/DC-STAMP promoter model, the 5' end of the promoter was sequentially truncated and the resulting constructs (Fig. 17a) were analyzed in detail for their potential to drive gene expression in various cell types of the immune system (for gating strategy see Fig. 26, APPENDIX). Two different analysis methods were used: histogram overlays illustrate the results in the best way, but lack the possibility of statistical analysis (Fig. 17b). As an alternative, the mean values of percentage and intensity of gene expression were determined (Fig. 18).

The CD11c promoter constructs between 2000 and 750bp were found to be comparable in their gene expression profiles: GFP expression could be observed in all DC populations. The CD8 positive DC subset produced the highest levels of the transgene in comparison to CD8 negative DCs or plasmacytoid DCs (pDCs). The latter were lowest for GFP, but this is in accordance with their intermediate expression of CD11c. In contrast, all other cell types tested expressed only negligible amounts of GFP. In T and B cells expression was absent and very low promoter activity was detected in monocytes, neutrophils and NK cells (Fig. 18).

Strikingly, expression in conventional DCs was lost completely in the pCD11c^{400bp} fragment, whereas some residual activity in pDCs remained. These data identify a region of 350bp, located between 750 and 400bp and containing the CD11c/DC-STAMP-model, as core promoter region driving CD11c gene transcription. The fact that gene expression is abrogated without the described region experimentally validates the importance of the CD11c/DC-STAMP-model, which had been found by computational analysis.

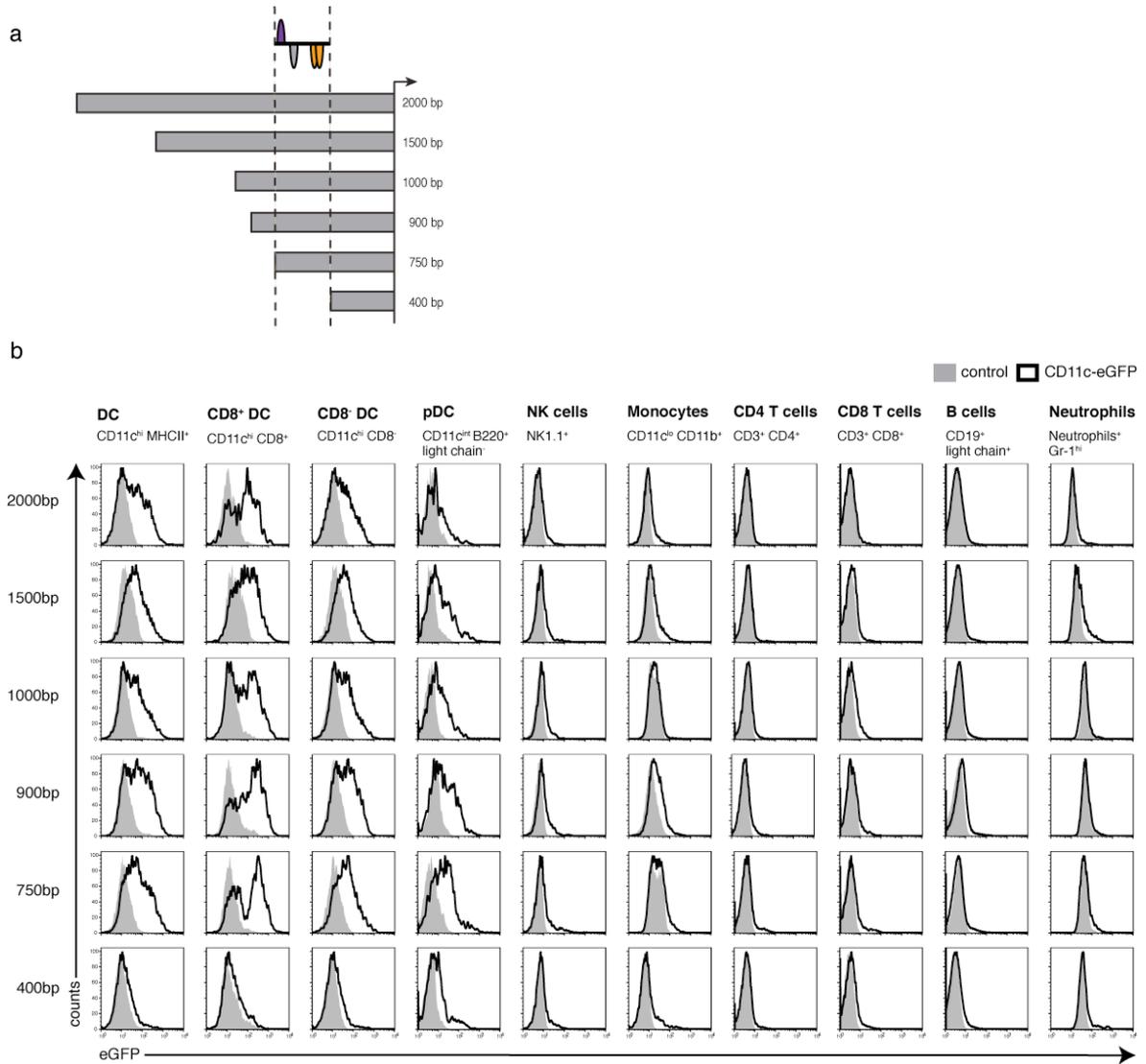


Figure 17 Sequential deletion analysis of the CD11c promoter *in vivo*.

(a) Schematic illustration of pCD11c deletion constructs with the respective localization of the CD11c/DC-STAMP promoter model. (b) Promoter activity of different sizes of the mouse CD11c promoter measured by flow cytometry. Spleen cells from bone-marrow chimeras generated with HSCs transduced with lentiviral constructs carrying the indicated pCD11c-segment were analyzed for GFP expression. The different cell types were identified based on the expression of the indicated markers. Overlays were generated after gating on the relevant population using cells from C57BL/6 mice as negative controls. For every promoter construct, the data shown are representative of two independently performed experiments with 4-5 mice per group.

