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Antigen-specific tolerance induction by transcriptional targeting of dendritic cells with a novel lentiviral vector

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Contents

1. Abbreviations

AAV	adeno-associated virus
Ag	antigen
APC	antigen presenting cell or allophycocyanin
AIRE	auto-immune-regulator protein
Bdnf	brain-derived neurotrophic factor
blastn	nucleotide blast
blastp	protein blast
BM	bone marrow
bp	base pairs
CD	cluster of differentiation
CMV	cytomegalovirus
CFA	complete Freund's adjuvant
CFSE	carboxyfluorescein-diacetate-succinimidylester
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
cTECs	cortical thymic epithelial cells
CTL	cytotoxic T lymphocyte
dNTP	desoxyribonucleotidtriphosphate
DC	dendritic cell
DC-STAMP	dendritic cell-specific transmembrane protein
DLI	donor lymphocyte infusion
E. coli	*Escherichia coli*
eGFP	enhanced green fluorescent protein
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
Fc, FcR	fragment crystallizable, Fc-Receptor
FITC	fluoresceinisothiocyanate
forw	forward
Foxp3	transcription factor forkhead box P3

5-FU	5-Fluoro-Uracil
GVHD	graft versus host disease
GVL	graft versus leukemia
HLA	human leukocyte antigen
HSC	hematopoietic stem cells
HSV	herpes simplex vírus
HSVgB	herpes simplex vírus glycoprotein B
i.e.	*id est*, from Latin that is.
Ig	immunoglobulin
IKDC	interferon-producing killer dendritic cell
IL	interleukin
IFN-I	interferon type I (alfa and beta)
IFN-α/β	interferon alfa/beta
IFN-γ	interferon-gama
IL2RG	γ-chain of the interleukin-2 receptor
i.p. / i.v.	intraperitoneal / intravenous
kb	kilobase
LTR	long terminal repeat
NK cell	natural killer cell
µg	microgram
µl	microliter
MHC	major histocompatibility complex
MFI	mean fluorescent intensity
MNC	mononuclear cells
MOI	multiplicity of infection
mTECs	medullary thymic epithelial cells
OD	optical density
O/N	over night
ORF	open reading frame
OVA	ovalbumin
pBS	plasmid Blue Script
PBS	buffered saline solution

PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PerCP	peridinin-Chlophyll-a Protein
qPCR	quantitative PCR
rev	reverse
RIP	rat insulin promoter
RNA	ribonucleic acid
RT	room temperature
SA	streptavidin
s.c.	subcutaneous
SCID	severe combined immunodeficiency
SFFV	Spleen-focus forming virus
SIN	self-inactivating
SIINFEKL	$OVA_{257-264}$
SSIEFARL	$HSVgB_{498-505}$
Ta	annealing temperature
TCR	T cell receptor
TGF-β	transforming growth factor beta
TLR	toll like receptor
Tm	melting temperature
TNF-α	tumor necrosis factor alfa
trOVA	transmembrane OVA
Treg	regulatory T cell
TSA	tissue-specific antigen
TU	transducing units
UTR	untranslated region
UV	ultraviolet
vs.	versus
v/v	volume per volume
X-SCID	X-linked form of severe combined immunodeficiency

WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
w/v	weight per volume
w/w	weight per weight

2. Abstract

Dendritic cells (DC) are the most powerful antigen presenting cells (APCs) of the immune system. Since DCs can induce both tolerance and immune responses, there is an increased interest in understanding the biology of DCs for basic research and clinical applications. Different DC subpopulations have been described and several attempts have been made trying to correlate these DC subsets with different functions. However, the difficulties to manipulate DC *ex vivo* or *in vitro* without changing their original phenotypic and functional characteristics are major obstacles in DC-research. In this study, we developed a novel lentiviral vector allowing DC-selective transgene expression after hematopoietic stem cell transduction. We show that this gene-therapy approach yields DC-selective expression, which is maintained long-term. When we analyzed the *in vivo* functionality of this method, we were able to show induction of antigen-specific $CD4^+$ and $CD8^+$ T cell tolerance to the virally encoded transgene expressed by DCs. This tolerogenic state was not reverted even after immunization and was sufficient to avoid development of autoimmune disease. In addition, our preliminary data *in vitro* show that this system also targets human DCs. Together, this data supports the delivery of transgenes specifically to DCs using viral vectors as a promising tool in gene therapy.

Zusammenfassung

Dendritische Zellen (DZ) sind die besten antigenpräsentierenden Zellen des Immunsystems. Da sie sowohl Toleranz wie auch Immunantworten induzieren können, ist es von großem Interesse die Biologie der DZ für Grundlagenforschung und klinische Anwendungen zu verstehen. Es wurden verschiedene DZ Subpopulationen beschrieben und etliche Versuche unternommen diese DZ Subtypen mit den einzelnen

Funktionen in Zusammenhang zu bringen. Die Manipulation der DZ *ex vivo* oder *in vitro* ohne den ursprünglichen Phänotyp und funktionelle Charakteristiken dabei zu verändern stellt jedoch ein großes Hindernis dar. In dieser Studie haben wir einen neuen lentiviralen Vektor entwickelt, der Expression von Transgenen nach der Transduktion hämatopoetischer Stammzellen selektiv in DZ ermöglicht. Wir zeigen, dass dieser Gentherapie-Ansatz zu DZ-selektiver Expression führt, welche langfristig erhalten bleibt. Bei der funktionellen Analyse dieser Methode *in vivo* konnten wir zeigen, dass in CD4- und CD8-positiven T-Zellen antigenspezifische Toleranz gegen das in DZ exprimierte und viral kodierte Transgen induziert wird. Die Toleranz konnte selbst durch Immunisierung nicht aufgehoben werden und war ausreichend um Autoimmunität zu verhindern. Zudem weisen vorläufige Ergebnisse *in vitro* darauf hin, dass dieses System auch in humanen DZ funktionieren kann. Lentivirale Vektoren dazu zu nutzen Transgene spezifisch in dendritische Zellen einzuführen, könnte ein vielversprechendes Hilfsmittel in der Gentherapie sein.

3. Introduction

3.1 A brief introduction to immunology

3.1.1 Innate and adaptive immunology

The immune system has been classified into a more simple “innate” and into a more developed and complex “adaptive” (or acquired) immune system. The innate immune responses are present in both invertebrates and vertebrates, as well as in plants, and are the first line of defense of an organism. In contrast, the adaptive response is present only in vertebrates and involves more sophisticated mechanisms of defense.

3.1.2.1 The innate immune system

The innate immune system is crucial in the first hours and days after exposure to a new pathogen. Single bacteria for example, with an exponential rate of duplication, can produce around 20 million of progeny in a single day. Therefore, the innate immunity does not rely on pathogen-specific recognition, but rather on recognition of common patterns. The principal components of the innate immune system are: physical barriers, phagocytic cells, natural killer (NK) cells, complement system and cytokines.

3.1.2.2 The adaptive immune system

The adaptive system is highly specific for each pathogen. It can generate long lasting protection, so called immunological memory, that responds more vigorously and faster to a repeated exposure with the same pathogen. This principal is exploited by vaccination. The adaptive immune system is able to recognize and react to a universe of microbial and non-microbial substances and any molecule capable of eliciting an adaptive immune response is referred to as an “antigen” (Ag). There are basically two types of adaptive immune responses: cellular and humoral.

Although classified as innate and adaptive, both components of the immune system form an integrated system of host defense with numerous cells and molecules functioning cooperatively (Fearon and Locksley 1996). For example, dendritic cells (DCs) as part of the innate system recognize and phagocytose pathogens and, subsequently, as part of the adaptive system, they process and present the respective pathogen-derived antigens to other cells of the immune system. It is equally important to note that besides the aim of both systems to sense the presence of "non-self" patterns and antigens (or the presence of anomalous antigens when considering cancer), both systems are able to react against "self" tissues, which can lead to a process of autoimmunity. To avoid such responses to self molecules it is of cardinal importance that the immune system is always under surveillance to guarantee "self tolerance".

3.1.2 Antigen presentation

Activated T cells proliferate and differentiate into effector cells only when antigen is displayed on the surface of antigen presenting cells (APCs). This occurs because T cells only recognize fragments of proteins that have been processed by APCs into peptides. These peptide fragments are then presented on the surface of the APC on so called "major histocompatibility complex" (MHC) molecules. T cells recognize such MHC-antigen complexes through their T cell receptor (TCR). There are two main types of MHC gene products, called class I MHC (MHC-I) and class II MHC (MHC-II) molecules, which present different pools of protein Ag. Intracellular (or cytosolic) antigens are presented by the MHC-I (present in all nucleated cells) to $CD8^+$ cytotoxic T lymphocytes (CTL), while extracellular antigens that have been endocytosed are presented by MHC-II (found only in professional APCs, such as DCs, monocytes and B cells) to $CD4^+$ helper T lymphocytes. There is also a mechanism called "cross presentation" that is restricted to DCs, in which extracellular antigens are presented by the MHC-I to $CD8^+$ T cells (Bevan 1976; Bevan 2006).

Under normal conditions, self-proteins are presented constantly by MHC molecules. However, self-proteins can be also recognized by T cells leading to

autoimmunity. To avoid self-reactivity several mechanisms have evolved to establish self-tolerance.

3.2 Tolerance

Tolerance means inability to respond to a certain antigen. This characteristic is essential to avoid destruction of self tissues and subsequent autoimmunity. At least two mechanisms control the “education” of the immune system: central and peripheral tolerance.

3.2.1 Central tolerance

Central Tolerance is induced at the primary sites of lymphocyte development: thymus for T cells and bone marrow for B cells. The main process responsible for T cell central tolerance is clonal deletion, in which T cells with high affinity for self-antigens die due to apoptosis. But there are other processes of tolerance induction in the thymus that do not involve removal but the generation of regulatory T cells; this process is called “non-deletional tolerance”. Although the second process is much less understood than the first, its importance in avoiding auto-reactivity and preventing autoimmunity has become clear in the last few years.

3.2.1.1 Deletional tolerance

T lymphocytes originate from a common hematopoietic stem cell (HSC) progenitor. Developing T cells in the thymus are called “thymocytes”. In the cortical region of the thymus, cortical thymic epithelial cells (cTECs) present different Ags to the thymocytes and those expressing low avidity TCR binding to self-antigen/MHC complexes survive, a process called “positive selection” (von Boehmer 1994). These cells migrate to the medulla, where DCs and medullary thymic epithelial cells (mTECs) present several different tissue-specific antigens (TSAs) to the T cells. Those T cells that recognize self-antigen/MHC complexes with high avidity are deleted by apoptosis, a process called “negative selection”.

The expression of TSAs in the thymus depends, at least in part, on the autoimmune regulator protein (AIRE), and such TSA expression seems to be restricted to TECs (Derbinski, Schulte et al. 2001). AIRE is a transcriptional regulator controling the expression of tissue-specific genes. AIRE-deficiency results in severe autoimmunity in both humans and mice (Anderson, Venanzi et al. 2002; Ramsey, Winqvist et al. 2002). Although AIRE and TSAs are absent or expressed only at low levels in DCs, these cells pick up the antigens expressed by the TECs and present them to T cells. Therefore, DCs play an important role in central tolerance, being the most important cells able to delete auto-reactive $CD4^+$ T cells in the thymus (Gallegos and Bevan 2004).

3.2.1.2 Non-deletional tolerance

While it is accepted that central tolerance is mediated mainly by negative selection or clonal deletion in the thymus (Starr, Jameson et al. 2003), several studies showed that some of the self-reactive T cells undergo a process called non-deletional central tolerance. During this process, self-reactive T cells become anergic or give rise to regulatory T cells [Tregs; (Sakaguchi, Fukuma et al. 1985; Ramsdell and Fowlkes 1990; Sakaguchi 2004; Fontenot and Rudensky 2005)], initially called immunosuppressive T cells (Modigliani, Thomas-Vaslin et al. 1995). It is believed that $CD4^+CD25^+$ Tregs are a distinct lineage of mature T cells and that cTECs alone are sufficient for their development (Bensinger, Bandeira et al. 2001). About 10% of the peripheral repertoire of $CD4^+$ T cells are $CD25^+$ and display suppressive function. For example, “scurfy” mice, which present a spontaneous mutation in the forkhead box transcription factor P3 (FOXP3- known to be present in Tregs), suffer from severe autoimmunity (Fontenot, Gavin et al. 2003; Khattri, Cox et al. 2003). Although Treg cells arise in the thymus, their suppressive function is seen mainly in the periphery contributing to peripheral tolerance.

3.2.2 Peripheral tolerance

Central tolerance is a very efficient but not entirely sufficient mechanism to eliminate all self-reactive lymphocytes, as not all TSA present in an organism are

expressed in the thymus (Mathis and Benoist 2004). The mechanisms responsible for peripheral tolerance are: (i) functional inactivation, called anergy; (ii) apoptotic cell death, called deletion; and (iii) suppression of lymphocyte activation by Tregs.

3.2.2.1 Anergy

When T cells recognize Ags presented in absence of co-stimulation (such as B7), they are incapable of responding to the antigen, even if later the antigen is presented by a competent (or activated) APC. This state of non-responsiveness is called “anergy”. Anergy can be induced artificially, for example by administrating Ag without inflammatory signals. Anergy may also be induced if the T cell recognizes Ag in the context of inhibitory molecules such as PD-1 or CTLA-4. However, the regulation of such active tolerization is poorly understood, as these negative regulators are expressed and even upregulated also throughout productive immune responses (Walker and Abbas 2002).

3.2.2.2 Apoptosis

Repeated stimulation of T lymphocytes by persistent Ags results in death of activated cells by apoptosis. In $CD4^+$ T cells repeated activation leads to the expression of two molecules, a death-inducing receptor called Fas and its ligand, FasL. This leads to activation of intracellular proteases, called caspases, causing cell death. It is believed that this kind of apoptosis is responsible for the elimination of T cells specific for abundant peripheral self-antigens (Siegel, Chan et al. 2000). The same pathway of apoptosis is involved in the elimination of self-reactive B cells, but seems not to be involved in apoptosis of $CD8^+$ T cells. Mice with defects in the expression of Fas or FasL, and humans with mutations in Fas develop autoimmune diseases.