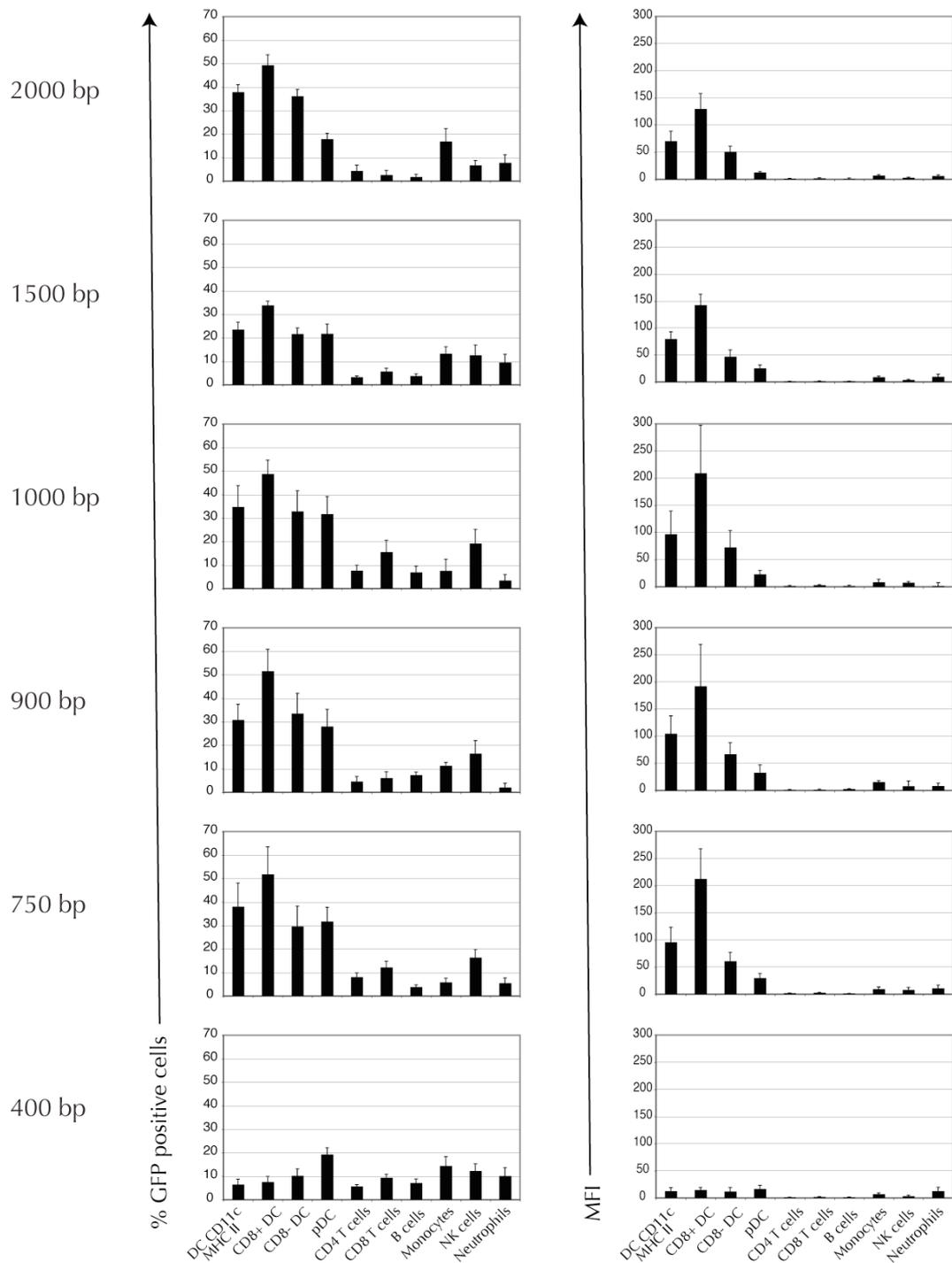


Figure 18 Statistical analysis of *in vivo* promoter activity.

Various cell types from bone marrow chimeras generated with different fragments of the mouse CD11c promoter driving GFP expression were analyzed. Percentage and level (MFI) of GFP expression are displayed. The MFI (mean fluorescence intensity) represents the whole respective cell population and is calculated as $MFI^{CD11c-GFP} - MFI^{C57BL/6}$. The representative overlay data shown in Figure 17 are statistically analyzed with all animals per group ($n=6$ for $CD11c^{2000bp}$ and $n=5$ for $CD11c^{400-1500bp}$). Error bars represent standard deviations (SD). For each promoter construct, experiments were repeated independently 2-3 times.

5.3.4 Segmental analysis of the CD11c/DC-STAMP promoter model

To further prove the transcriptional potential of the CD11c/DC-STAMP-model we next tested whether the relevant region is still capable of driving DC-specific expression, when taken out of its original genomic context. Therefore the pCD11c^{750-400bp} region was cloned into a lentiviral backbone upstream of the minimal SV40 promoter and GFP as a reporter gene (Fig. 19a).

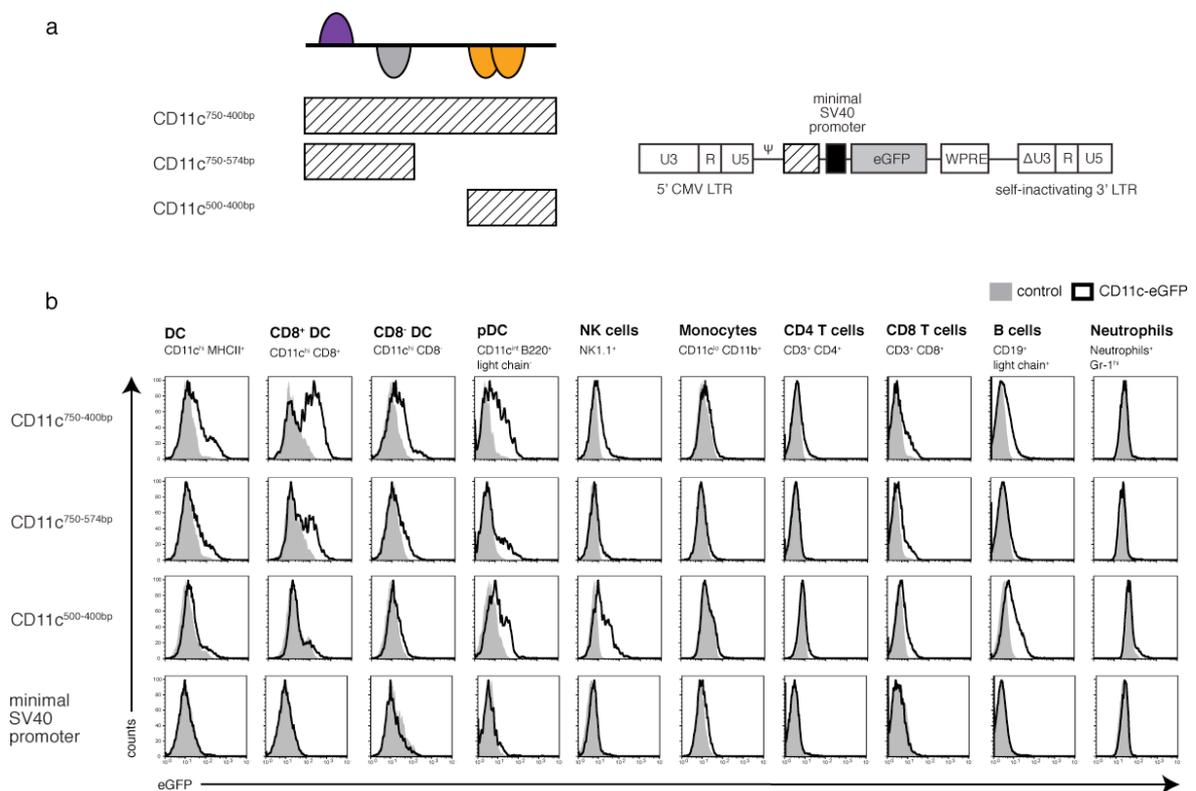


Figure 19 Segmental analysis of the CD11c/DC-STAMP promoter model.

(a) Schematic illustration of the different parts of the CD11c/DC-STAMP promoter model and their respective location. pCD11c^{750-400bp}, pCD11c^{750-574bp} and pCD11c^{500-400bp} were cloned upstream of a SV40 minimal promoter into a lentiviral vector. (b) *In vivo* promoter analysis of the three pCD11c fragments indicated and the minimal SV40 promoter alone. Spleen cells from bone-marrow chimeras were analyzed for GFP expression by flow cytometry. Shown are histogram overlays of cells from C57BL/6 mice as negative controls. Data shown are representative of three independently performed experiments (n=4-5).

For the minimal SV40 promoter alone, no *in vivo* activity was detected, but the presence of the pCD11c^{750-400bp} region resulted in GFP expression preferentially in DCs (Fig. 19b), which was comparable to the longer CD11c promoter fragments

(750-2000bp). The fact that this 350bp region can also act isolated in the context of a minimal promoter identifies the sequence as a DC-specific enhancer.