3.2.2.3 Suppression by Tregs

Tregs express a TCR able to recognize self-antigens with the difference that instead of being activated to generate autoimmunity, they inhibit effector T cells to maintain self-tolerance. The mechanism of action of Tregs is still not well established,

but it is known that secretion of immuno-supressive cytokines such as IL-10 and transforming growth factor-ß (TGF-ß) are involved (Shevach 2002).

3.3 Dendritic cells

DCs are specialized in capturing, processing and presenting Ags to T cells. There are many distinct DC subtypes with different localization and function in the immune system and most of these subpopulations have a short lifespan. Therefore, they need to be continuously renewed (Kamath, Henri et al. 2002). Although DCs have received a lot of attention from immunologists since their discovery, DC development, differentiation and their exact role in immune response vs. tolerance is still a very difficult and controversial subject in the field.

3.3.1 Dendritic cell function

DCs are among the most central components of the immune system, being the main professional APCs and contributing to both innate and adaptive immunity. They play a critical role in sensing pathogens, and several independent studies have shown their role in the interaction between the two immunological arms, regulating the strength, quality and persistence of Ag-specific adaptive immune responses (Shortman, 2002). DCs can be found in primary and secondary lymphoid organs, as well as in almost every peripheral tissue. Within the thymus, DCs play an important role in thymocyte negative selection and central tolerance induction. In the periphery DCs are able to pick up Ags and migrate to the lymphoid organs to present the processed Ags to other cells of the immune system, inducing immune responses or tolerance. A simplified view is that signals received through receptors such as toll like receptors (TLRs), cytokine receptors and chemokine receptors, lead DCs towards a maturation process that enables them to induce T lymphocytes proliferation and immune response (Villadangos and Schnorrer 2007). On the other hand, in the absence of such signals, DCs remain in an immature state, inducing deletion or anergy of self-reactive T cells. It is through these mechanisms that DCs are thought to mantain peripheral tolerance (Banchereau and Steinman 1998; Dhodapkar and Steinman 2002). However, the

concept that mature DCs always promote immunity has been questioned, since even in this state they can induce tolerance (Albert, Jegathesan et al. 2001; Sporri and Reis e Sousa 2005; Reis e Sousa 2006).

3.3.2 Dendritic cell sub-populations

A large variety of DCs have been described and their proportion can vary accordingly with the different organs and tissues (Table 1). In mice, one can divide lymphoid tissue resident DCs into three major populations according to molecular markers, function and cytokine production: $CD8^-$, $CD8^+$ and plasmacytoid DCs. $CD8^-$ $CD11b^+$ DCs can be further classified in $CD4^+$ and $CD4^-$ (double negative) DCs. $CD8^+$ DCs are the only cells able to cross-present Ags on MHC-I molecules (den Haan, Lehar et al. 2000; Pooley, Heath et al. 2001; Heath, Belz et al. 2004). Plasmacytoid DCs (pDCs) are distinguished from the other subtypes by their expression of the CD45 isoform B220, and are characterized by their potent ability to produce type I interferon (IFN-I) after viral infection (Asselin-Paturel, Boonstra et al. 2001; Liu 2005). Some immunologists classify pDCs as a DC precursor (together with monocytes), as after inflammatory stimuli they may develop some of the antigen-processing and antigen-presentation properties characteristic to the conventional DCs (Shortman and Naik 2007). It is important to notice that other DC types have been described in the literature during the last few years. Examples include the interferon-producing killer dendritic cell [IKDC; (Taieb, 2006)] and the $CD19^+$ pDCs (Munn, Sharma et al. 2004). However, as they are present in the organism only under specific circumstances and in lower numbers, they are not considered further here. For an overview of the most important murine DC population cell markers see table 2.

DC sub-population	Thymus	Spleen	Lymph nodes	Bone marrow
$CD8^+$ DCs	++	+	+	+
$CD8^-$ DCs	+	++	+	+
pDCs	+	+	+	++

Table 1: Distribution of the murine DC sub-populations. DC, dendritic cell; pDC, plasmacytoid dendritic cell; +, present; ++, primary sub-type.

DC sub-population	Markers
$CD8^+$ DCs	$CD11c^+$, $CD8^+$, $CD205^+$, $CD11b^-$, $CD4^-$
$CD8^-$ DCs	$CD11c^+$, $CD11b^+$, $CD8^-$, $CD4^{+/-}$,
pDCs	$CD11c^{+/low}$, $B220^+$, $CD11b^-$, $PDCA^+$, $Gr\text{-}1^+$, $MHC\text{-}II^{low}$,

Table 2: Markers that identify the different murine DC sub-populations. DC, dendritic cell; pDC, plasmacytoid dendritic cell;+, present; -, absent; low, low expression

3.3.3 Dendritic cell origin

Although extensively investigated, the origin of the different DC sub-populations is still controversial. As all other blood cells, DCs have their ultimate origin in a hematopoietic stem cell (HSC) progenitor. One of the earliest steps of hematopoietic differentiation is to a common myeloid progenitor (CMP) or to a common lymphoid progenitor (CLP) (Akashi, 2000; Kondo, 1997). Already at this early stage, it is still not known at which point the DC subtypes diverge. For a long time it was believed that all DCs were derived from myeloid origin, since they have several similarities with macrophages and can even be differentiated from monocytes. Only after a series of several studies it was finally shown that CLP and CMP can give rise to both “conventional” and “lymphoid” DCs (Manz, 2001; Wu, 2001; Traver, 2000). The current view is that most of the DCs present in the peripheral lymphoid tissues are from myeloid origin and that many thymic DCs derive from an early T-lineage precursor. In accordance with this idea, it was shown that around half of the DCs found in the thymus, but only a small number of DCs residing in the spleen and lymph nodes, have immunoglobulin heavy-chain gene D-J rearrangements (Corcoran, Ferrero et al. 2003). Interestingly, about half of all pDCs, independent on the tissue where they are found, have such IgH D-J rearrangements (Corcoran, Ferrero et al. 2003; Shigematsu, Reizis et al. 2004). Because of these findings, DC subtype commitment seems to be dictated downstream of the early lymphoid or myeloid progenitors. Recently, a common bone marrow precursor of DCs and macrophages was isolated (Fogg, Sibon et al. 2006). This precursor has been shown to have the capacity to generate $CD8^-$ and $CD8^+$ DCs, but not pDCs. These findings indicate that the pDC pathway branches off before this common macrophage/DC precursor, but it still remains to be determined whether this precursor is the only route of DC development. While a precursor able to give rise to

$CD8^-$ and $CD8^+$ DCs but not pDCs has been described, there is no identification of a pDC precursor unable to produce $CD8^-$ and $CD8^+$ DCs. The current model for pDC development in spleen and lymph nodes suggests a bone marrow progenitor, but this still remains to be determined.

3.4 Autoimmunity

The immune system possesses the important function of protecting the host against infectious diseases and tumors, but in the event of failure of self-tolerance, the immune responses can be redirected against autologous antigens, leading to the development of autoimmune diseases. How self-tolerance fails and self-reactive lymphocytes are activated are fundamental issues in autoimmunity and likely the basis for understanding mechanisms of tolerance. The knowledge of autoimmune diseases cause and development has increased greatly in the last two decades, mainly because of the development of a variety of animal models and the identification of genes that might be involved in and/or cause predisposition to a particular disease. Nevertheless, the etiology of most autoimmune diseases remains obscure and understanding these disorders is a major challenge in immunology. Autoimmunity is an important cause of disease in humans, representing, in developed countries, the third major cause of morbidity and mortality after cancer and atherosclerosis (Chatenoud 2006). The current therapeutic approach is essentially anti-inflammatory and/or immunossupressive therapy, which are not specific to the antigens involved in the pathogenesis. These therapies lead to global suppression of the immune system and as consequence, increase the risk of infection and carcinogenesis, as well as other serious side effects such as osteoporosis. Moreover, such broad immunosuppression is only of transient therapeutic benefit. These are the reasons for the growing attention towards new biological agents and methods, including immuno and gene therapy, which present a great potential for rescuing antigen-specific tolerance.

3.5 Immunotherapy

Immunotherapy is the treatment of a disease with therapeutic agents, as for example antibodies, cytokines or (modified) cells of the immune system that promote or

inhibit immune responses. Immunotherapy has primarily been applied to treat several different types of cancer. One example of immunotherapy to treat autoimmune diseases or avoid transplant rejection is the use of proteins, such as interferons (IFNs) and several different cytokines, and the use of monoclonal antibodies against leukocyte specific antigens. Examples of such antibodies include anti-CD3 (Herold, Hagopian et al. 2002; Belghith, Bluestone et al. 2003; Chatenoud 2003; Trucco 2005), anti-CD40 ligand alone or in combination with anti-CTLA-4 (Larsen, Elwood et al. 1996; Kirk, Harlan et al. 1997; Abbas 1999; Kirk, Burkly et al. 1999), anti-CD52 (Keating, Flinn et al. 2002; Cohen and Nagler 2004), anti-CD4 (Moreland, Pratt et al. 1995; Choy, Schantz et al. 1998; Schulze-Koops and Lipsky 2000) and anti-TNF (Feldmann 2002). The consequence of this approach is generalized immunosuppression and other associated risks, since not only the auto-reactive lymphocytes are targeted. Moreover, cytokines and antibodies are expensive and have a short half-live, necessitating frequent administration. Furthermore, when the treatment stops the disease may rebound (van der Meide, de Labie et al. 1998). One potential alternative in achieving tolerance by eliminating only the pathogenic cells includes gene therapy. For example, DCs can be genetically modified with the objective to rescue self-tolerance leaving the other functions of the immune system unperturbed.

3.5.1 Gene therapy

Gene therapy comprises the delivery of new genetic material through different vectors into the cells of an individual for therapeutic purposes. In basic research, the same methodology can be used with investigatory objectives. To date, more then 3000 patients have already been treated with gene therapy worldwide (American Society of Gene Therapy, 2007). Several children with SCID have been treated with this methodology, where a retroviral vector was used to deliver a functional copy a defective gene in some HSC, which reconstituted the lymphoid system and cured the immunodeficiency (Cavazzana-Calvo, Hacein-Bey et al. 2000; Aiuti 2002; Gaspar, Parsley et al. 2004). Unfortunately, some of the children with the X-linked form of the disease (X-SCID) were later diagnosed with T-cell leukemia, considered to be a

consequece of the activation of the oncogene LMO2 as a result of retrovirus vector integration (Hacein-Bey-Abina, Von Kalle et al. 2003). Although no similar effects were found in children with another kind of SCID, this result raised serious concerns about the safety of the technique. The syndrome X-SCID is caused by faulty expression of the γ-chain of the interleukin-2 receptor (IL2RG) and gene therapy is used to restore IL2RG. Recently, it was shown in a murine model of X-SCID that the gene IL2RG itself can contribute to the development of T cell lymphoma and not the vector insertion in the genome (Woods, Bottero et al. 2006). However, the validity of this observation has been questioned concerning its extrapolation to humans (Pike-Overzet, 2006; Thrasher, 2006). While the safety of gene therapy is still controversial and the development of improved therapeutic viral vectors is of fundamental importance, gene therapy still reflects an important tool in the treatment of diseases that cannot be treated by standard therapies or for which treatment causes severe side-effects.

3.5.1.1 Commonly used vectors in gene therapy

Efficient gene delivery is central to the success of gene therapy. Non-immunogenic vectors are required because otherwise the cells transfected or tranduced with these vectors, and consequently their beneficial effect, are destroyed. Besides DNA, which can be delivered to cells either naked or complexed with liposomes, viral vectors are preferable vehicles as they have several advantages, such as intrinsic mechanisms for cell entry (DNA needs transfection techniques like electroporation or gene gun), integration into the host genome and long-term expression. Viral vectors commonly used in gene therapy include those derived from adenovirus, adeno-associated virus (AAV), herpes simplex virus (HSV), retrovirus and lentivirus (for features of the main vectors used in gene therapy see table 3). Lentivirus is part of the retrovirus family, but with the aim of simplicity, commonly in gene therapy the term lentivirus is used as an independent virus type. As the vectors derived from retroviruses and lentiviruses are the only vectors that mediate DNA insertion into the host genome, and the aim of this work was to modify HSCs permanently so that their progeny would still present the transgene, the following background information and methodology will be restricted to these two vectors.

Vector	Vector genome	Transgene capacity	Immu no-genici -ty	Genomic integration	Duration of expression	Advanta -ges	Disadvanta-ges
Naked DNA	DNA	Unlimited	Low	No	Short term	Easy, fast and cheap to produce; support large transge -nes	Lacks intrinsic mechanisms for cell entry; short term expression
Adeno-virus	DNA	30 Kb	High	No	6 weeks	Highly stable; support large transge-nes; can be produ -ced at high titers	Does not infect lymphocytes; more than 50% of humans have pre-existing antibodies
Adeno-associa -ted virus	DNA	4.5 Kb	low	possible	Long term	Infect qiescent cells; site specific integra -tion in the host genome	Low transgene capacity; low rate of integration in the host genome
Herpes simplex virus	DNA	50 Kb	High	No	At least 6 months	Support large trans -genes; infect neuronal cells	Induces cellular toxicity and inflammation; time consuming production
Retro-virus	RNA	7-8 Kb	Low	Yes	For the life of the cell	Integra -tion in the host genome	Can cause insertional effects; small transgene capacity; does not infect quiescent cells
Lenti-virus	RNA	7-8 Kb	Low	Yes	For the life of the cell	Integra-tion in the host genome; Infect qiescent cells; can be produ -ced at high titers	Can cause insertional effects; small transgene capacity

Table 3: Features of the vectors used in gene therapy. Modified from Chernajovsky, 2004.

3.5.1.2 Retroviral and lentiviral vectors

3.5.1.2.1 Retrovirus

Retroviruses are enveloped viruses containing a single stranded RNA molecule as a genome. Following infection, the viral genome is reverse transcribed into double stranded DNA, which integrates into the host genome and can express viral proteins (Fig. 1, top). The viral genome is approximately 10 Kb, containing three genes: *gag*, coding for core proteins; *pol*, coding for reverse transcriptase; and *env*, coding for the viral envelope protein. At each end of the genome are long terminal repeats (LTRs) which include promoter/enhancer regions and sequences involved in genomic integration. In addition, there are sequences required for packaging the viral RNA (Ψ or psi) and RNA splice sites in the *env* gene.

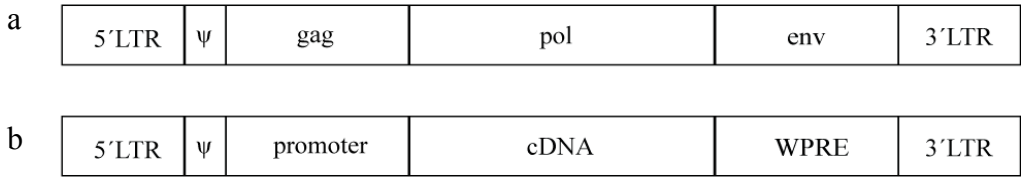

Figure 1. Schematic representation of a retrovirus (MoMLV) and a retroviral vector. (a) Wild type retrovirus contains genes encoding viral elements. (b) Some of the genes present in the retrovirus are replaced with cDNA encoding gene of interest, giving rise to the retroviral vector. LTR, long terminal repeat; MoMLV, Moloney murine leukaemia virus; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

Standard retroviral vectors are mostly based on Moloney murine leukaemia virus (MoMLV), which have the viral genes (gag, pol and env) replaced with the transgene of interest (Fig.1). As these viral genes are essential for virus production, they are expressed on plasmids in the packaging cell line. Transgene expression can be driven by the promoter/enhancer region in the 5´LTR or by alternative viral or cellular promoters. Though transgene expression is usually adequate, prolonged expression is difficult to mantein because the viral promoters tend to be inactivated. To avoid this silencing mechanism the use of host cell promoters has been a valuable approach. The use of cell specific promoters has other advantages as well, as it allows the expression of the transgene to be restricted to target cells or tissues of choice and at physiological levels. The cell-specific transcriptional targeting can be problematic since the viral promoter/enhancer present in the LTR interferes with the activity and regulation of cis-

acting elements inserted in the virus backbone between the two LTRs (Emerman, 1984; Emerman 1986). To overcome this problem the use of self inactivating (SIN) retro- and lentiviral vectors has become a popular tool in gene therapy. SIN vectors lack the regulatory elements present in the U3 region of the 3´LTR of the viral RNA genome and after the process of reverse transcription to DNA and integration into the host genome, the internal promoter is the only one able to give rise to transcripts (fig 2; Yu, 1986; Yee, 1987).

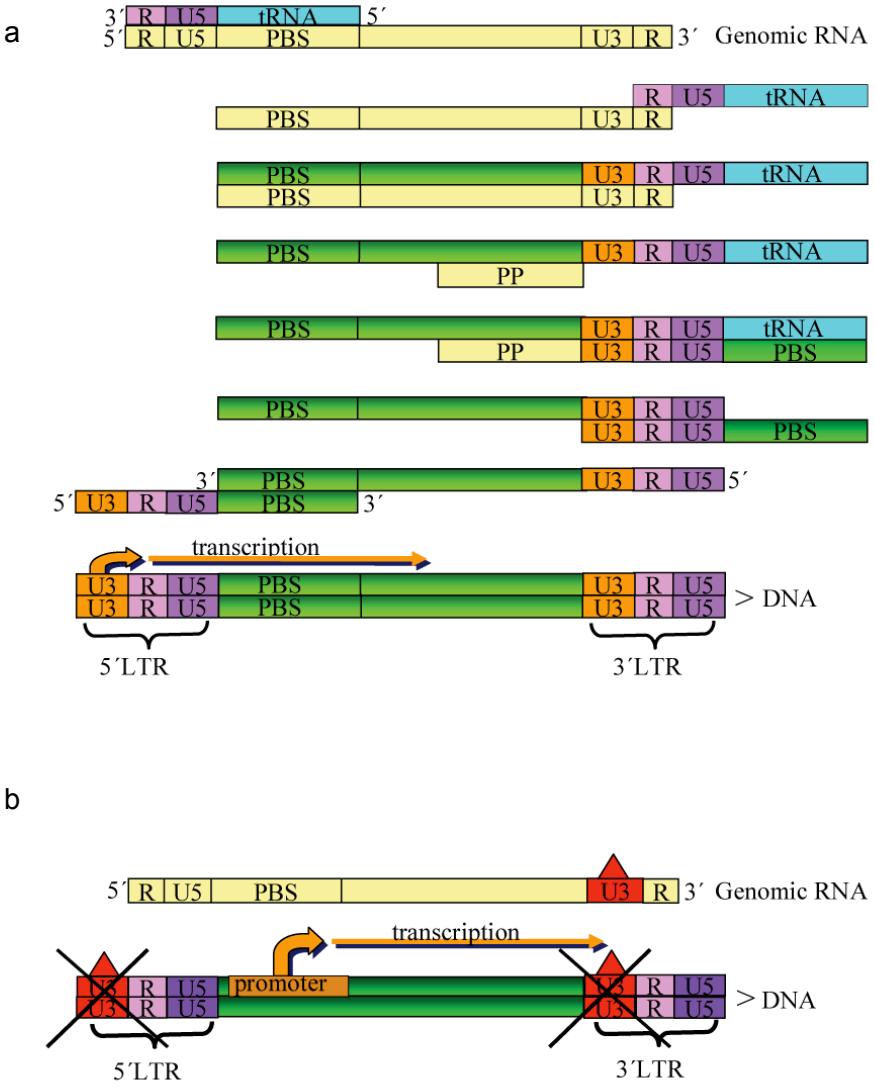

Figure 2. Schematic representation of reverse transcription of a retroviral genome. (a) The genomic RNA of the retrovirus (yellow) is packed in the virion with a retrovirus-specific cellular tRNA (blue) hybridized to its PBS. This tRNA works as a primer that gives rise to the process of transcription of the viral RNA into DNA through the enzyme reverse transcriptase. RNaseH digests the RNA strand in a DNA-RNA hybrid. The entire process consists in serial events of reverse transcription, RNA digestion and "jump"/anneling to complementary sequences. The process yields a double strand DNA with identical

LTRs at each end. (b) The same process of reverse transcription occurs when the genomic RNA is deriving from a self inactivating retroviral vector, resulting in deletion in both LTRs (in red). LTR, long terminal repeat; PBS, primer-binding site; PP, polypurine tract; tRNA, transfer RNA; ▲, deletion. Modified from Coffin et al, 1997.

Viruses differ with respect to their tropism. Therefore, by replacing the *env* gene with that of another virus, the host range can be extended by a technique known as pseudotyping. Frequently the vesicular stomatitis virus G (VSVG) protein is used as the envelope, since it is relatively stable and its tropism is broad. Regarding the expression of the transgenes, it is unknown for both retro- and lentiviruses if it is necessary for the RNA to be either unspliced or partially spliced to be efficiently exported to the cytoplasm. As it is difficult to place splice sites in the virus vector backbone without affecting the efficacy of virus production, the Woodchuck hepatitis virus posttranscriptional element (WPRE) can be employed. WPRE stimulates nuclear exportation of intronless RNA, improving transgene expression from retro- or lentiviral vectors (Donello, Loeb et al. 1998; Zufferey, Dull et al. 1998). One requirement for retroviral integration is that the target cells is dividing. This restricts the use of this kind of vector to cells that are able to proliferate and excludes its use in non-dividing target cells such as hematopoietic stem cells and neurons. To overcome this limitation, lentiviral vectors can be used.

3.5.1.2.2 Lentivirus

Lentiviruses are a subclass of retroviruses, which are able to infect both proliferating and non-proliferating cells. They can integrate into the genome of non-proliferating cells due to two virion proteins: *matrix* and *vpr*. These proteins interact with the nuclear import machinery and mediate the active transport of the viral pre-integration complex through the nucleopore (Bukrinsky, MI et al. 1993; Naldini, L et al.1996). Lentiviruses have a high complexity and additional genes including *tat*, *rev*, *vpr*, *vpu*, *nef* and *vif*. The production of lentiviruses differs from the production of retroviruses in regard to the packaging cell lines. In the packaging cell lines used for lentiviral production, viral genes are not kept permanently but are transiently induced via plasmids providing the pseudotyped env gene and the structural and regulatory genes

in trans. The transgene construct, however, is similar to that used to produce MoMLV based retrovirus vectors. Current lentiviral vectors are derived from the human immunodeficiency virus (HIV) and their safety profile seems to be approximately the same as for the retroviral vectors.

3.5.2. Dendritic cells and immuno/gene therapy

DCs play essential roles in both priming immune responses and in generation of central and peripheral tolerance. While DCs ability to initiate and stimulate effector cells have been extensively exploited in anti-tumor therapy, the regulatory functions of DCs in maintaining tolerance have generated considerable interest in harnessing them for Ag-specific immunotherapy of autoimmune diseases, allergic hyper-sensibility and transplantation. Indeed, several attempts using DCs for the treatment of cancer and autoimmune diseases have shown promising results. Among the different approaches, one of the most widely applied consists of loading DCs isolated from peripheral blood or differentiated from monocytes or bone marrow precursors, with a known Ag and then transferring them to the individual to be treated. Administration of genetically modified DCs with genes encoding immunoregulatory molecules or the Ag involved in the immune response is also an attractive strategy to circumvent undesired and/or exaggerated immunity. This approach has been used for example with: (I) DCs expressing Fas ligand, prolonging cardiac allograft survival in mice (Min, Gorczynski et al. 2000), (II) DCs expressing IL-4, resulting in suppression of murine arthritis (Kim, Kim et al. 2001), (III) and DCs expressing IL-12p40 or IL-10, suppressing collagen-induced arthritis (Nakajima 2006). Besides the difficulty of obtaining a sufficient amount of cells, a very negative aspect of these methodologies is the extensive manipulation and consequently undesired modification of the DCs. Moreover, considering that different subpopulations of DCs play specific functions in the immune system and that it is still not known how to differentiate or isolate all these different cells, the results obtained by such *ex vivo* manipulation are even more uncertain. Current possibilities to modify DCs *in vivo* without such a need for extensive handling are: (I) DCs targeted *in vivo* through antibodies against receptors expressed

mainly by DCs, as in the case of anti-DEC-205 antibodies conjugated to a protein to be processed and presented in context of the MHC (Bonifaz, 2002), (II) virus vaccination to transduce DCs *in vivo* (He, 2006), or (III) promoters that drive transgene expression specifically in certain DC populations, such as the fascin promoter that transcriptionally targets gene expression to cutaneous mature DCs (Ross, Sudowe et al. 2003). The disadvantages of these techniques are lack of long term transgene expression, unwanted immune responses against the virus used in the vaccination, and targeting of only some DC populations or states of maturation, which might result in restricted immune responses. Although all available techniques to modify DCs with clinical objectives present some faults, the positive results already achieved in the treatment of immune disorders have encouraged immunologists to continue investing this field, but have also reinforced the necessity of improvement of the current methodologies.

3.6 Goals of the project

The two basic objectives underlying this work were:

(I) Transcriptionally target gene expression to DCs through the use of a viral vector suitable for gene therapy;

(II) to test the use of the developed viral vector for induction of antigen-specific tolerance *in vivo*.

The project involved the following main steps:

(I) Identification of a DC-specific promoter and its ability to drive transgene expression in different DC subpopulations *in vivo*;

(II) Comparison of retrovirus and lentivirus as optimal vectors;

(III) Test the efficiency of the system in inducing CD4 and CD8 T cell tolerance *in vivo* in different murine strains;

(IV) Test the functionality of the system in human DCs *in vitro*.

4. Material and Methods

Both material and methods are listed by alphabetical order.

4.1 Material

4.1.1 Antibodies

Specificity (anti-mouse)	Conjugate	Clone	Source of supply
B220	FITC	RA3-6B2	BD Pharmingen (San Diego, CA, USA)
CD3	PE	17A2	BD Pharmingen
CD4	PE	H129.9	BD Pharmingen
	PerCP		
CD8	PerCP	53-6.7	BD Pharmingen
CD11b	PE	M1/70	BD Pharmingen
CD11c	APC	HL3	BD Pharmingen
CD19	PE	1D3	BD Pharmingen
	APC		
CD24	PE	M1/69	BD Pharmingen
CD25	PE	PC61	BD Pharmingen
CD44	PE	IM7.8.1	Caltag
	APC	IM7	BD Pharmingen
CD45.1	FITC	A20	BD Pharmingen
CD62L	FITC	Mel14	BD Pharmingen
	APC		
CD69	PE	H1.2F3	BD Pharmingen
DX5	PE	DX5	BD Pharmingen
Foxp3	APC	FJK-16s	eBioscience (San Diego, CA, USA)
Gr-1	PE	RB6-8C5	BD Pharmingen
I-A^b	FITC	AF6-1201	BD Pharmingen
	PE		
NK1.1	APC	PK136	BD Pharmingen
PDCA-1	PE	JF05-1C2.4.1	Miltenyi Biotec
Vα2 TCR	FITC	B20.1	BD Pharmingen
	PE		
Vβ5.1/5.2 TCR	FITC	MR9-4	BD Pharmingen
	PE		
Specificity (anti-human)	**Conjugate**	**Clone**	**Source of supply**
CD1a	APC	HI149	BD Pharmingen
CD14	PerCP	M5E2	BD Pharmingen
CD19	APC	HIB19	BD Pharmingen

Tabele 3: Antibodies used in flow citometry. All antibodies were titrated before use.

The MHC tetramers H-2k^b/SIINFEKL (OVA257-264), H-2k^b/SSIEFARL (HSVgB498-505) and APC- conjugate were purchased from ProImmune (Oxford, UK).

4.1.2 Chemicals

All buffers and solutions were prepared using double distillated water. If not stated differently, all chemicals (maximal degree of purity) were purchased from Merck (Darmstadt), Roth (Karlsruhe) or Sigma (St. Louis, MO, USA).

4.1.3 Consumable supplies

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 26 G x 1/2“ (Terumo Medical Corporation, Tokyo, Japan), disposable syringes (Braun, Melsungen, Germany), reactions container 0,2 ml (Nunc, Wiesbaden,Germany), reactions container 1,5 ml und 2 ml (Eppendorf, Hamburg, Germany), reaction tubes 5 ml (Becton, Dickinson & Co., Franklin Lakes, NJ, USA), reaction tubes 15 ml und 50 ml (Greiner, Frickenhausen, Germany)

Other materials and plastic wares were purchased from Falcon, Becton Dickinson (Franklin Labs. NJ, USA), Nunc (Wiesbaden, Germany) und Greiner (Frickenhausen, Germany).