To dissect the roles of the four TFBSs in the CD11c/DC-STAMP model, we investigated the two halves containing two TFBS elements each, again employing the minimal SV40 promoter (Fig. 19a). The first part (pCD11c^{750-574bp}) was capable of inducing some expression in DCs, but not to the same extent as the full region (Fig. 19b). In contrast, the second part (pCD11c^{500-400bp}) could not drive any expression in conventional DCs, but did lead to expression in pDCs and other cell types like NK cells and B cells. This shows that the second half of the CD11c enhancer with the putative two binding sites for the transcription factors of the Brn POU family does not play a major role in gene transcription in DCs, whereas the region containing the binding sites for the STAT family and the Homeodomain transcription factors is of significant importance. Notably, the best promoter activity was achieved when the whole 350bp region was used and all four elements could work together.

5.3.5 Identification of coregulated genes using a combinatorial approach

The characterization of the CD11c/DC-STAMP model consisting of TFBSs in a defined orientation can be used to search for novel genes, which have the same organization of regulatory elements within their promoters and have not been linked to DC biology before. To this end, a database of 72.900 mouse promoters was screened (*ModelInspector*, Genomatix). Of those, only 49 promoter sequences (0.07% of all sequences, Table 4, APPENDIX) were found to contain the CD11c/DC-STAMP model. To create an additional line of evidence, we employed another platform, the “immunological genome project” (www.immgen.org) (80). In this approach, gene expression data of various cell types across the immune system are compared and gene correlation is calculated by principal component analysis. Using this database two genes from the promoter search were linked to CD11c: Pftk1 and Ppef2. According to the “immunological genome project” Pftk1 is correlated to CD11c, while Ppef2 shows correlation to Pftk1 (Fig. 20).

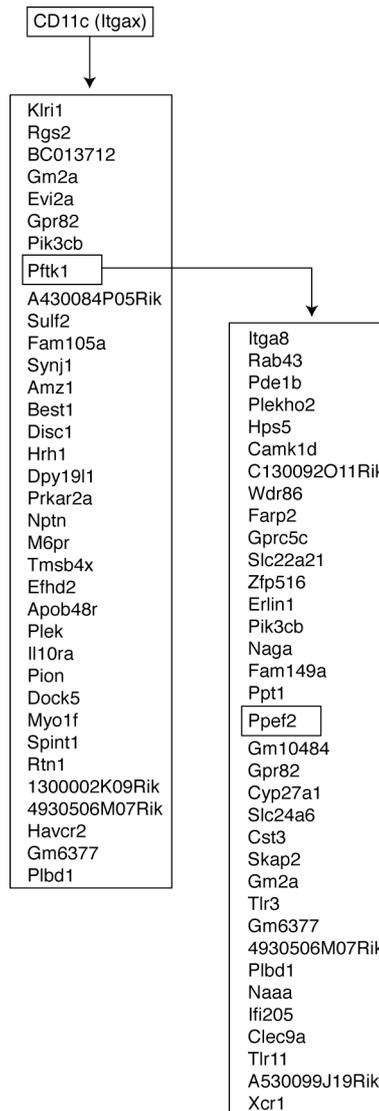


Figure 20 Gene constellation analysis based on gene expression profiles.

Immgen software was used to find the best 35 genes correlated with CD11c. As Pftk1 was found in parallel in the mouse promoter database search, its gene constellation was analyzed subsequently and led to the identification of Ppef2.

The promoter sequences of the two genes were screened across species to define evolutionary conservation and the precise location of the CD11c/DC-STAMP promoter model. Regarding the Pftk1 promoter, the CD11c/DC-STAMP-model was partly conserved across species (Fig. 21a): three of four elements were still present in the rat promoter and in the human orthologue these two elements, which were considered to be of higher importance for CD11c promoter activity (Fig. 19), were found. Remarkably, the CD11c/DC-STAMP-model is rotated by 180° in the Ppef2 promoter (Fig. 21b), but this should not influence the activity, as promoters are three-dimensional structures.

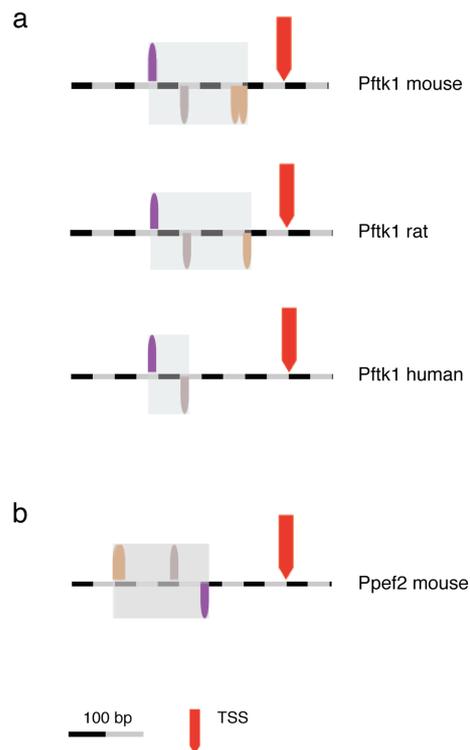


Figure 21 Identification of Ppef2 and Pftk1 as novel candidate genes.

The *ModelInspector* program (Genomatix) was used to scan DNA sequences for the CD11c/DC-STAMP model. In the first place a database of mouse promoters of annotated genes was screened. Then orthologous promoters, which were identified using a comparative genomics tool (*EIDorado*, Genomatix), were analyzed by the same method. Location of the CD11c/DC-STAMP-promoter model in Pftk1 promoters across species (a) and in the mouse Ppef2 promoter (b). In the Ppef2 promoter, the two BRNF binding sites are so close together (10bp) that they appear as one.

To confirm the expression of the two genes in DCs, mRNA isolated from sorted primary cells was analyzed by real-time PCR. Both Pftk1 and Ppef2 were expressed in DCs, particularly in the CD8 positive subset, but not in CD8 T cells from spleen (Fig. 22a). However, as coexpression of genes can occur by unrelated events, this result was not sufficient to support the idea of a similar transcriptional mechanism. We therefore investigated coregulation of these genes by analyzing samples at different time points during *in vitro* DC generation from bone marrow precursor cells, in which the percentage of DCs increases with duration of the GM-CSF culture. Accordingly, the expression of CD11c and DC-STAMP increased with time (Fig. 22b and (87)). In addition, DCs were matured by LPS-stimulation, a major hallmark of DC-biology, which resulted in down regulation of both CD11c and DC-STAMP (Fig. 22b and (87)). Ppef2 showed a parallel gene expression pattern, whereas Pftk1 was already expressed in bone marrow cells and stayed

constant during the culture (Fig. 22b). However, Pftk1 mRNA levels decreased similarly with DC stimulation.

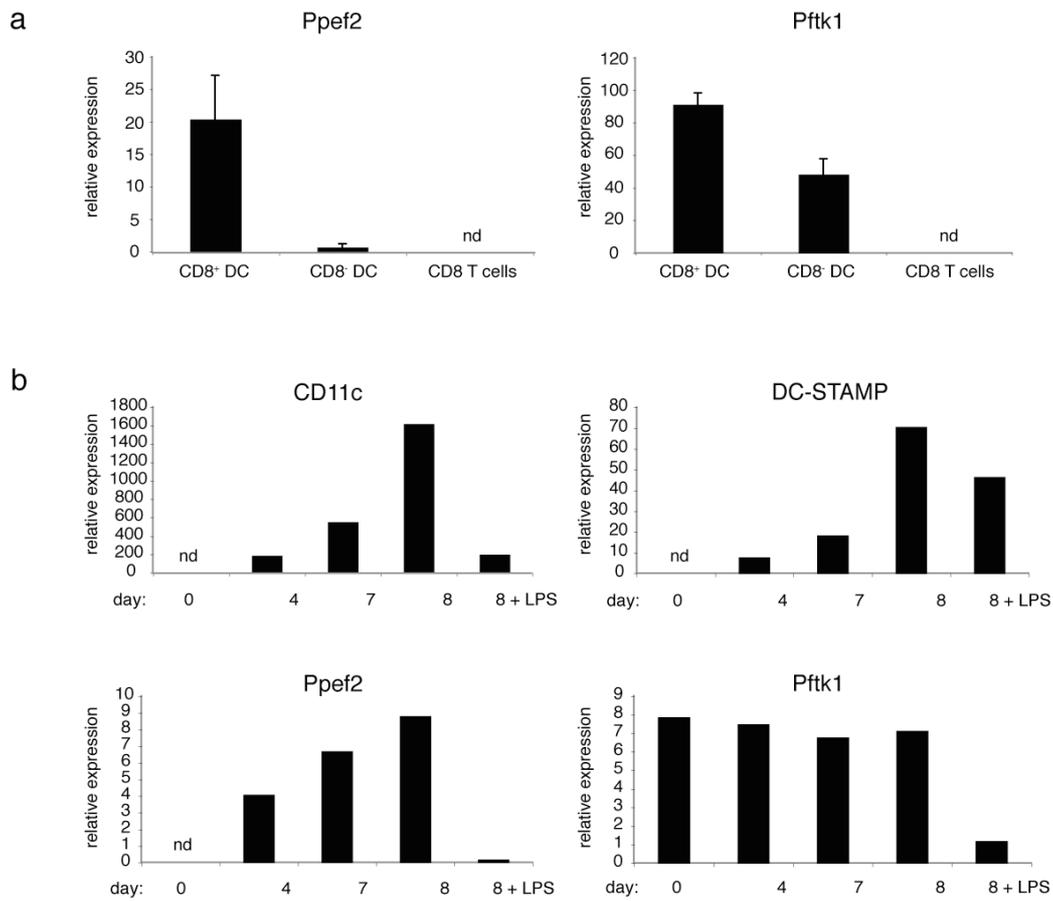


Figure 22 Expression of Ppef2 and Pftk1 in DCs.

(a) Gene expression profiling of *ex vivo* spleen cells. After density enrichment, DCs were sorted on a BD FACS^{Aria} instrument based on their expression of CD11c, MHC-II and CD8. CD8 T cells were identified as CD3⁺, CD8⁺ and CD4⁻ cells. Gene expression levels were calculated relative to Ubiquitin C. Error bars correspond to standard deviations between two independently performed experiments. (b) Gene expression profiling of DCs generated *in vitro*. Real-time PCR analysis of RNA from samples isolated at day 0, 4, 7 and 8 during DC culture. At day 7 parts of the cultures were stimulated over night with LPS (2 μ g/ml). Shown is relative gene expression after normalization to Ubiquitin C. Data are representative of two independently performed experiments. Nd, not detectable.

In summary, common promoter frameworks between CD11c and DC-STAMP promoters were found using comparative analysis and confirmed by *in vivo* promoter analysis, identifying a DC-specific enhancer region. The information about promoter organization (Genomatix) was combined with gene expression data (Immgen) and lead to the identification of two candidate genes with potential roles in DC biology: Ppef2 and Pftk1.

6 DISCUSSION

6.1 Tolerance induction

We have demonstrated that expression of a model antigen specifically in DCs mediates both central and peripheral tolerance. However, we frequently observed that central tolerance was not as effectively induced as peripheral tolerance (Fig. 10b). Differences in transgene expression between thymic and splenic DCs could be one possible explanation for this observation. In DC-STAMP-GFP chimeras (76) there was a lower percentage of GFP positive DCs in the thymus compared to the spleen. However, analysis of fluorescent intensities did not indicate significant differences between these populations. This suggests that transgene expression levels might not be the cause of the discrepancy in tolerance induction, but rather the distinct developmental origins of the thymic DC compartment. One subset ($CD8^+$, $SIRP\alpha^-$), comprising 50% of all thymic DCs, is derived from an early intrathymic precursor, whereas pDCs and the $CD8^- SIRP\alpha^+$ DC subset arise from an extrathymic precursor that homes partially differentiated to the thymus (96). In parabiosis experiments (97) it was not possible to replace this thymus-resident precursor and it is unclear whether the irradiation protocol used in our setting depletes it. The fact that the percentage of GFP positive DCs was reduced in the thymus argues for there being difficulties in replacing the early intrathymic precursor and the resulting DC subset, similar to other radio-resistant DC-subsets such as Langerhans cells of the skin (98). In thymic DC stainings we did not distinguish between resident and migratory populations and can therefore not ultimately answer this question.

Concerning the DC-STAMP-OVA model, it cannot be assumed that all thymic DCs must express antigen to induce central tolerance under normal conditions. However, as we used TCR transgenic T cells present in artificially high frequencies, antigen expression on many DCs could still be necessary to ensure optimal deletion.

6.2 Peripheral T cells in the thymus

In accordance with previous findings (59, 60, 62) we showed that mature OT-I T cells isolated from lymph nodes are able to home back to the thymus in irradiated hosts. These cells were found already at day 5 after transfer as well as at later time points (8 weeks). Our results did not distinguish whether the T cells, once in the thymus, persist there or if it is a circulating movement, in which they come and go. Thymic re-entry of OT-I T cells was possible both in the presence (Fig. 7,9,13) or absence of their cognate antigen (Fig. 8). However, re-entry seemed to be more efficient when antigen was present: In the OT-I BM→RIPmOVA experiment (Fig. 7), about 60 000 mature OT-I T cells that naturally occur in the BM were transferred. As this number of mature CD8 T cells occurs under physiological circumstances in the BM, it served as a basis for all following experiments.

In contrast, in the OT-I→B6 experiment (Fig. 8) much more (6.5×10^6) OT-I T cells were injected together with syngenic BM. In RIPmOVA hosts, 1×10^5 OT-I T cells were detected in the thymus at day 8 after transfer, whereas in B6 hosts - even though more cells were transferred - only 1×10^4 cells were found to have re-entered at day 11. These findings clearly indicate that OT-I T cells favor thymic re-entry in the presence of antigen. In line with this, it has previously been observed that allogenic compared to syngenic T cells preferentially home to the thymus (99).

The phenomenon that mature T cells re-enter the thymus has been described before and various functions for these cells were proposed (61), e.g. induction of positive selection (64), maintenance of mTECs (100) or induction of negative selection to antigens they passively acquire through their TCRs (68, 101).

As we have developed a model to induce central tolerance by transcriptional targeting of OVA as a model antigen to DCs (76), we decided to address the question whether re-entering T cells would interfere with negative selection in the thymus. We were able to show that the presence of mature re-entered OT-I T cells in the thymus indeed prevented negative selection of both TCR transgenic CD8⁺ and CD4⁺ T cells (OT-I and OT-II) and polyclonal CD8 T cells with OVA specificity. This was due to Ag-specific eradication of DCs and mTECs by re-entered mature OT-I T cells. However, this observation is restricted to OT-I T cells, which express a transgenic TCR with a defined affinity. It will be interesting to

investigate whether CD8 T cells with different specificities are also able to delete APCs in the thymus.

We demonstrated that mTECs can also be targets of re-entering CD8 T cells in the Δ CD8 OT-I BM \rightarrow RIPmOVA model (Fig. 13), in which the BM was either spiked with mature OT-I T cells or not. In this system, OVA expression is controlled by the insulin promoter and can therefore be considered as a tissue restricted antigen (TRA), which is expressed by mTECs. In the presence of mature re-entered OT-I T cells significantly more endogenous OT-I T cells developed in the thymus, which indicates less central deletion and indirectly shows the removal of OVA expressing mTECs. To provide more direct evidence for Ag-specific deletion of mTECs, it would be necessary to compare mTECs from the two groups by qPCR analysis of OVA mRNA. mTECs are a rare cell type, which makes isolation of sufficient material for analysis very difficult. The fact that each TRA is only expressed by a minor subset of mTECs (102) further complicates this approach.