4.1.4 Devices

Analytic scale (Adventurer, Ohaus Corp., Pine Brooks, NJ, USA), bench centrifuge (Centrifuge 5415 D, Eppendorf, Hamburg, Deutschland), “β-Counter“ (Wallac, Perkin Elmer, Turku, Finnland), centrifuge (Rotixa RP, Hettich, Tuttlingen, Deutschland), chemical scale (Kern, Albstadt), Flow cytometer (FACSCalibur von Becton Dickinson), incubator (Hera cell, von Heraeus Kendro Laboratory Products, Hanau, Deutschland), laminar airflow cabinet (Heraeus), magnetic stirrer (Ika Labortechnik, Staufen, Deutschland), PCR-machine (Biometra) pH-Meter (Inolab, Weilheim, Deutschland), pipettes (Gilson, Middleton, WI, USA), automatic pipettors (Integra Biosciences, Baar, Schweiz), power Supply (Amersham Pharmacia, Piscataway, NJ, USA), vacuumm pump (KNF Neuberger, Munzingen, Deutschland), vortex-Genie2 (Scientific Industries,

Bohemia, NY, USA), water bath (Grant Instruments Ltd., Barrington Cambridge, England). All other devices are mentioned in “methods” section.

4.1.5 Medium and solutions

ACK-Buffer	8,29 g NH_4Cl 1 g $KHCO_3$ 37,2 mg Na_2EDTA H_20 ad 1 l pH 7,2-7,4 adjusted with1 N HCl and sterilized by 0,2µm filtration
PBS	150 mM NaCl 10 mM Na_2HPO_4 2 mM KH_2PO_4 pH 7,4 adjusted with 5 N NaOH
PBS-FBS	Dulbecco’s PBS (Invitrogen, San Diego, CA,USA) without Ca^{2+}/Mg^{2+} 2% FBS (v/v) (Invitrogen, San Diego, CA, USA)
FACS-buffer	PBS 2% FBS (v/v) 0,01% NaN3 (v/v)
5-Fluoro-uracil (5-FU)	20 mg/ml in Dulbecco’s PBS (Gibco) pH 10-11 adjusted with NaOH vortexed until completely dissolved pH 7,5 adjusted with HCl Sterilized by 0,2µm filtration Stored at –20°C
MACS-buffer	Dulbecco’s PBS (Invitrogen, San Diego, CA, USA) without Ca^{2+}/Mg^{2+}

	0,5 % BSA (m/v)
	pH 7,4 adjusted with 5 N NaOH
RFI	15% Glycerin (v/v)
	100 mM KCl
	50 mM $MnCl_2$
	30 mM C_2H3KO_2
	10 mM $CaCl_2$
	pH 5,8 adjusted with 0,2 mM acetic acid
	Sterilized by 0,2µm filtration
	Stored at 4°C
RFII	15% Glycerin
	10 mM MOPS
	10 mM KCl
	75 mM $CaCl_2$
	pH 6,8 adjusted with 1 N NaOH
	Sterilized by 0,2µm filtration
	Stored at 4°C
50x TAE-buffer	242g Tris
	57,1 ml 100% (v/v) acetic acid
	100 ml 0,5 M EDTA (pH 8,0)

<u>Solutions used for transfection</u>

2xHBS	50 mM HEPES
	280 mM NaCl
	1,5 mM Na2HPo4-Dihydrat
	pH 7,05 adjusted with NaOH
	Sterilized by 0,2µm Filtration
	Stored at –20°C (≤ 6 months)
$CaCl_2$	2,5 M $CaCl_2$
	Sterilized by 0,2µm filtration
	Stored at –20°C

Cell culture media

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

DC-Medium	Iscove's Modified Dulbecco's Medium (IMDM) 5% FBS (inactivated, v/v) 500 mM β-Mercaptoethanol 100 U/ml Penicillin 100 µg/ml Streptomycin 25 ng/ml GM-CSF
Freezing-Medium	90% FBS 10% DMSO
HSC-Medium	*Stemline hematopoietic stem cell expansion medium* (Sigma-Aldrich, St. Louis, USA) 100 U/ml Penicillin 100 µg/ml Streptomycin 50 ng/ml hIL-6 10 ng/ml mIL-3 50 ng/ml mSCF
Phoenix-Medium	Dulbecco's Modified Eagle Medium (DMEM) with Glutamax-I 10% FBS (inactivated, v/v) 100 U/ml Penicillin 100 µg/ml Streptomycin
Phoenix-transfection medium	same as Phoenix-medium, plus 2.5mM Chloroquine (Sigma)
293T-Medium	DMEM Glutamax-I

	10% FBS (inactivated, v/v) 100 U/ml Penicillin 100 µg/ml Streptomycin 0.1 mM MEM non essencial aminoacids 10 mM HEPES 500ug/ml Geneticin 2 mM L-glutamin
293T-transfection medium	same as 293T medium, without geneticin

4.1.6 Mouse strains

All mice were maintained in the mouse facilities of the Institute of Immunology. LMU, Munich

C57BL/6 and B6SJL

The MHC-haplotype of this mouse strain is H-2^b. Mice from the C57BL/6 strain express the allele Ly5.2 in all leukocytes. The congenic strain B6SJL is genetically identical to the C57BL/6 strain, except for expressing the allele Ly5.1 in all leukocytes.

OT-I

OT-I mice express a transgenic Va2/Vb5 TCR specific for the $OVA_{257–264}$ peptide in the context of MHC-I H2-K^b (Hogquist, Jameson et al. 1994). These mice were bred onto the C57BL/6 and B6SJL background, i.e., the OT-I cells express the allele Ly5.2 and Ly5.1, respectively.

OT-II

OT-II mice have a transgenic Va2/Vb5 TCRs specific for the $OVA_{323-339}$ peptide that can be recognized in the context of MHC-II I-A^b. (Robertson, Jensen et al. 2000). These mice were bred on the C57BL/6 background.

RIP-OVA_{lo}

RIP-OVA_{lo} mice express a membrane-bound form of OVA under control of the rat insulin promoter [RIP (Blanas and Heath 1999)]. In the pancreas and testis OVA is

expressed as a model auto-antigen. When RIP-OVA_{lo} mice receive OT-I cells and are immunized, they develop diabetes. The progress of diabetes can be monitored by measuring the glucose concentration in the urine (Diabur 5000, Roche Diagnostic, Rotkreuz, Switzerland).

4.1.7 Peptide, Protein and Oligonucleotides

Chicken-ovalbumin (OVA *albumin, chicken egg*, *Grade* V) was purchased from Sigma (St. Louis, MO, USA). The peptides $OVA_{257-264}$ and HSVgB $_{498-505}$ were purchased from Neosystems (Strassburg, France)

The following nucleotides were purchased from MWG-Biotech AG (Ebersberg, Germany)

Bdnf forw: 5'-ACGACATCACTGGCTGACAC-3'

Bdnf rev: 5'-CATAGACATGTTTGCGGCATC-3'

DC-STAMP forw: 5'-GCTGAGAGGCCTGAAAACAC-3'

DC-STAMP rev: 5'-CAGAGAGTACTTTTAAACCTGTCTTCT-3'

qPCR forw: 5'-TGAAAGCGAAAGGGAAACCA-3'

qPCR rev: 5'-CCGTGCGCGCTTCAG-3'

All sequencings were carried out by Sequiserve (Vaterstetten, Germany).

4.1.8 Vectors

4.1.8.1 Cloning vector

For subcloning, the Plasmid pBluescript-II-KS^+ (pBS; Stratagene, Amsterdam, Netherlands) was used.

4.1.8.2 Herpes Simplex Vector

The recombinant, replication deficient vector HSV-OVA was produced by P. Marconi (University of Ferrara, Italy).

4.1.8.3 Viral Vectors

Retroviral vectors

The retroviral vector used in this work was constructed based on SIN-SF (Kraunus, Schaumann et al. 2004). In this vector, the promoter/enhancer-containing region located in the 3'LTR was deleted. To generate DCSTAMP-eGFP-SIN-retrovirus, the DC-STAMP promoter was amplified by PCR from total genomic DNA of C57BL/6 mice using specific oligonucleotide primers (DC-STAMP forw and DC-STAMP rev) to amplify a 2552bp-fragment. The latter was digested with BbsI resulting in a product of 1704bp covering the region between -1565bp and +131, considering +1 as the first base pair of transcription initiation of DC-STAMP. This promoter containing region was cloned into SIN-CD19-TfrOVA-W (Werner-Klein, Dresch et al. 2007), previously digested with *Not* I/Klenow blunt ended/*Nru* I.

Lentiviral vectors

The lentiviral vectors used in this work are based on FUGW. In this vector, the promoter/enhancer-containing region located in the 3'LTR was deleted (Lois, Hong et al. 2002). To generate DCSTAMP-eGFP-SIN-lentivirus, the DC-STAMP promoter was isolated from DCSTAMP-eGFP-SIN-retrovirus through *Pst* I/Klenow blunt ended/*Age* I. This sequence was cloned into FUGW, that was digested with *Pac I*/Klenow blunt ended/*Age* I. DCSTAMP-mock-SIN-lentivirus was generated by digesting DCSTAMP-eGFP-SIN-retrovirus with *Xba* I/*Age* I/ Klenow blunt ended and followed by religation. To generate DCSTAMP-trOVA-SIN-lentivirus, a plasmid containing the chimeric transferrinreceptor-OVA-cDNA (produced by Henning Lauterbach, subcloned into pBS and designated here trOVA-pBS), was digested with *Sac* II/Klenow blunt ended/*Eco* RI. This cDNA was then cloned into DCSTAMP-eGFP-SIN-lentivirus, which was digested with *Age* I/ Klenow blunt ended/ *Eco* RI.

4.2 Methods

4.2.1 Cellular and immunological methods

4.2.1.1 Adoptive cell transfer

This method allows tracing antigen specific T cells *in vivo*. The T cell population of interest is isolated from spleen and/or lymph nodes of a donor and transferred into the

recipient in sufficient amounts to be detected by flow cytometry. MACS (negative selection) was used for the isolation of the T cells to be transferred. The purity of T cells was determined, before transfer, by flow cytometry.

4.2.1.2 Cell culture

4.2.1.2.1 Culture and transduction of HSC

Bone marrow cells of at least 6 weeks old C57BL/6, OT-I or OT-II mice were harvested 4 days after intravenous (i.v.) injection of 5-FU (150 mg/kg body weight, Amersham Pharmacia, Uppsala, Sweden). The cells were cultured in 100 mm plates in a total amount of $10x10^6$ cells/10 ml at 37°C and 5% CO_2. Before tranduction, the cells were prestimulated for 2 days in serum-free Stemline Hematopoietic stem cell expansion medium (Sigma-Aldrich, St. Louis, USA), supplemented with penicillin-streptomycin (Gibco BRL, Invitrogen Corporation, Carlsbad, CA) and a growth factor cocktail containing human IL-6 (25 ng/ml), murine IL-3 (10 ng/ml) and murine SCF (50 ng/ml). Recombinant growth factors were purchased from Strathmann Biotech (Hannover, Germany). Cells were transduced by spin-infection (300xg, 2 hours, 32°C) with cell-free stocks of lentiviral vectors (MOI of 1) in the presence of protamine sulfate (4 µg/ml). If desired the transduction procedure was repeated 20-26 hours after the first round.

4.2.1.2.2 Culture of dendritic cells

For differentiation of DCs *in vitro*, $1x10^6$/ml bone marrow cells were cultured in DC-medium, in a total amount of 10 ml per 100 mm plate at 37°C and 5% CO_2. Each 2-3 days, fresh medium was added. DCs are viable under these conditions until day 9 of culture. When desired, transduction was performed at day 2 of culture with $1x10^6$ cells/ml, in a total of 2 ml per well in 6 well plates. The transduction protocol was the same for NIH3T3 cells. The human bone marrow cells (Cambrex, Walkersville, USA) were differentiated into DCs *in vitro* in RPMI medium supplemented with penicilin-streptomicin, 10% foetal bovine serum and a cytokine cocktail containing human GM-CSF (100ng/ml), IL-4 (20ng/ml) and TNF-α (20ng/ml), all purchased from Strathmann Biotech (Hannover, Germany).

4.2.1.2.3 Culture of 293T, Phoenix-eco and NIH3T3 cells

Phoenix-eco and NIH3T3 cells were cultured in Phoenix-medium on 100 mm cell culture plates at 37°C and 5% CO_2. 293T cells were cultured in 293T-medium on 100 mm cell culture plates at 37°C and 10% CO_2. All cells were split so that a confluence of less than 75% was mantained. Phoenix-eco cells stored at -180 °C longer than 6 months, were selected during 2 weeks with 1 µg/ml Diphteria-Toxin (Calbiochem-Novabiochem, San Diego, CA, USA) and 500 µg/ml Hygromycin B (CNbiosciences LTD., Beeston, UK). In the case of 293T, only cells with less than 30 passages were used.

Management of NIH3T3 cells for virus titration

NIH3T3 cells were plated at a concentration of $4x10^4$ cells/well in 24-well cell culture plates 18-24 hours before transduction. A total of 9 wells per virus stock to be titrated were necessary to achive dilutions of 1/10, 1/50, 1/100 1/500, 1/1000, 1/5000, 1/10.000, 1/25.000 and 1/50.000. An additional 3 wells were plated for counting the number of cells per well at the time of transduction. Each well was transduced with 500-1000ul of virus supernatant dilution in the presence of 8 ug/ul of polybrene (Hexadimethrine-Bromid, Sigma, St. Louis, MI, USA). The plates were centrifuged at 300g, 32°C for 2 hours and incubated at 32°C and 5% CO_2 for a further 4 hours. The virus supernatant was then replaced with Phoenix-medium. After 24-48 hours, cells were harvested with Trypsin/EDTA and total genomic DNA was extracted for qPCR analysis.

4.2.1.3 CFSE staining

CFSE (*carboxyfluorescein-diacetate-succinimidylester)* staining is used with the aim of tracking cell division both *in vitro* and *in vivo*. CFSE binds to intra and extra cellular proteins and after each cell division, the dye is divided between the daughter cells and the intensity of the fluorescence (analyzed by flow cytometry) is reduced 50%. The number of cell divisions can be identified by the number of times that the stain was reduced by half. For the staining procedure, the single cell suspension to be labeled is depleted of erythrocytes (with ACK buffer), and washed two times with PBS. The cell pellet is resuspended in PBS (without FBS, since this inhibits the staining reaction) and 5 uM CFSE is added per 1-$50x10^6$ cells. The cells are incubated for 10 minutes at 37°C

and protected from light. The reaction is stopped by addition of equal amount of FBS. The cells are washed 2 times with PBS and resuspended in the desired amount of PBS or culture medium.