In our setting, thymic cell numbers were diminished by the irradiation procedure and were further compromised under diabetic conditions. Therefore it was not possible to isolate sufficient amounts of mTECs with high purity from these mice. Nevertheless, when T cells developed in a RIPmOVA thymus in the absence of mature peripheral OT-I T cells, which allowed normal encounter of OVA expressed by mTECs, their surface marker expression was significantly different (Fig. 13d): half of the T cells displayed strongly reduced levels of Thy1.1. This surface molecule is widely used as a standard T cell marker, but has also been implicated to function in fine-tuning T cell activation (103). Two reports (84, 85) provide evidence that Thy-1 is downregulated in response to antigen. This, together with the observed CD69 upregulation (Fig. 13d), suggests that the developing T cells indeed recognized OVA in the thymus. In contrast, the presence of mature OT-I T cells in the thymus prevented Thy-1 downregulation and reduced CD69 upregulation on developing T cells, indicating the removal of OVA-expressing mTECs.

Our data demonstrate that thymus re-entry of mature CD8 T cells can lead to Ag-specific removal of mTECs and DCs with concomitant loss of central tolerance and the resulting appearance of self-reactive peripheral T cells.

Of course, it was also of interest to ascertain whether the self-reactive CD8 T cells are able to exert effector functions. Those OT-I T cells, which could escape thymic deletion in DC-STAMP-OVA chimeras due to the presence of mature OT-I T cells, had a surface phenotype similar to naive OT-I T cells in the spleen (Fig. 11c). But there were still some OVA expressing DCs left in the spleen (Fig. 11d), which could exert a tolerogenic effect on those escaped OT-I T cells. However, lentiviral modification generates an artificially high frequency of OVA-expressing DCs and under normal conditions they would probably be completely removed. Further experiments showed that the escaped OT-I T cells can indeed be restimulated to produce cytokines like IFN- γ (Fig. 14d), which suggests that they could function as effector cells.

Also in the OT-I \rightarrow RIPmOVA mice it was difficult to address the question of cell functionality. There was a higher frequency of endogenous OT-I T cells in the thymus, if mature OT-I T cells acted to remove OVA-expressing mTECs (Fig. 13). However, simultaneously with endogenous OT-I T cells leaving the thymus, the adoptively transferred OT-I T cells had already destroyed the pancreas. As such, the self-reactive potential of those OT-I T cells, which escaped thymic deletion, could not be investigated. Other experimental approaches are needed to clarify this issue.

Given that thymus escaping self-reactive T cells would indeed be functional, this result has important implications for bone marrow transplantation settings. In allogeneic bone marrow transplantation, donor T cells can broadly damage the host thymus (thymic GVHD). This can be mediated by secretion of IFN γ (104) or by mechanisms involving FasL and TRAIL (99). This overall destruction of the thymic epithelium impairs T cell development from transplant BM, which is essential for long-term immunity and survival of the patients. But there is not only a failure to reconstitute the T cell compartment, the damage can even lead to the loss of negative selection and appearance of T cells with anti-host reactivity (105). Our findings suggest that, in addition to overall thymic destruction, adoptively transferred T cells can eliminate antigen-presenting cells (DCs or mTECs) in a selective and Ag-specific manner. The elimination of APCs presenting self-Ag allows an increase in the generation of self-reactive T cells. As a consequence, endogenous self-reactive T cells might contribute to the continuation of GVHD and autoimmunity after BM-transplantation.

It is also possible that thymus re-entering T cells could play beneficial roles in therapy such as the highly successful adoptive transfer of tumor-specific T cells in immune depleted cancer patients (106). Here, the removal of tumor- (self-) Ag presenting thymic DCs and mTECs (102, 107) might positively enhance the endogenous anti-tumor repertoire of the patients. However, as single mTECs express many other self-Ags besides those recognized by tumor-specific T cells, mTEC eradication could lead to a collateral loss of self-tolerance. It will be interesting to investigate the potential relevance of our findings to adoptive therapy treatments in clinical settings.

6.3 *In vivo* promoter analysis

It has been previously shown in transgenic mice that a 5.5kb-fragment upstream of the transcriptional start site of the mouse CD11c gene targets transgene expression to DCs (86). Although this approach was successfully adopted by many different labs (108-112), and the human CD11c promoter has been studied extensively (reviewed in (113)), there has to date been no information about the location of regulatory elements in the mouse orthologue.

The comparison of CD11c and DC-STAMP promoters by a computational approach identified a promoter model and drew attention to a shared 250bp region. By *in vivo* promoter analysis using lentiviral vectors (Fig. 16), we confirmed that this particular region in the CD11c promoter is important to drive DC-specific expression (Fig. 17). Recent findings of Ni et al. (114) support our results, as they successfully used a 700bp fragment of the CD11c promoter in a vaccination approach against tumor antigens. The promoter model we described here is still present within this 700bp region. The fact that gene expression driven by pCD11c constructs between 2000 and 750bp results in similar expression profiles (Fig. 17) argues against other positive or negative regulatory elements within this region.

Some cell types, such as pDCs, monocytes, macrophages and NK cells display low expression of CD11c. Likewise, we observed only low-level promoter activity in monocytes and splenic macrophages (Fig. 18 and data not shown). In NK cells, the CD11c promoter also drove only weak transgene expression in our experiments

(Fig. 18). This is in accordance with previous findings in transgenic mice using the 5.5kb promoter (111). However, it has been demonstrated that this low expression level can still be sufficient to change the NK cell phenotype (115). In contrast, pDCs display intermediate expression levels, but are nevertheless resistant to diphtheria toxin, if the corresponding receptor is expressed under the control of the CD11c promoter (116). These findings emphasize that the type of application and transgene have a strong impact on the outcome of CD11c-driven gene expression.

We investigated both CD11c and DC-STAMP by *in vivo* promoter analysis (Fig. 5 and 17). A comparison of the tissue specificity of the two promoters revealed an interesting difference: the 1.7kb DC-STAMP promoter fragment displayed only minor activity in pDCs. Most publications regarding DC-STAMP do not distinguish between cDCs and pDCs in their experiments. Therefore it is unclear whether our findings correspond to the mouse DC-STAMP promoter in its genomic context and DC-STAMP is indeed not expressed in pDCs. Alternatively, the missing gene expression in pDCs could be attributed to the reduced promoter length used in our experiments. If this is the case, it would mean that regulatory elements driving gene expression in pDCs are located outside the 1.7kb region we tested. In relation to the DC-STAMP-OVA model, this suggests that although pDCs are able to induce tolerance (117), they are dispensable for this process in our setting.

Both CD11c and DC-STAMP promoters were found to drive gene expression with a similar intermediate strength, although the CD11c promoter displayed a slight tendency to have superior activity. However, DC-STAMP mRNA levels are so much lower, close to the detection limit in quantitative PCR and microarray assays that a major difference in gene expression levels would be expected. These findings imply differences in post-transcriptional regulation of CD11c and DC-STAMP mRNA that influence their stability.

Most of the existing knowledge on transcription factors that are related to DC biology concerns the development of the various DC subsets from hematopoietic stem cells. Using transcription factor knockout mice both positive and negative effects on DC development have been described (Fig. 23). Only recently the transcription factor E2-2 has been defined as a molecular switch between pDC and

cDC development (118): in its absence pDCs develop into cDC-like cells, thus E2-2 negatively regulates the default cDC pathway (Fig. 23).

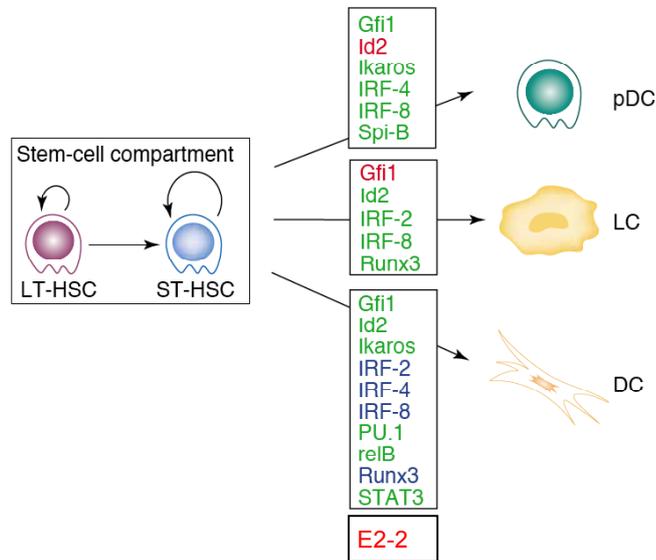


Figure 23 Transcription factors in DC development.

Different transcription factors are necessary for the development of conventional DC, Langerhans cells (LC) and plasmacytoid DC (pDC) from HSCs. Activating and inhibitory transcriptional activity is illustrated in green and red, respectively. LT, long-term; ST, short-term. Modified from (119).

From the TF families comprised in the CD11c/DC-STAMP promoter model in this study, none of the members has previously been linked to DC biology or found to be preferentially expressed in DCs. The HOMF (homeodomain transcription factors) and BRNF (Brn Pou domain factors) families display a very wide tissue distribution and only the STAT (signal transducer and activator of transcription) family shows a more restricted activity, namely in breast and hematopoietic cells (*MatBase*, *Genomatix*). However, considering the variety of promoter sequences within the genome and the limited number of transcription factors, it is likely that transcriptional regulation is a concerted action that is dependent on the combination and availability of particular factors.

An interesting characteristic of the BRNF family is the tendency to homodimerize (120). This is also likely to occur in the CD11c/DC-STAMP promoter model, as two members of this family bind in close proximity (Fig. 15b).

Our results assigned a prominent role to a STAT family member in the regulation of CD11c, as deletion of this particular binding sequence resulted in loss of DC-specific expression (Fig. 19). This finding is supported by the fact that interleukin

(IL)-4, which signals via STAT6 (121) and is added in some culture conditions to generate DCs, does indeed enhance the expression of DC-STAMP and CD11c (87, 122).

After refinement of the CD11c/DC-STAMP promoter model, it was used to screen a mouse promoter database, which revealed 49 genes predicted to contain the CD11c/DC-STAMP model in their promoter regions (Table 4, APPENDIX). We combined this result with information about gene expression, which is used to calculate so called gene constellations (Immgen). The two candidate genes Pftk1 and Ppef2 were found together with CD11c in both approaches (Fig. 24). DC-STAMP could not be included in gene expression analysis, as mRNA levels are too low for detection in microarray assays.

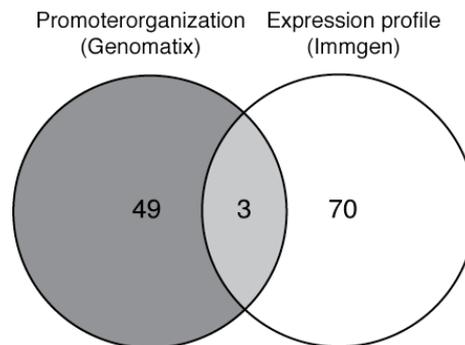


Figure 24 Combinatorial approach for promoter analysis.

The combination of two computational tools led to the identification of three common genes: CD11c, Pftk1 and Ppef2. Searching a mouse promoter database with the CD11c/DC-STAMP model resulted in 49 genes. Analyzing gene constellations based on expression profiles resulted in 70 correlated genes (35 with CD11c, 35 with Pftk1). DC-STAMP could not be included, as expression levels are too low for detection in microarray analysis.

We assume that Pftk1 and Ppef2 could have functional and so far undiscovered roles in DC biology. In the mouse, Pftk1 is highly expressed in brain and testis, but its function is unclear. It is also known as Cyclin-dependent Kinase (Cdk)-14 and for human Pftk1 a role in cell cycle progression and cell proliferation has been demonstrated (123). In addition, Pftk1 interacts with Cyclin D3 (123) and Cyclin Y, a novel membrane-associated cyclin (124). This function seems to not be rather necessary for DCs, as they were originally considered to be an end-stage, non-dividing cell type. However, there is now evidence that DCs undergo at least a limited number of divisions in spleen and lymph nodes (125, 126). Moreover, CD8 positive DCs, which have higher levels of Pftk1 mRNA, also have a faster turnover

compared to other DC subsets. Furthermore, Pftk1 is also expressed in post-mitotic cells (127), which suggests additional functions beyond the cell cycle. Pftk1 expression does not follow the same kinetics like CD11c and DC-STAMP (Fig. 22b), as it is already present in bone marrow cells, as suggested by public microarray data (*GEO profiles*, NCBI). Therefore it is most likely that other regulatory elements might be active in the Pftk1 promoter in addition to the CD11c/DC-STAMP promoter model. It is of interest to note that one member of the BRNF family, namely Brn3a, has already been described to be important for transcriptional regulation of the Pftk1 gene, although in a different tissue (128).

Ppef2 is a serine/threonine-protein phosphatase with two EF-hand calcium binding domains at the C-terminus that interacts with calmodulin via its N-terminus (129), thereby providing a dual regulation mechanism by calcium. Recently, it has been shown that Ppef2 binds and negatively regulates ASK-1 (apoptosis signal regulating kinase) (130), which is activated by oxidative stress and can transduce apoptotic or inflammatory signals (131). The fact that DCs are 10 times more resistant to reactive oxygen species (ROS) than T lymphocytes (132) could be explained by the presence of Ppef2. Another possible role of Ppef2 in DCs could be in the control of cytokine production, as ASK-1 is required for the IL-6 production in response to LPS (133). We have shown that Ppef2 is downregulated after LPS stimulation (Fig. 22b), potentially clearing the way for effective cytokine production in the mature DC. It will be interesting to assess how these newly identified genes are in fact functionally linked to DCs in future studies.

Interestingly, all of the pCD11c constructs between 750 and 2000bp showed higher gene expression in CD8 positive DCs than in other subsets (Fig. 17 and 18). This is consistent with results in transgenic mice using the 5.5kb promoter (112). Even when the enhancer region alone was used together with the SV40 minimal promoter, this characteristic persisted (Fig. 19). In addition, the same trend was observed for DC-STAMP (76) and the newly identified genes, Pftk1 and Ppef2, at least regarding mRNA levels (Figure 22a and *GEO profiles*, NCBI). This argues for the idea that the enhancer region we described is not only specific for DCs, but also shows a higher activity in CD8 positive DCs. As this subset is important for cross-presentation (134, 135) and thereby for the generation of optimal cytotoxic immune responses, this feature makes the enhancer an attractive tool for engineering of vaccination vectors.

Common promoter frameworks between the CD11c and DC-STAMP promoters were found using comparative analysis and confirmed by *in vivo* promoter analysis, identifying a DC-specific enhancer region. This information was subsequently used to detect genes previously not associated with DC biology. As such, a transcriptional network could be defined that orchestrates the expression of coregulated genes in DCs. The power of this combinatorial approach will help to face the challenge of dissecting complex transcriptional networks.

6.4 Conclusion

The lentiviral gene transfer system developed in this study offers multiple interesting applications, as stable gene expression is achieved without the need to produce transgenic mice. The generation of bone marrow chimeras with lentivirally transduced HSCs is substantially less time consuming than making novel transgenic mice. As such, important biological questions can be addressed in a relatively short period of time *in vivo*.