4.2.1.4 Extraction of blood and harvest of organs from mice

a) Lymphocyte enrichment from peripheral blood

Before blood extraction, the mice were kept under an infrared lamp to achive vasodilatation. A small cut was made in the tail so that 3-10 drops of blood could be extracted and mixed with 50 µl of Heparin-sodium (25000 I.E./5 ml, Ratiopharm, Ulm, Germany). Next, 2 ml of FACS buffer was added and mixed into each blood sample and, 1 ml of lymphocyte separation medium (PAA Laboratories, Linz, Austria) was added slowly to the bottom of the tube, so that the blood/buffer suspension was located on the upper part of the reaction tube. After centrifugation (30 minutes at 25°C and 450g), the lymphocytes were harvested from the intermediate phase. The lymphocytes were then washed and resuspended in 50 µl of FACS buffer.

b) Organs harvesting and preparation of single cell suspension

Lymph nodes and spleen were harvested with fine tweezers and kept in FACS buffer on ice. For single cell preparation, organs were placed in a 100 µm cell strainer (BD Biosciences, Erembodegem, Belgium) and smashed through with a syringe plunger. The cells were resuspended in FACS buffer and centrifuged for 5 minutes at 4°C and 300 g. The cells extracted from lymph nodes could be then resuspended in the desired amount of buffer or medium. Spleen cells were depleted of erythrocytes with ACK buffer.

Bone marrow was extracted from femurs and tibias of mice. The extremities of the bones were cut off with scissors and the bone marrow was flushed out with medium using a needle and syringe. Cells were centrifuged for 5 minutes at 4°C and 300 g and resuspended in culture medium.

c) Erythrocyte lysis

After centrifugation, the single cell pellet was resuspended in 4 ml of ACK buffer and left for 4 minutes at RT. Afterwards, 10 ml of FACS buffer was added and the cell

suspension was centrifuged for 5 minutes at 4°C and 300 g and then resuspended in culture medium or FACS buffer.

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

Flow cytometry permits simultaneous measurements of multiple parameters in single cells. Specific molecules or cluster of differentiation (CD) that are differentially expressed in certain leukocyte sub-populations, can be assessed by staining with fluorochrome-coupled monoclonal antibody specific for the surface molecules of interest.

Staining procedure

The identification of the cell populations and subpopulations using different antibodies were made according to a FACS marker profile for each cell type. Before staining, 50μl of a cell suspension was washed in 5 ml FACS-buffer at 300 g for 5 minutes. The supernatant was discarded and cells were resuspended in 100μl of antibody-containing buffer. The tubes were then incubated in the dark at 4°C for 20 minutes. The cells were washed 2 times to remove the excess of unbound antibodies and the supernatant discarded. Before acquisition, 200μl of PBS was added to the tubes. When intracellular staining was necessary, the intracellular Staining Set (eBioscience, San Diego, CA, USA) was used and staining was performed according to instructions of the manufacturer. The measurements were performed using a FACSCalibur^{TM}-Flow Cytometer (Becton, Dickinson & Co., Franklin Lakes, NJ, USA) with two lasers. The data was acquired with CellQuest Software, Version 3.4 (Becton, Dickinson & Co., Franklin Lakes, NJ, USA) and analyzed with CellQuest- or FlowJo -Software (TreeStar, Ashland, OR, USA).

4.2.1.6 Generation of bone marrow chimeras

Bone marrow cells of at least 6 week old C57BL/6, OT-I or OT-II mice were harvested 4 days after intravenous (i.v.) injection of 5-FU (150 mg/kg body weight, Amersham Pharmacia, Uppsala, Sweden). The cells were stimulated for 2 days in serum-free HSC medium. Cells were transduced and after the final transduction 1-3x10^6 cells/mouse were injected i.v. into lethally irradiated (550rad day -2 and day 0; Cesium-137, Model

G.C. 40; Type B (4); Atomic Energy of Canada Limited, Kanata ,Ontario, Kanada) C57BL/6 recipients. When stated, $CD8^+$ cells were depleted by magnetic sorting before injection. Recipient mice received drinking-water containing neomycin (1,17g/l) for 3 weeks after reconstitution.

4.2.1.7 Immunization

a) Immunization with antibody immuno-complexes

Mice were immunized with rIgGαOVA-ovalbumin (or rIgG in the mock controls) immune-complexes and 20μg/mouse of CpG nucleotides (InvivoGen, USA). The complexes were formed with 25μg of rIgGαOVA (ICN Pharmaceuticals, USA) and 1μg of ovalbumin (Sigma, USA) for 30 minutes at 37°C.

b) Immunization with recombinant Herpes Simplex Virus Type 1 (rHSV-1)

The stock of virus was thawed on ice and resuspended with ultrasonic waves for 10 seconds (Ultrason E, Greiner, Frickenhausen, Germany). The virus concentration was adjusted with PBS and $4x10^6$ pfu of rHSV-1 expressing OVA was injected i.v. per mouse.

4.2.1.8 *in vivo* killer assay

This method permits the evaluation of the cytotoxic effector function of $CD8^+$ T cells *in vivo* (Coles, Mueller et al. 2002). First, C57BL/6 erythrocyte-depleted splenocytes were incubated in the presence or absence of 10 μM of $OVA_{257-264}$ peptide or $HSVgB_{498-505}$ peptide for 2 h at 37°C and 5% CO_2. Peptide-loaded cells were labeled with a high (1.7 μM) concentration of CFSE (Molecular Probes, USA), whereas unloaded cells, used as internal control, were labeled with a low concentration (0.2 μM) of CFSE. Equal numbers of $CFSE^{high}$ and $CFSE^{low}$ cells were mixed and analyzed by flow cytometry. $2x10^7$ total cells/mouse were administered i.v. 15-18 h later, mice were sacrificed and spleen cell suspensions were analyzed for the loss of peptide coated population by flow cytometry. The specific lysis is calculated as follows:

Percentage of specific lysis (PSL)= 1- (r of unimmunized mouse/ r of immunized mouse) x 100.

r = (percentage $CFSE^{low}$/ percentage $CFSE^{high}$)

4.2.1.9 Magnetic cell sorting (MACS)

Magnetic cell sorting (MACS, Miltenyi Biotech, Bergisch-Gladbach, Germany) is a technique that allows isolation of different cell- subpopulations based on their expression of different antigens or CDs on the cell surface. For MACS separation, the mononuclear cells are incubated first with MACS colloidal super-paramagnetic MicroBeads conjugated to a specific monoclonal antibody with specificity towards the CD expressed by the cell-subpopulation to be isolated (positive selection) or to be eliminated (negative selection). 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. The MS column is used for positive selection for up to 10^7 cells. Labeled cells are retained by the magnetic field inside the column, while the unlabeled ones (negative fraction) are eluted. The column is washed three times with MACS buffer to remove the excess cells of the negative fraction. After removal of the column from the magnetic field, the cells retained in the column can be eluted and collected as the positive fraction. MACS separation was applied to purify DCs (CD11c Microbeads) and $CD8^+$ T cells ($CD8^+$ T cell Isolation Kit) from cells isolated from spleen, lymph nodes and thymus. All procedures were performed according to the instructions of the manufacturer.

4.2.1.10 Production of supernatant containing viral vectors

a) Lentivirus production

293T cells were plated 14-18 hours before transfection ($6x10^6$ cell per 100 mm cell culture plate) and kept at 37°C in a 10% CO_2 incubator. Before transfection, 10 ml of pre-warmed 293T-transfection medium replaced the normal cell culture medium in each plate. For the transfection solution, 20 μg of vector plasmid, 15 μg of pCMVdR8.2 and 10 μg of VSV-G were mixed with 100 μl of $CaCl_2$ and water sufficient for 1 ml of total volume. 1 ml of HBS 2X was added while vortexing. This solution was carefully added in the plates containing the Phoenix cells and incubated for 3-5 hours. Afterwards, cells were washed with pre-warmed PBS and cultured in 10 ml of 293T-medium. Cells were kept at 37°C in a 10% CO_2 incubator and after 24, 36 and 48 hours post-transfection,

medium was harvested and filtered (0.45 μm filter, Nalgene, Rochester NY). Virus was concentrated by filtration (Centricon Plus-70, Millipore, Bredfoard, MA, USA). Until use, the virus containing supernatant was stored at –80°C.

b) Retrovirus production

Phoenix-eco cells were plated 18 hours before transfection ($7{,}5x10^6$ cell per 100 mm cell culture plate) and kept at 37°C in a 5% CO_2 incubator. Before transfection, 8 ml of pre-warmed Phoenix-transfection medium replaced the medium of each plate. For the transfection solution, 60 μg of vector plasmid was mixed with 75 μl of $CaCl_2$ and water sufficient for 750 μl. While vortexing, 750 μl of HBS 2X was added. This solution was carefully added to the plates containing the Phoenix cells and incubated for 4-6 hours. Afterwards, cells were washed with pre-warmed PBS and 10 ml of Phoenix-medium was added. Cells were kept in a 32°C, 5% CO_2 incubator and after 24, 36 and 48 hours post-transfection, medium was harvested and filtered (0.45 μm filter, Nalgene, Rochester NY, USA). Until use, the virus containing supernatant was stored at –80°C.

4.2.1.11 T cell proliferation *in vitro*

Splenocytes from OT-1 mice were prepared as a single cell suspension and T cells were isolated by MACS with CD8 microbeads. Afterwards, T cells were stained with CSFE and resuspended in culture medium. DCs were differentiated *in vitro* from BM isolated from chimeric mice. DCs that have been in culture for 6-8 days were used for the assay. As a positive control, DCs were loaded with 1 μg/ml of SIINFEKL peptide during 2 hours at 37°C and 5% CO_2. Subsequently, 0.5×10^6 DCs were culture together with 0.5×10^6 T cells. After 72 hours the cells were harvested, stained and analyzed by flow cytometry.

4.2.2. Molecular biology methods

4.2.2.1 Agarose gel electrophoresis

This technique was used to identify and isolate DNA fragments. The amount of agarose used depended on the size of the DNA fragment to be identified or isolated (0.8-2%

w/v). The samples were compared to a 100bp or 1kb ladder (Invitrogen, Carslbad, CA, USA). The separation of the DNA fragments was obtained under a constant voltage of 80 in an electrophoresis chamber. (Repair workshop, Institute of Immunology, Munich, Germany). The visualization of the DNA was achived using ethidium bromide (0.005% added in the gel) under UV light (312 nm, Intas, Göttingen, Germany).

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 sequences for cloning. All restrictions enzymes were purchased from New England Biolabs (Beverly, MA, USA) and were used according to instructions of the manufacturer.

4.2.2.3 Culture of bacteria

Transformed bacteria were cultured in LB-medium (ICN Biomedicals, Aurora, Ohio, USA) at 37°C O/N. Since all plasmids and vectors contained an ampicilin resistance gene, 100μg/ml of ampicilin was added in the LB medium. For culture in solid medium, plates containing LB-agar were used (7.5g Agar/500 ml LB-Medium, containing 100μg/ml of ampicilin).

4.2.2.4 DNA and RNA isolation and purification

The following kits were used for the respective objectives according to instructions of the manufacturer. All kits were purchased from Qiagen (Qiagen GmbH, Hilden, Germany), unless stated otherwise:

Purification of DNA fragments from agarose gel QIAquick® Gel Extraction Kit

Isolation of small amounts (up to 20μg) of plasmidial DNA	QIAprep Spin Miniprep Kit
Isolation of large amounts of plasmidial DNA	QIAfilter Plasmid Maxi Kit
Isolation of genomic DNA	DNeasy Tissue Kit
Isolation of total RNA	PureLink Micro-to-Midi (Invitrogen, Carlsbad, CA, USA)

4.2.2.5 Ligation of DNA fragments

The ligation reaction was carried out using 100 ηg of vector DNA and 300-400ηg of insert DNA in ligase buffer with 400U T4-Ligase (New England Biolabs, Beverly, MA, USA). The reaction was performed at RT for 30 minutes or at 4°C O/N.

4.2.2.6 Polymerase chain reaction (PCR)

a) PCR for cloning

When a DNA sequence was amplified for cloning, *Pfu* DNA-Polymerase was used (Stratagene, La Jolla, CA, USA). Compared to the other thermostable polymersases normaly used, Pfu amplifies DNA with a higher fidelity. The error rate is six-fold lower than when, for example, *Taq* polymerase is used. The PCR product was purified and sequenced.

PCR reaction conditions

5-50 ηg DNA

0,5 μM primer forw

0,5 μM primer rev

1 x Pfu buffer

200 μM dNTP mix (10 mM each)

2,5 U *Pfu* DNA polymerase

H_2O sufficient for 50 μl

The approximated melting temperature (T_M) and the annealing temperature (T_A) used initially, and adjusted if necessary, were calculated accordingly with the following formulas:

$T_M = [(G+C) \times 4°C] + [(A+T) \times 2°C]$

$T_A = T_M - 5°C$

The amplification conditions were as follows

5 min 95ºC

30 cycles
- 30 sec 95ºC
- 30 sec T_A (varied according to the primers used)
- 1-4 min 72ºC (2 min/kb)

10 min 72ºC

b) Quantitative PCR (qPCR) for virus titer determination

NIH genomic DNA was purified and resuspended in 100μl of water. Real-time qPCR was performed with the LightCycler System (Roche Diagnostics, Penzberg, Germany). The virus backbone and the single-copy housekeeping gene Bdnf (used as internal standard control) were amplified using specific primers. Each sample was measured in duplicates using SYBR green I (Roche, Indianapolis, USA). Standard curves were generated using serial dilutions of DNA from a plasmid containing the region amplified with the respective primers.

qPCR Reaction conditions

200 ηg DNA

750 ηM primer forw (1000 ηM for Bdnf)

200 ηM primer rev (1000 ηM for Bdnf)

1x Master SYBR Green I mix

3 mM $MgCl_2$

H_2O sufficient for 20 μl

The amplification conditions were as follows:

2 min 50ºC

10 min 95°C

40 cycles → 10 sec 95ºC, 5 sec 58°C, 10 sec 72ºC

1 min 65ºC

30 sec 40°C

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

$$\text{Virus titer (TU/ml)} = \frac{\text{No. of NIH cells transduced x No. virus copy per genome}}{\text{volume of virus supernatant (ml)}}$$

$$\text{MOI} = \frac{\text{volume of virus supernatant (ml) x virus titer (TU/ml)}}{\text{No. of cells}}$$

4.2.2.7 Production of chemo competent bacteria

One single colony from a culture of *E. coli* DH5α or Stbl3™ (Invitrogen, Carlsbad, CA, USA) was incubated O/N in LB medium without ampicilin. The following day, 1ml of this culture was diluted in 99ml of LB medium containing 10mM of $MgCl_2$ and incubated at 37°C in a shaker, until an OD_{600} of 0,4-0,6 was reached. The bacteria-containing medium was then left on ice for 10 minutes, followed by centrifugation at 3000g, 4ºC for 25 minutes. The pellet was resuspended in 40ml of RFI medium and left on ice for 15 minutes. After centrifugation, the pellet was resuspended in 4ml of RFII medium and incubated on ice for another 15 minutes. Aliquots of 100μl were stored at – 80°C.

The *Escherichia coli* (*E. coli*) DH5α strain was used for transformation of SIN-retrovirus and pBS. The Stbl3™ E. coli strain is designed for cloning direct repeats found in lentiviral expression vectors. These cells reduce the frequency of unwanted homologous recombination of LTRs found in lentiviral vectors.

4.2.2.8 Transformation of $CaCl_2$-competent bacteria:

For each transformation, 100 μl of frozen cells was thermed on ice for 10 minutes. Afterwards, the plasmid DNA was added to the bacteria and the whole mix was chilled on ice for 30 minutes. The cells were then heat shocked at 42 ºC for 30 - 45 seconds and the vial placed directly back on ice before being transferred into 0.9 ml of LB agar and grown for 1 hour at 37 ºC with shaking. The cells were then plated on agar/ampicillin plates and incubated O/N at 37 ºC.

4.2.3 Sequence analysis

Database searches for homologous genes and proteins were performed using the BLASTp and BLASTn algorithms (Altschul, Madden et al. 1997) at the server of the NCBI (http:www.ncbi.nlm.nih.gov/Blast). Promoter analysis was performed using the Genomatix software (www.genomatix.de). Primers were designed with the assistance of Primer3 software (http:www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). For general manipulation of the sequences, the tools of the BMC Search Launcher (http:searchlauncher.bcm.tmc.edu/seq-util/seq-util.html) were used.

4.2.4 Statistical Analysis:

Data were analyzed using the Student's *t* test (GraphPad Prism 4.03; GraphPad Software). A value of $p<0.05$ was considered significant. All experiments consisted of at least 3 mice per group, unless otherwise stated.

5. Results

5.1 The murine DC-STAMP promoter presents all basic properties required to drive transgene expression from a viral vector.

The first step to achieve lentivirus-mediated transgene expression specifically in DCs, was to identify a protein which is synthesized specifically in these cells. The DC-specific transmembrane protein (DC-STAMP) was first identified in monocyte-derived human DCs (Hartgers, Vissers et al. 2000), and later in murine DCs (Eleveld-Trancikova, Triantis et al. 2005). Although preferentially expressed in DCs, the role of this molecule has so far only been described as being involved in osteoclastogenesis (Kukita, Wada et al. 2004; Yagi, Miyamoto et al. 2005). DC-STAMP was chosen because as opposed to other molecules expressed on DCs, DC-STAMP was described not simply as being expressed preferentially by these cells but also as being expressed in both immature and mature DCs. In addition, it is highly conserved between human and mice (Hartgers, Vissers et al. 2000; Eleveld-Trancikova, Triantis et al. 2005), suggesting that its promoter could also be used to target human DCs. Moreover, when mRNA expression of DC-STAMP was compared with CD11c (a beta 2 integrin expressed mainly in DCs), DC-STAMP showed a higher expression level (Hartgers, Vissers et al. 2000), suggesting that this promoter may be strong.

Because DC-STAMP was previously described only in human cells, a computational analysis was performed to find its murine homologue. The full-length human DC-STAMP transcript of 1954 bp and its corresponding open reading frame (ORF) of 1410 nucleotides were previously described (Hartgers, Vissers et al. 2000). The encoded amino acid sequence (GenBank access no. AF305068) was used to perform a protein-protein blast (blastp) search (Altschul et al., 1997) against the nr (non-redundant) database of the GenBank to detect the murine ortholog. A unique protein (GenBank access no. NM_029422) showing a high level of homology (74% of identity and 86% of similarity) with human DC-STAMP was identified. (Fig. 3). (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=10090).

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transmembrane 7 superfamily member 4 [Mus musculus] Length=470 aa

Identities = 349/468 (74%), Positives = 403/468 (86%),

Human  1    MGIWTSGTDIFLSLWEIYVSPRSPGWMDFIQHLGVCCLVALISVGLLSVAACWFLPsiia  60
            M +WT GT IFL LW  YV PRSP W+DFIQHLGVCC VA +SV L S A  W LP +
Mouse  1    MRLWTLGTSIFLRLWGTYVFPRSPSWLDFIQHLGVCCFVAFLSVSLFSAAFYWILPPVAL  60

Human  61   aaaswiiTCVLLCCSKHARCFILLVFLSCGLREGRNALIAAGTGIVILGHVENIFHNFKG 120
             ++ W+ITCV LCCSK ARCFILL  LSCGLREGRNALIAAGTG+VI GHVENIF+NF+G
Mouse  61   LSSVWMITCVFLCCSKRARCFILLAVLSCGLREGRNALIAAGTGVVIFGHVENIFYNFRG 120

Human  121  LLDGMTCNLRAKSFSIHFPLLKKYIEAIQWIYGLATPLSVFDDLVSWNQTLAVSLFSPSH 180
            LLD MTCNLRAKSFS+HFPLLK+Y EAIQWIYGLATPL++FDDLVSWNQTL VSLFSPSH
Mouse  121  LLDSMTCNLRAKSFSVHFPLLKRYTEAIQWIYGLATPLNLFDDLVSWNQTLVVSLFSPSH 180

Human  181  VLEAQLNDSKGEVLSVLYQMATTTEVLSSLGQKllafaglslvllgTGLFMKRFLGPCGW 240
             LEA +ND++GEVL VL+ M  TTE+L+S+GQKLLA AGL L+L+ TGLF+KRFLGPCGW
Mouse  181  ALEAHMNDTRGEVLGVLHHMVVTTELLTSVGQKLLALAGLLLILVSTGLFLKRFLGPCGW 240

Human  241  KYENIYITRQFVQFDERERHQQRPCVLPLNKEERRKYVIIPTFWPTPKERKNLGLFFLPI 300
            KYEN+YIT+QFV+FDE+ERHQQRPCVLPLNK+ER+KYVI+P+   TPKE+K LGLFFLP+
Mouse  241  KYENVYITKQFVRFDEKERHQQRPCVLPLNKKERKKYVIVPSLQLTPKEKKTLGLFFLPV 300

Human  301  LIHLCIWVLFAAVDYLLYRLIFSVSKQFQSLPGFEVHLKLHGEKQGTQDIIHDSSFNISV 360
            L +L +WVLFAAVDYLLYRLI S++KQFQSLPG EVHLKL GEKQGTQ ++HDS+FNIS+
Mouse  301  LTYLYMWVLFAAVDYLLYRLISSMNKQFQSLPGLEVHLKLRGEKQGTQGVVHDSAFNISM 360

Human  361  FEPNCIPKPKFLLSETWVPlsvillilvmlglllssilmQLKILVSASFYPSVERKRIQYL 420
            FEP+CIPKP+  +SETWVPLS+ILL L++LGLLSS+LMQLKILVS SFYP VER+RI+YL
Mouse  361  FEPSCIPKPRLSVSETWVPLSIILLTLIILGLLSSMLMQLKILVSVSFYPKVERERIEYL 420

Human  421  HAKLLKKRSKQPLGEVKRRLSLYLTKIHFWLPVLKMIRKKQMDMASAD  468
            HAKLL+KRSKQPL E   + SLY  KIHFW PVLKMIRKKQ   A+ D
Mouse  421  HAKLLEKRSKQPLREADGKPSLYFKKIHFWFPVLKMIRKKQTIPANED  468
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Figure 3: Results of a blastp alignment of the human DC-STAMP protein and the Mus musculus homologue (transmembrane 7 superfamily member 4). Identical amino acid positions between the two sequences (74%) are labeled in bold. Conserved amino acids (86%) between the two DC-STAMP proteins are assigned as (+). aa, amino acid.

The location of the nucleotide sequence encoding the murine DC-STAMP protein and its mRNA were identified in the genome (Fig. 4). This allowed us to design primers to amplify the 5' untranslated region (UTR) where the promoter regulatory sequences were expected. A sequence of 1704bp covering the region between -1565bp and +131 (considering +1 the transcriptional start site) was chosen and subcloned into the SIN-retrovirus and the SIN-lentivirus vectors.

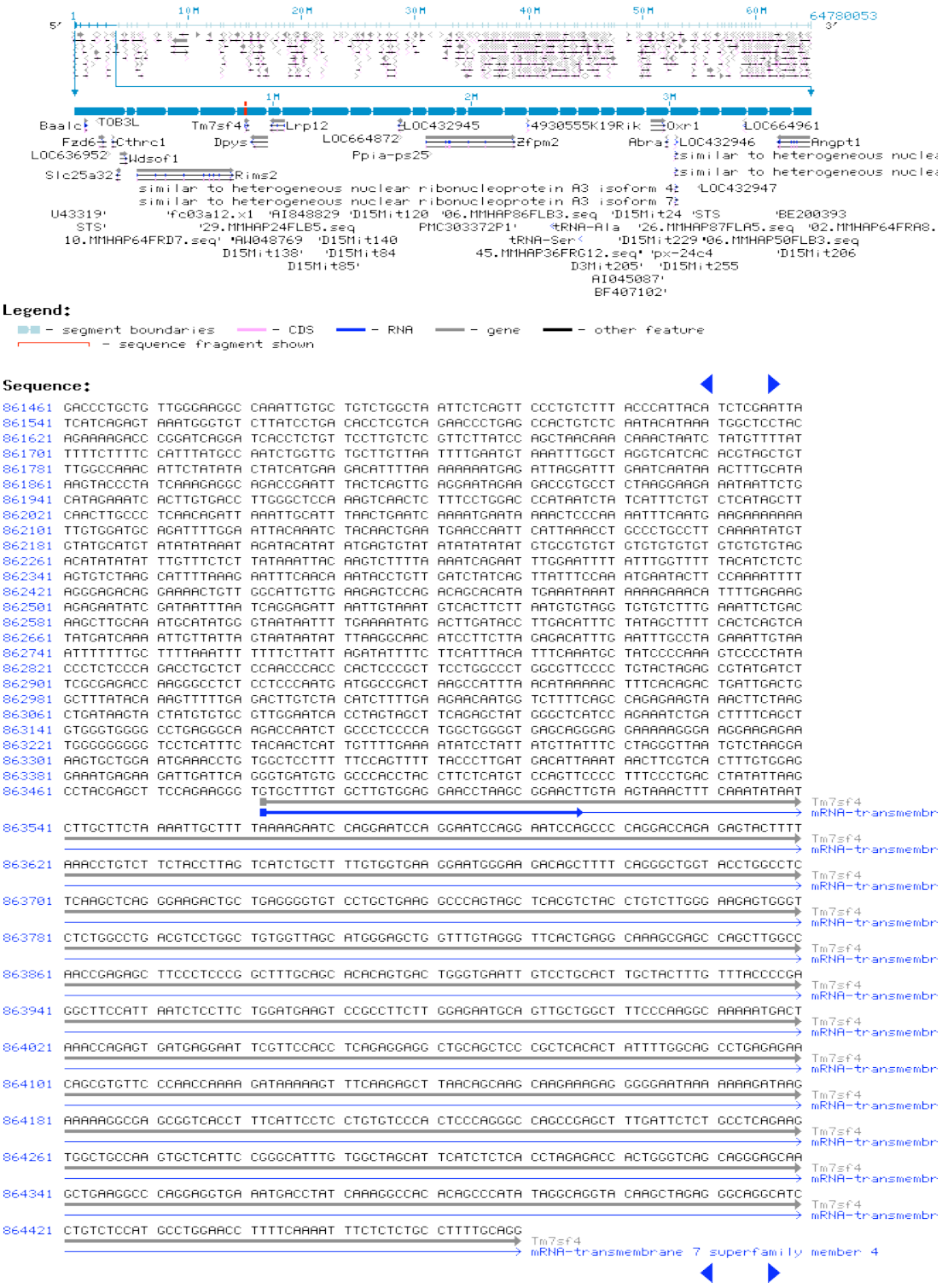

Figure 4: Mouse database browser display of the nucleotide sequence of the mus musculus chromosome containing the DC-STAMP gene. DC-STAMP is annotated in the database as Tm7sf4. The mRNA is indicated by a blue arrow over the sequence (mRNA-transmembrane). The region upstream of the mRNA (and the initial 131 nucleotides) contains the promoter region used in our studies. Numbers refer to the nucleotide positions in chromosome 15. Mouse sequences are available at (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=10090).

5.2 The murine DC-STAMP promoter confers DC specific transgene expression *in vivo* when delivered by a lentiviral vector, but not by a standard retroviral vector.

The great advantage of using retroviral vectors for gene transfer is that the transgene sequences are integrated into the genome of the infected cell, thereby conferring stable transgene expression. Stability is particularly important when the cells to be transduced are HSC with the aim of targeting transgene expression to differentiated HSC-progeny. In order to develop a retrovirus allowing transgene expression mainly in DCs, we replaced the human CD19 promoter from a retroviral vector intended to target expression in B cells (Werner, Kraunus et al. 2004) with the DC-STAMP promoter to generate DCSTAMP-eGFP-SIN-retrovirus (Fig. 5a).