DC-specific gene expression by transcriptional targeting using the mouse CD11c or DC-STAMP promoters can be used to further investigate aspects of DC biology. These results could help to develop novel protocols for immunomodulation, which potentially contribute to improving vaccination, autoimmunity and transplantation treatments in the long run.

A second application of the lentiviral gene transfer system is the novel method of *in vivo* promoter analysis. In the field of promoter analysis, most approaches are conducted *in vitro* and involve cell lines, as primary cells are difficult to manipulate for gene transfer. This artificial setting can only give a first indication of promoter function, but cannot reflect the physiological situation.

In our *in vivo* system, promoter analysis is performed with primary cells that have developed from lentivirally infected HSCs. The analyzed cells are derived from a natural environment and have not been directly manipulated. Furthermore, promoter activity can be compared in one cell type in different organs, e.g. DCs in the spleen, lymph nodes and thymus.

7 APPENDIX

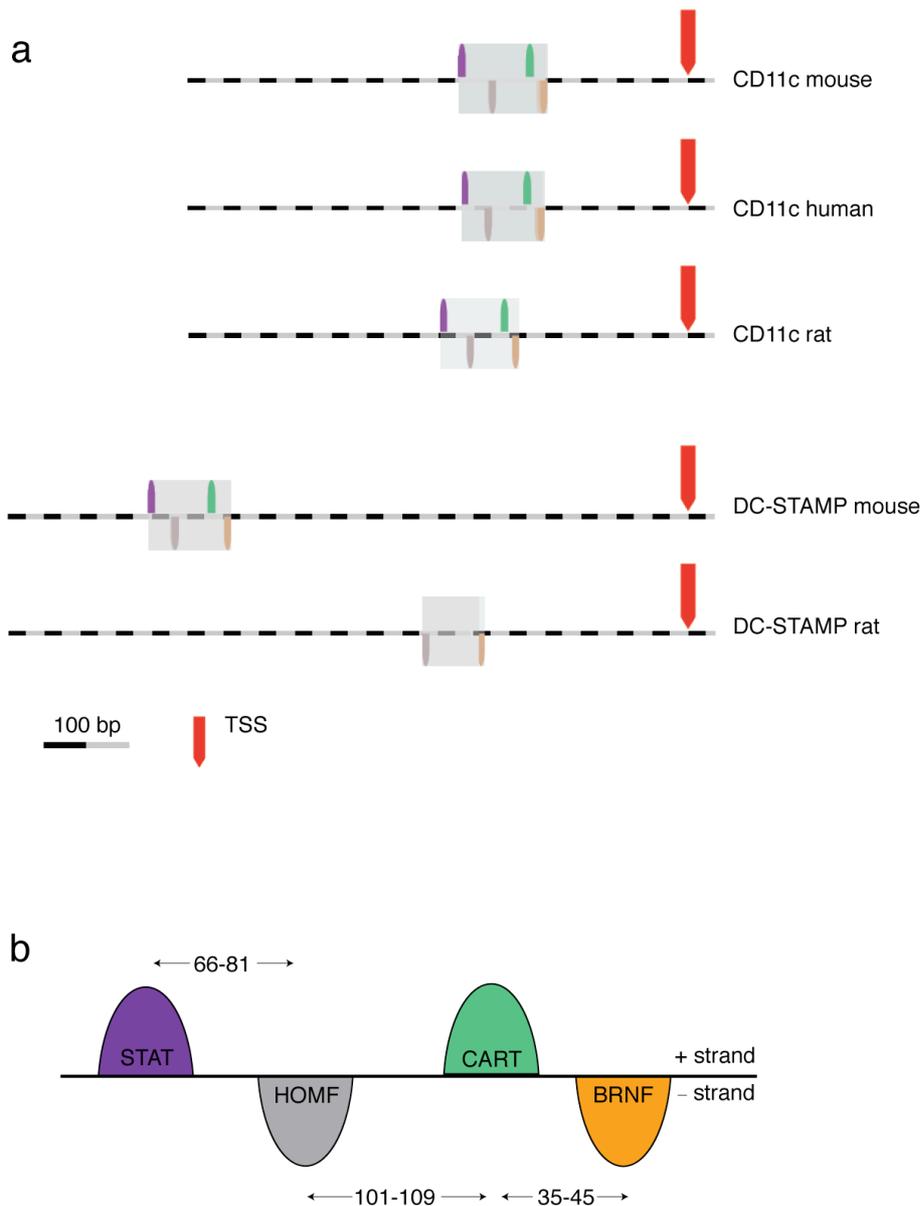


Figure 25 Alternative framework in the CD11c and DC-STAMP promoters.

In comparison to the model presented in Fig. 15, the only difference is evident on position three: a CART binding site replaces the first of the two BRNF binding sites. (a) The location of the alternative CD11c/DC-STAMP framework in CD11c promoters and DC-STAMP promoters across species is displayed. TSS, transcription start site. (b) Detailed representation of the alternative CD11c/DC-STAMP promoter framework, which contains four elements over a length of 250bp. The transcription factor families and their respective orientation on the + and - strand are displayed. The indicated distance ranges result from the refinement of the framework in the rat CD11c promoter. STAT (signal transducer and activator of transcription), HOMF (Homeodomain transcription factors), CART (cartilage homeoprotein-1), BRNF (Brn POU domain factors).