One of the most important limitations of the SIN retrovirus is the relatively low virus titer obtained. This titer is inversely proportional to the size of the transgene inserted into the backbone. Low titers from SIN retroviral vectors have been associated with inefficient polyadenylation of the viral RNA due to extensive deletions made to the U3 region of the 3' LTR. Such deletions included the TATA box affecting the nearby R region, which is implicated in polyadenylation (Yee JK, 1987; zufferey R, 1998). As in HIV the main cis-acting element governing polyadenylation is located in a region distal to the TATA box (DeZazzo JD, 1991; Valsamakis A, 1991; Valsamakis A, 1992). HIV–derived vectors tolerate large U3 deletions without altering viral titers (Zufferey R, 1998). Since we were not able to reach good viral titers with the SIN-retrovirus vector, we decided to try a parallel approach using a SIN-lentivirus vector. To this end, we replaced the human ubiquitin promoter from a SIN-lentiviral vector (Lois, Hong et al. 2002) with the DC-STAMP promoter to construct the DCSTAMP-eGFP-SIN-lentivirus (Fig. 5b). As expected, by transducing mouse bone marrow derived DC cultures with identical volumes of DCSTAMP-eGFP-SIN-lentivirus or DCSTAMP-eGFP-SIN-retrovirus, we were able to show that the titers of the SIN-lentivirus vector stocks were much higher than those of the SIN-retrovirus (Fig. 5).

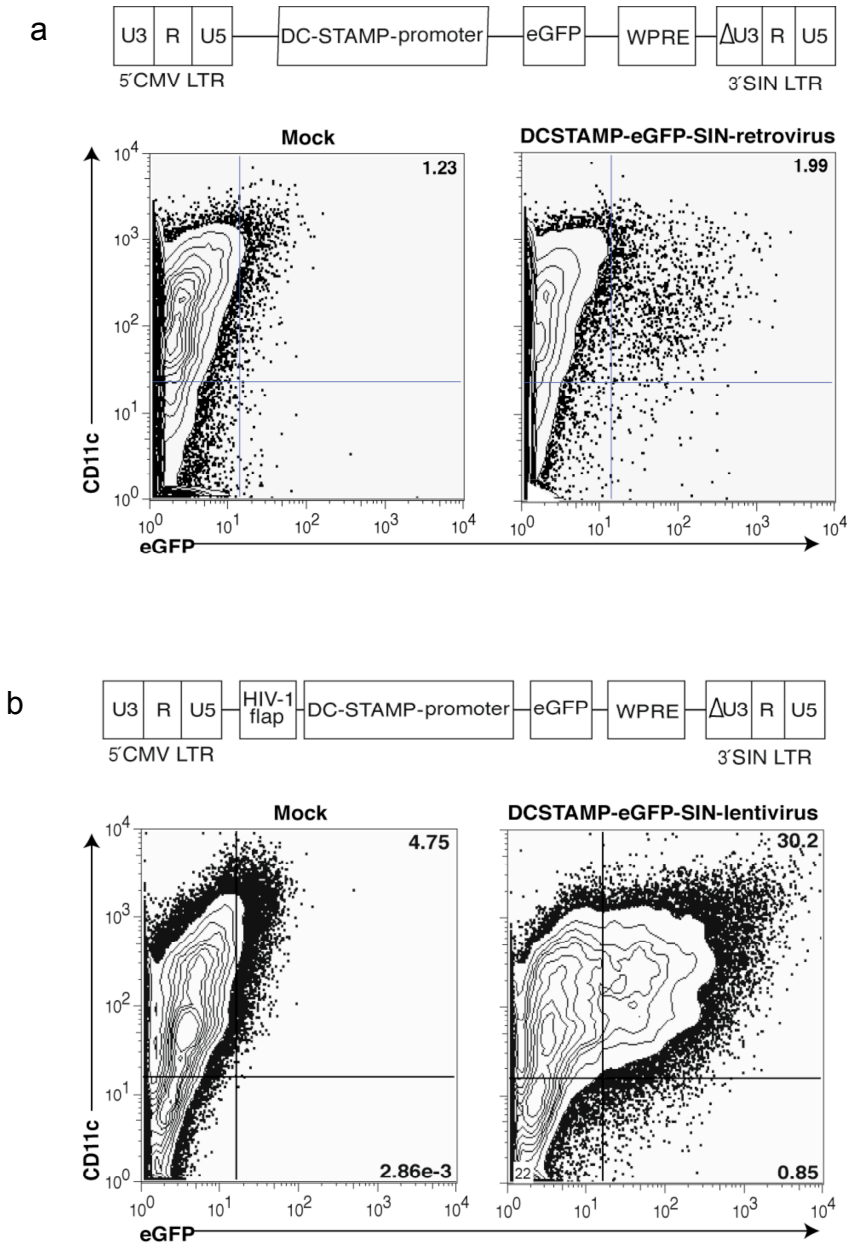

Figure 5: DCSTAMP-eGFP self-inactivating retroviral and lentiviral vectors confer transgene expression in DCs *in vitro*. Schematic representation of (a) retroviral and (b) lentiviral-based SIN-vector, with the murine DC-STAMP promoter to control expression of eGFP-cDNA. Bone marrow cells from C57BL/6 mice were cultured in presence of GM-CSF and 2 days later, transduced with DCSTAMP-eGFP or DCSTAMP-mock (encoding no cDNA) retroviral or lentiviral vectors. Cells were analyzed for eGFP-expression by flow cytometry at day 7 of culture. Numbers in quadrants represent percentages of cells. CMV, cytomegalovirus; SIN, self-inactivating; LTR, long terminal repeat; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; △U3, deletion in the U3 region.

In order to evaluate the specificity and potency of the DC-STAMP promoter *in vivo,* we generated mouse BM chimeras by reconstituting lethally irradiated mice with hematopoietic stem cells transduced two times with DCSTAMP-eGFP-SIN-retrovirus or

DCSTAMP-eGFP-SIN-lentivirus. 8 weeks after transplantation, we analyzed eGFP expression in the main populations of splenocytes (Fig. 6). While in the chimeras generated with DCSTAMP-eGFP-SIN-retrovirus only unspecific background of fluorescence in all cells was detected (Fig. 6, bottom), in the chimeras produced with DCSTAMP-eGFP-SIN-lentivirus eGFP expression was mainly restricted to DCs, with significant expression also in monocytes. However, no significant levels of eGFP expression could be observed in T cells, B cells or NK cells (Fig. 6, top).

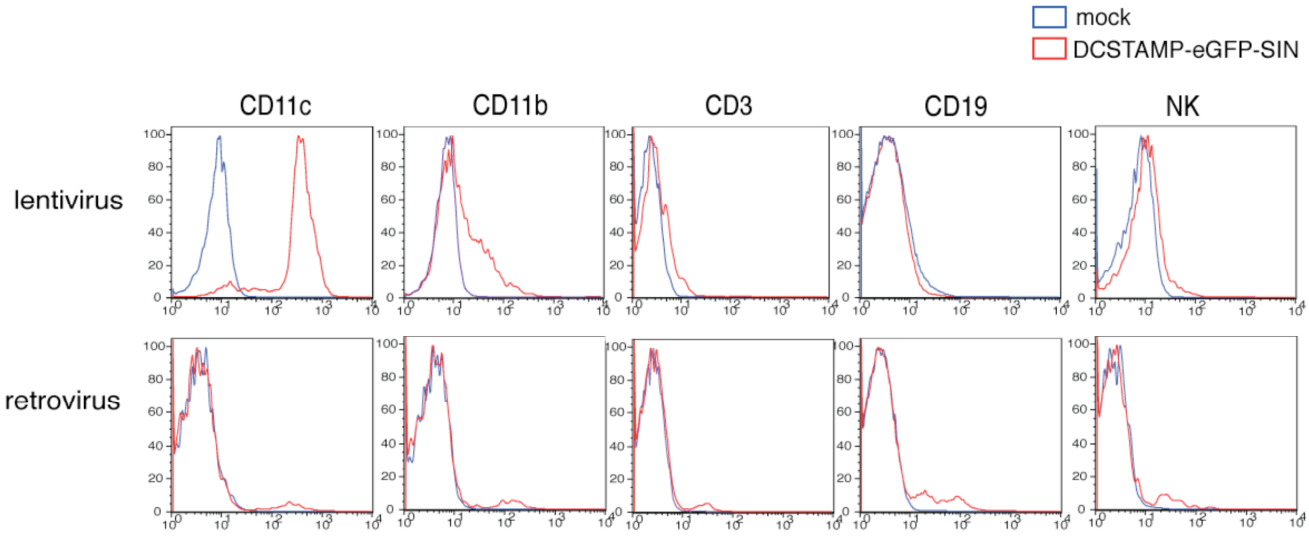

Figure 6: Murine DC-STAMP promoter targets transgene expression to dendritic cells *in vivo* when delivered by a lentiviral, but not a retroviral vector. Bone marrow HSCs from 5-FU treated C57BL/6 donor mice was transduced twice with DCSTAMP-retroviral or DCSTAMP-lentiviral vectors encoding eGFP (DCSTAMP-eGFP-SIN, red) or no cDNA (mock, blue) and 1-3 x 10^6 cells were injected into lethally irradiated recipient mice. At 8 weeks post-transplantation, chimeras were sacrificed, and cells were analyzed for eGFP expression by flow cytometry. The histograms represent FACS analyses of the indicated leukocyte populations from spleen, gated for the corresponding markers. CD11b was gated in the $CD11c^-$ population. NK cells were identified as $DX5^+NK1.1^+$. At least 3 mice per group were used.

Since only DCSTAMP-eGFP-SIN-lentivirus but not -retrovirus gave promising results, we performed further detailed analysis only in chimeras generated with DCSTAMP-eGFP-SIN-lentivirus. The high eGFP MFI (mean of fluorescence intensity) indicates that besides being specific, DC-STAMP is also a strong promoter in the context of SIN-lentivirus (Fig. 7a, bottom). After two rounds of transduction of HSCs with virus concentrations ranging between MOI 0.2 and 1.5, the high DC-selectivity of transgene-expression was maintained. This specific expression could be observed in spleen, lymph nodes and thymus (Fig. 7 and data not shown). In addition, with

increasing viral titers, the expression levels of eGFP were amplified correspondingly (Fig. 7). Interestingly, despite an apparent tendency for the DC-STAMP promoter to drive transgene expression in myeloid cells, “lymphoid” $CD8^+$ DCs also expressed high levels of eGFP (Fig. 7a). Taken together, these data suggest that the DC-STAMP promoter region used in the SIN-lentivirus is suitable to target transgene expression to different DC subpopulations *in vivo*.

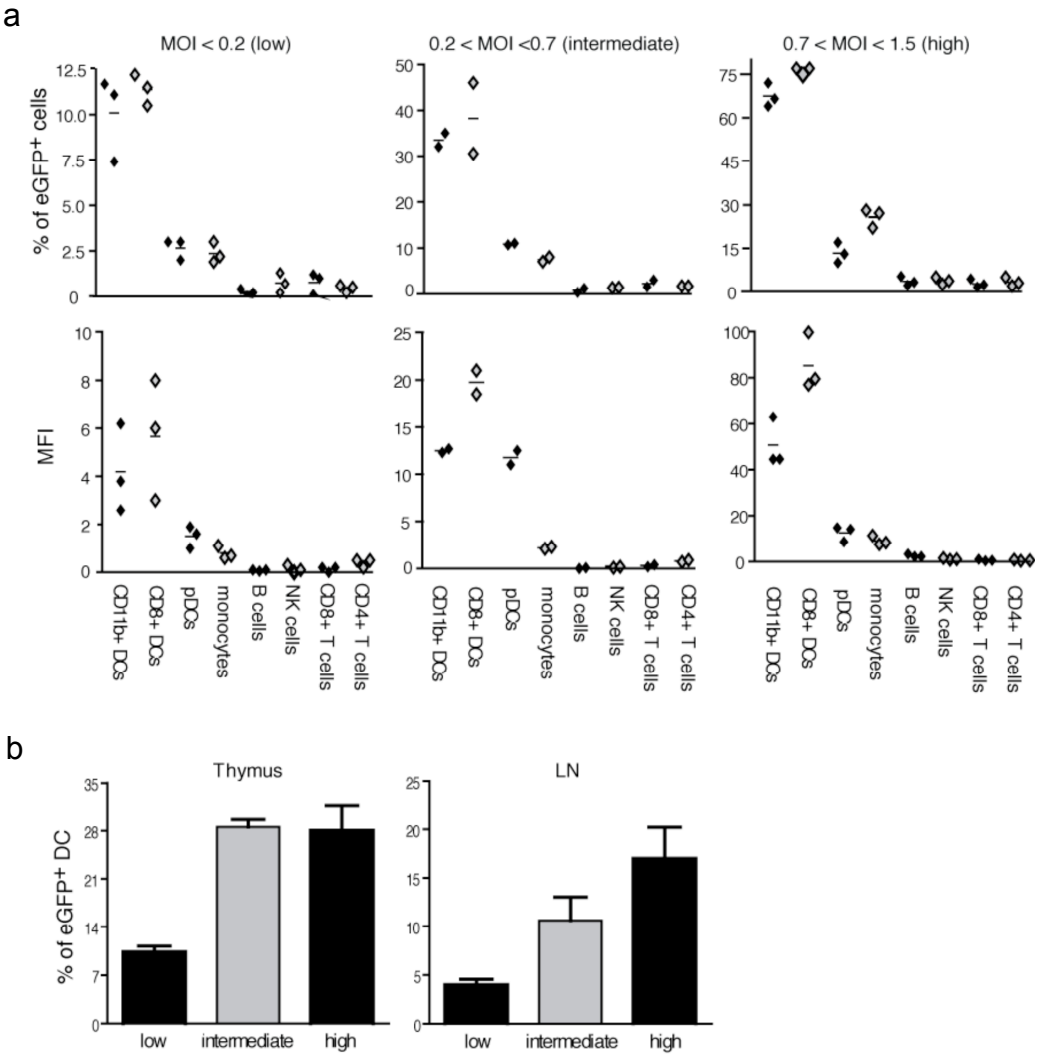

Figure 7. DC-selectivity of transgene expression is maintained *in vivo* after transduction with different MOIs. Bone marrow HSCs from 5-FU treated C57BL/6 donor mice were transduced twice with DCSTAMP-eGFP lentivirus vector, and 1-3 x 10^6 cells were injected into lethally irradiated recipient mice. At 8 weeks post-transplantation, chimeras were sacrificed, and cells were analyzed by flow cytometry. (a) Percentage (upper panel) and mean fluorescence intensity (MFI, lower panel) of eGFP positive cells within the indicated cell populations from chimeras generated with HSCs transduced twice with the indicated MOI. The leukocyte populations were gated accordingly with the indicated marker or as follow: DCs are correspondent to $CD11c^+$ cells; pDCs were identified as $CD11c^+B220^+$ cells; monocytes are correspondent to the $CD11b^+CD11c^-$ population; B cells were identified as $CD19^+$ $B220^+$ cells; NK cells were identified as $DX5^+NK1.1^+$. (b) Percentage of $eGFP^+CD11c^+$ cells in thymus and lymph nodes from the same chimeras as in (a).