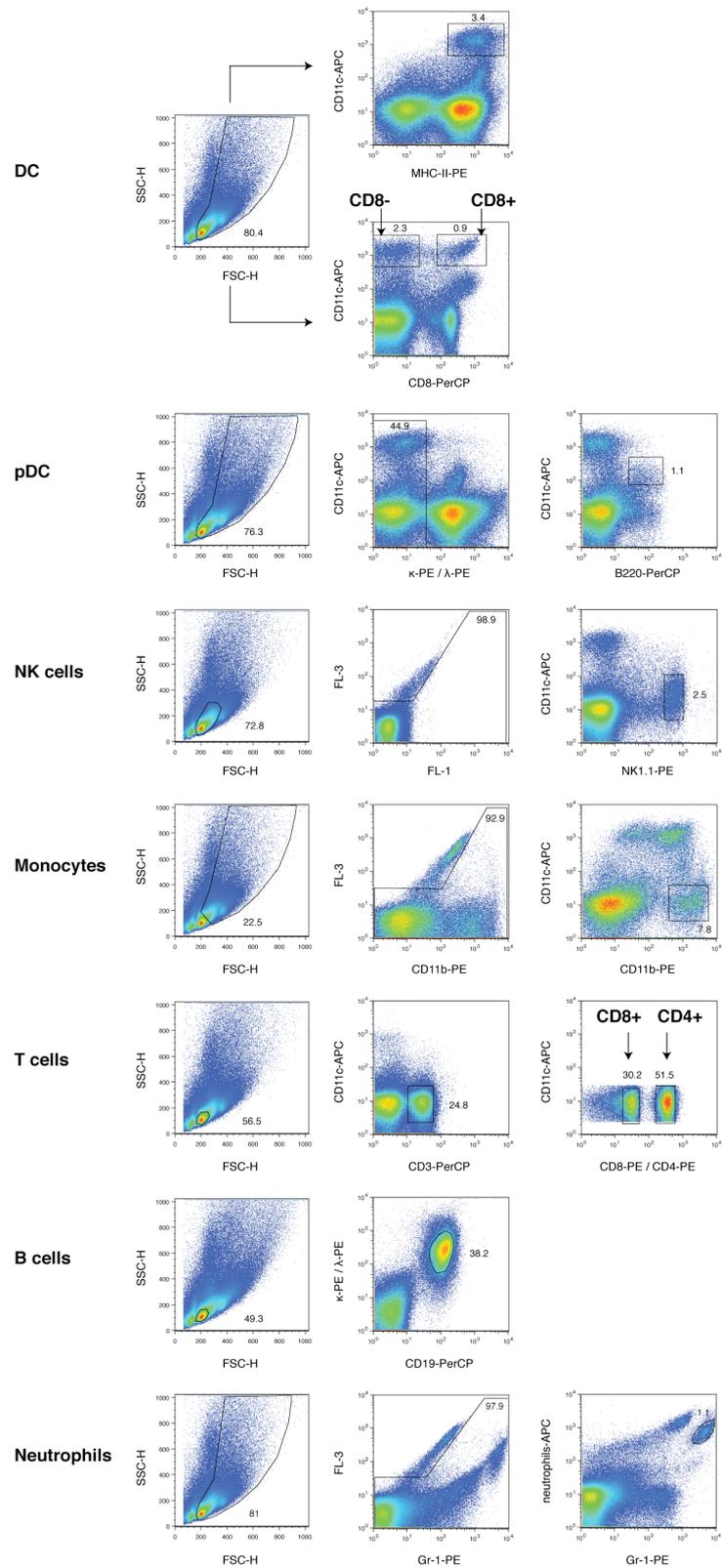


Figure 26 Gating strategy for *in vivo* promoter analysis.

Splenocytes were stained with different combinations of antibodies and the different cell types were identified by the progressive gating strategy shown from left to right. Representative blots of a C57BL/6 mouse are displayed. If possible, the FL-3 channel was spared to remove autofluorescent cells (middle column of NK cells, monocytes and neutrophils). The neutrophils-antibody was labeled with biotin and detected by streptavidin-APC.

Table 4 List of genes predicted by *ModelInspector* analysis.

These genes were found by screening a mouse promoter database and were predicted to contain the CD11c/DC-STAMP promoter model in their regulatory region.

Gene	Gene ID	Name
Nrk	27206	Nik related kinase
Braf	109880	Braf transforming gene
Strn3	94186	Striatin, calmodulin binding protein 3
Pmch	110312	Pro-melanin-concentrating hormone
Nck1	17973	Non-catalytic region of tyrosine kinase adaptor protein 1
Mecom	14013	MDS1 and EVI1 complex locus
Dsc1	13505	Desmocollin 1
Syt4	20983	Synaptotagmin IV
Ppef2	19023	Protein phosphatase, EF-hand calcium-binding domain 2
Olf1098	258842	Olfactory receptor 1098
Klra2	16633	Killer cell lectin-like receptor, subfamily A, member 2
Inpp4b	234515	Inositol polyphosphate-4-phosphatase, type II
Ntrk3	18213	Neurotrophic tyrosine kinase, receptor, type 3
Tchh1	71325	Trichohyalin-like 1
Olf1720	258387	Olfactory receptor 720
Zfp518	72672	Zinc finger protein 518
Smarcd2	83796	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2
Fn1	14268	Fibronectin 1
Mll3	231051	Myeloid/lymphoid or mixed-lineage leukemia 3
Abcb4	18670	ATP-binding cassette, sub-family B (MDR/TAP), member 4
Metap1	75624	Methionyl aminopeptidase 1
Brcc3	210766	BRCA1/BRCA2-containing complex, subunit 3
Olf1132	258833	Olfactory receptor 1132
Pde9a	18585	Phosphodiesterase 9A

Enpp5	83965	Ectonucleotide pyrophosphatase/ phosphodiesterase 5
Olf1370	258528	Olfactory receptor 1370
Pcm1	18536	Pericentriolar material 1
Stxbp5	78808	Syntaxin binding protein 5 (tomosyn)
Tnik	665113	TRAF2 and NCK interacting kinase
Eps15	13858	Epidermal growth factor receptor pathway substrate 15
Mdn1	100019	Midasin homolog (yeast)
Phrf1	101471	PHD and ring finger domains 1
Fryl	72313	Furry homolog-like (Drosophila)
Gm4190	100043046	Predicted gene 4190
Gm3114	100041051	Predicted gene 3114
Bet3l	692132	BET3 like (S.cerevisiae)
mCG 142697	71783	Ankhd1-eif4ebp3 readthrough transcript
Dst	13518	Dystonin
Mll5	69188	Myeloid/lymphoid or mixed-lineage leukemia 5
Spr2f	20760	Small proline-rich protein 2F
Pftk1	18647	PFTAIRE protein kinase 1
Fam174a	67698	Family with sequence similarity 174, member A
Pten	19211	Phosphatase and tensin homolog
Tgfbr1	21812	Transforming growth factor, beta receptor 1
Gm4836	22526	Predicted gene 4836
Gm5168	382275	Predicted gene 5168
Tet1	52463	Tet oncogene 1
Meg3	17263	Maternally expressed 3

8 ABBREVIATIONS

Ag	antigen
APC	1. antigen presenting cell 2. allophycocyanin
AIRE	auto-immune-regulator protein
Bdnf	brain-derived neurotrophic factor
BM	bone marrow
bp	base pair
CAGE	Cap-analysis gene expression
CD	cluster of differentiation
cDNA	complementary DNA
CFSE	carboxyfluorescein-diacetate-succinimidylester
CTL	cytotoxic T lymphocyte
dNTP	desoxyribonucleotidtriphosphate
DC	dendritic cell
DC-STAMP	dendritic cell-specific transmembrane protein
DNA	desoxyribonucleic acid
E. coli	<i>Escherichia coli</i>
EDTA	ethylenediamintetraacetic acid
eGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
Fc	fragment crystallizable of an antibody
FITC	fluoresceinisoithiocyanate
for	forward
5-FU	5-Fluoro-Uracil
GM-CSF	granulocyte-macrophage colony stimulating factor
GVHD	graft versus host disease
GVL	graft versus leukemia
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen

HSC	hematopoietic stem cell
HSV	herpes simplex virus
HSVgB	herpes simplex virus glycoprotein B
IDO	indoleamine 2,3- dioxygenase
IFN-I	interferon type I (alpha and beta)
IFN- α/β	interferon alpha/beta
IFN- γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
kb	kilobase
LPS	lipopolysaccharide
LTR	long terminal repeat
μg	microgram
μl	microliter
mRNA	messenger RNA
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
min	minute
MOI	multiplicity of infection
mTECs	medullary thymic epithelial cells
NK cell	natural killer cell
NKT cell	natural killer T cell
NLR	nod-like receptor
OD	optic density
ORF	open reading frame
OVA	ovalbumin
PAMP	pathogen associated molecular pattern
pBS	plasmid Blue Script
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PD-L1	programmed cell death ligand 1
PE	phycoerythrin

PerCP	peridinin-chlorophyll-a protein
pfu	plaque forming units
PRR	pattern recognition receptor
qPCR	quantitative PCR
rev	reverse
RIP	rat insulin promoter
RNA	ribonucleic acid
RRE	rev response element
RT	1. room temperature 2. reverse transcription
SA	streptavidin
SCF	stem cell factor
SIN	self-inactivating
SIINFEKL	OVA ₂₅₇₋₂₆₄
SSIEFARL	HSVgB ₄₉₈₋₅₀₅
SP	single positive (thymocyte)
STAT	signal transducer and activator of transcription
Ta	annealing temperature
TAP	transporter associated with antigen processing
TCR	T cell receptor
TFBS	transcription factor binding site
T _H cell	T helper cell
TGF- β	transforming growth factor beta
TLR	toll like receptor
Tm	melting temperature
TNF- α	tumor necrosis factor alpha
Treg	regulatory T cell
TRA	tissue-restricted antigen
TU	transducing units
UTR	untranslated region
UV	ultraviolet
vs.	versus
v/v	volume per volume
VSV-G	vesicular stomatitis virus glycoprotein G
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element

w/v	weight per volume
w/w	weight per weight
XCR1	XC chemokine receptor 1

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