5.3 Transgene expression controlled by the DC-STAMP promoter leads to deletion of autoreactive antigen-specific CD4$^+$ T cells *in vivo*.

As shown in previous studies, DCs are able to delete autoreactive CD4$^+$ T cells in the thymus (Brocker, Riedinger et al. 1997; Gallegos and Bevan 2004). In order to evaluate the functionality of our system, we wanted to test if mice reconstituted with bone marrow transduced with our lentiviral vector encoding ovalbumin (OVA) would develop OVA-specific tolerance. To this end we chose the TCR transgenic OT-II mouse strain in which CD4$^+$ T cells recognize OVA peptide in the MHC-II context (Barnden, Allison et al. 1998). We generated a lentiviral vector expressing cDNA encoding for a chimeric non-secreted membrane-bound form of OVA [trOVA; (Diebold, Cotten et al. 2001)] from the DC-STAMP promoter, giving rise to the DCSTAMP-trOVA lentivirus vector (Fig. 8).

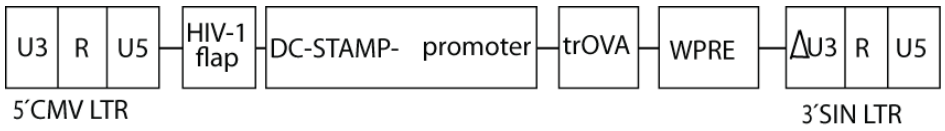

Figure 8: DCSTAMP-trOVA self-inactivating lentiviral vector. Schematic representation of lentiviral-based SIN-vector, with the murine DC-STAMP promoter controlling expression of trOVA-cDNA. CMV, cytomegalovirus; SIN, self-inactivating; LTR, long terminal repeat; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; △U3, deletion in the U3 region.

Next, we generated bone marrow chimeras from Ly5.2$^+$ OT-II donors transduced with DCSTAMP-trOVA or DCSTAMP-mock lentivirus and transferred these cells into lethally irradiated congenic Ly5.1$^+$ B6 recipients. In these chimeras, developing thymocytes and mature T cells would recognize their cognate antigen only on DCs, enabling us to monitor DC-functions and influence on CD4$^+$ T cells. We observed significant reduction in the frequency of CD8$^-$CD4$^+$ T cells specific for OVA peptide in the thymus of DCSTAMP-trOVA chimeras, when compared with DCSTAMP-mock transduced recipients ($p<0.0001$, Students's t test, Fig. 9). Furthermore, the frequencies of total CD8$^-$CD4$^+$ thymocytes were significantly decreased in DCSTAMP-trOVA chimeras, in contrast with DCSTAMP-mock chimeras ($p=0.0003$, Student's t test,

Fig. 9 left). This decrease in the OT-II compartment was not due to reduced chimerism, since at least 96% of thymocytes were from donor phenotype (Fig. 9, right).

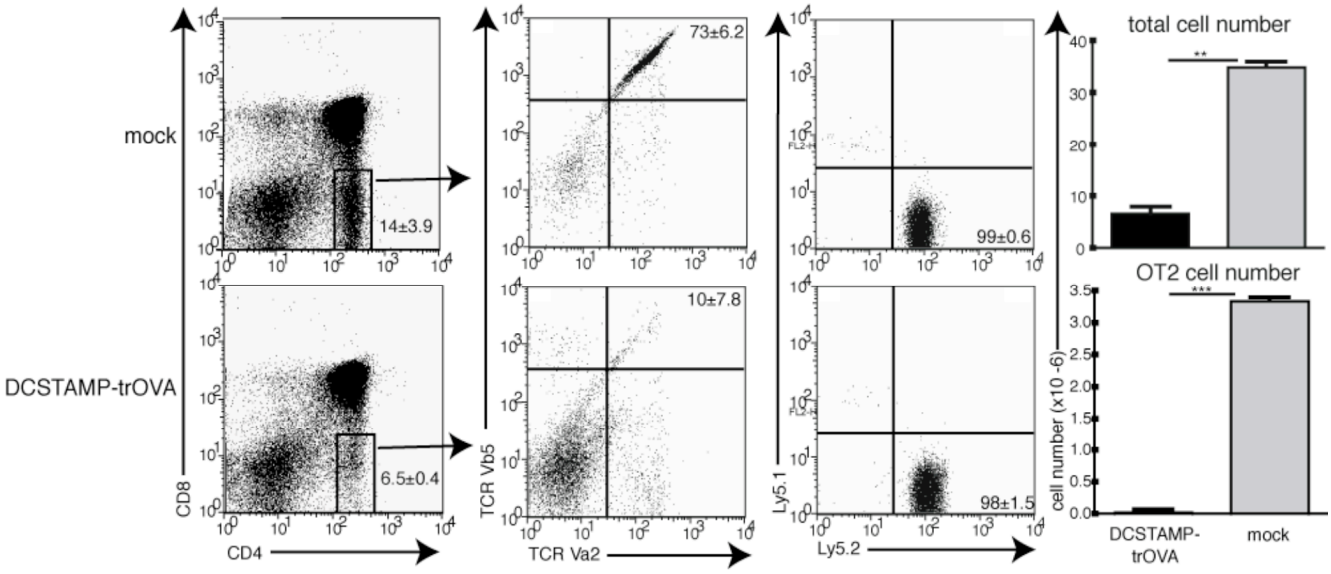

Figure 9: DC-STAMP promoter-regulated expression of OVA in dendritic cells leads to Ag-specific central tolerance induction in TCR-transgenic CD4$^+$ T cells. Bone marrow HSCs from OT-II mice were transduced with DCSTAMP-trOVA or DCSTAMP-mock lentivirus vector, and bone marrow chimeras were generated. At least 5 weeks after transplantation, chimeras were sacrificed, and cells were analyzed by flow cytometry. Thymocytes were gated on single positive CD4 cells, and OT-II cells were identified according to their expression of TCRVα2 and TCRVβ5.1/5.2; total numbers of thymocytes and total numbers of OT-II cells in both types of chimeras were compared (**, p= 0.004; ***, p=0.0003, Student's t test). At least 3 mice per group were analyzed.

Next, we analyzed spleens of these chimeras for presence of OT-II cells. The frequencies in OT-II cells were nearly 20-fold reduced in spleens from DCSTAMP-trOVA chimeras, resulting in an approximately 50-fold reduction in total numbers of OT-II cells, as compared to DCSTAMP-mock chimeras (Fig. 10a, right). The few remaining OT-II cells showed expression of specific surface activation markers, such as CD44, CD69 and CD25 and down-regulated CD62L (Fig. 10b), probably reflecting the constant interaction between OT-II and OVA-expressing DCs. The same reduction in the percentage of OT-II cells was still observed 30 weeks post-transplantation, indicating that the expression of the transgene is long lasting. Moreover, when the bone marrow from these 30 weeks post-transplantation chimeras were differentiated in DCs *in vitro,* and these cells were co-cultured with CD8$^+$ T cells expressing a transgenic TCR specific for OVA peptide in the context of MHC-I [OT-I cells; (Hogquist, Jameson et al. 1994)], only the T cells cultured with DCSTAMP-trOVA

derived DCs expanded (Fig. 10c). T cell proliferation was accompanied by IL-2 receptor (CD25) up-regulation, indicating T cell activation. The observed proliferation was nearly as strong as cell-division induced by mock-transduced DC loaded with the OVA-MHC class I peptide SIINFEKL (Fig. 10c). This data indicates that lentivirus vector mediated transgene expression was not silenced in DCs, as described previously in ubiquitous retroviral systems (Lindemann c, 2002).

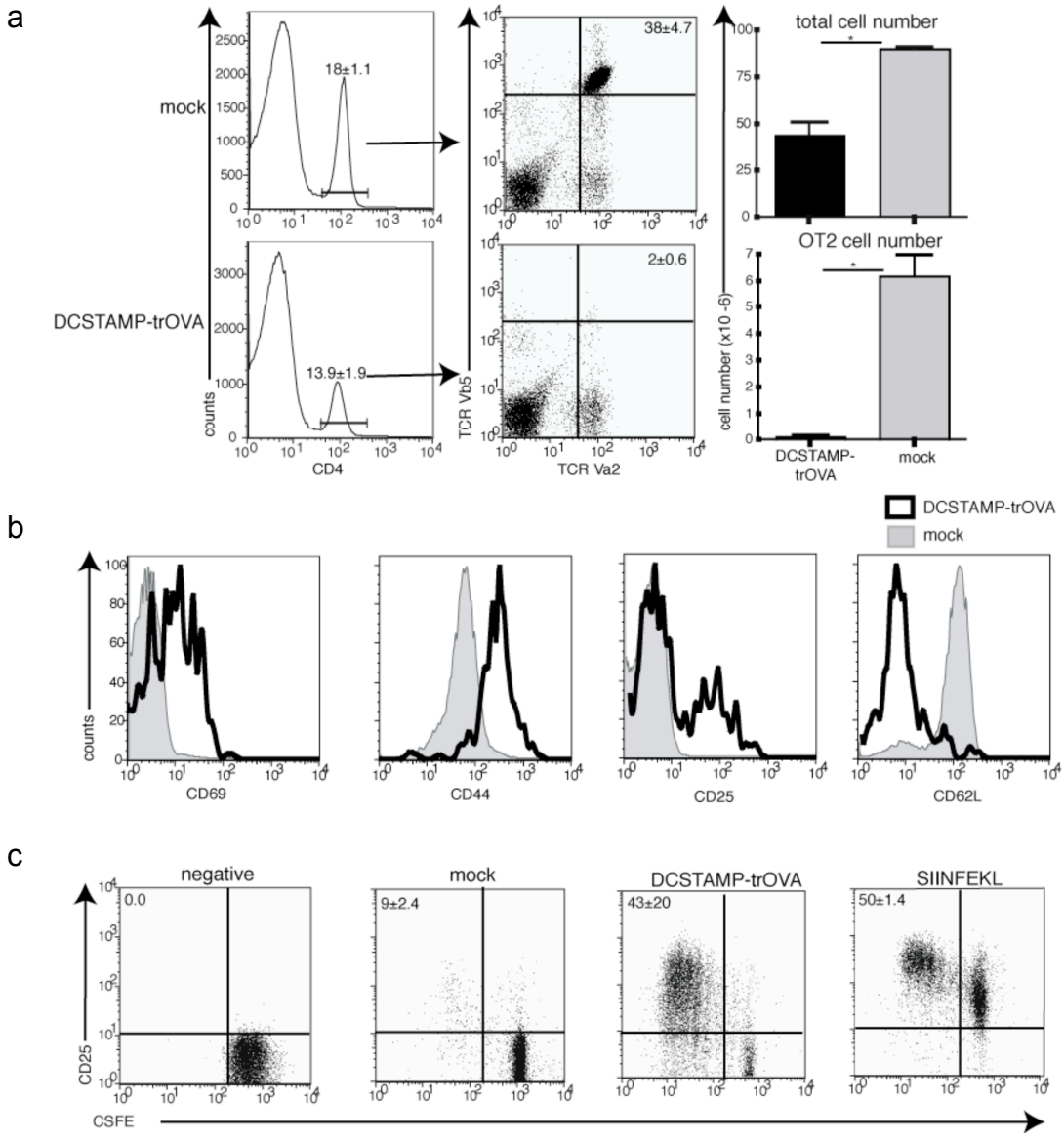

Figure 10: DC-STAMP promoter-regulated expression of OVA in dendritic cells leads to Ag-specific peripheral tolerance induction in TCR-transgenic CD4$^+$ T cells. Bone marrow HSCs from OT-II mice were transduced with DCSTAMP-trOVA or DCSTAMP-mock lentivirus vector, and bone marrow chimeras were generated. After at least 5 weeks following transplantation, chimeras were sacrificed and cells were analyzed by flow cytometry. (a) Expression of Vα2 and Vβ5.1/5.2 on CD4$^+$ OT-II cells from spleen; total numbers of splenocytes and total numbers of OT-II cells in both types of chimeras were compared (*p=0.0195; *p=0.0169, respectively, Student's *t* test). (b) OT-II cells from spleens were identified as described in (b) and expression of the indicated surface molecules was analyzed in

DCSTAMP-trOVA- (open histogram) and mock- (grey histogram) chimeras. (c) Bone marrow cells from DCSTAMP-trOVA, mock or normal B6 mice were differentiated into DCs *in vitro* with GM-CSF. At day 7 of culture, DCs were harvested and cultured (DCSTAMP-trOVA and mock), or loaded with 1 ug/ml of SIINFEKL peptide (SIINFEKL) and cultured with CSFE-labeled OT-I cells. As a control, OT-I cells were cultured alone (negative). At day 3 of culture, OT-I cells were analyzed by gating on $CD8^+$ T cells. At least 3 mice per group were analyzed.

Some studies indicate that DCs can promote the differentiation of Tregs (Watanabe, Wang et al. 2005). Because CD25 was up-regulated in the remaining OT-II cells in the peripheral lymphoid organs of DCSTAMP-trOVA chimeras (Fig.10b), we analyzed these cells for the presence of the transcriptional factor forkhead box P3 (foxp3) in $CD4^+TCRVa2^+$ T cells. Foxp3 is specifically expressed in $CD4^+$ Tregs (Hori, Shohei et al. 2003), and in normal mice approximately 10% of all peripheral $CD4^+$ T cells are $CD25^+Foxp3^+$. We observed significant increase in the frequency of $Foxp3^+CD4^+TCRVa2^+$ T cells in the splenocytes isolated from DCSTAMP-trOVA chimeras when compared with DCSTAMP-mock transduced recipients (p=0.0024, Student's t test, Fig. 11). However, no significant difference in total numbers of $Foxp3^+CD4^+TCRVa2^+$ T cells was observed (Fig.11). Although further studies are necessary to address in more detail the generation of Tregs by lentiviral vector-mediated antigen expression by DCs, these findings suggest that it does not lead to the differentiation of Tregs under steady state conditions. Nevertheless, efficient and long-lasting Ag-specific $CD4^+$ T cell deletion was induced.

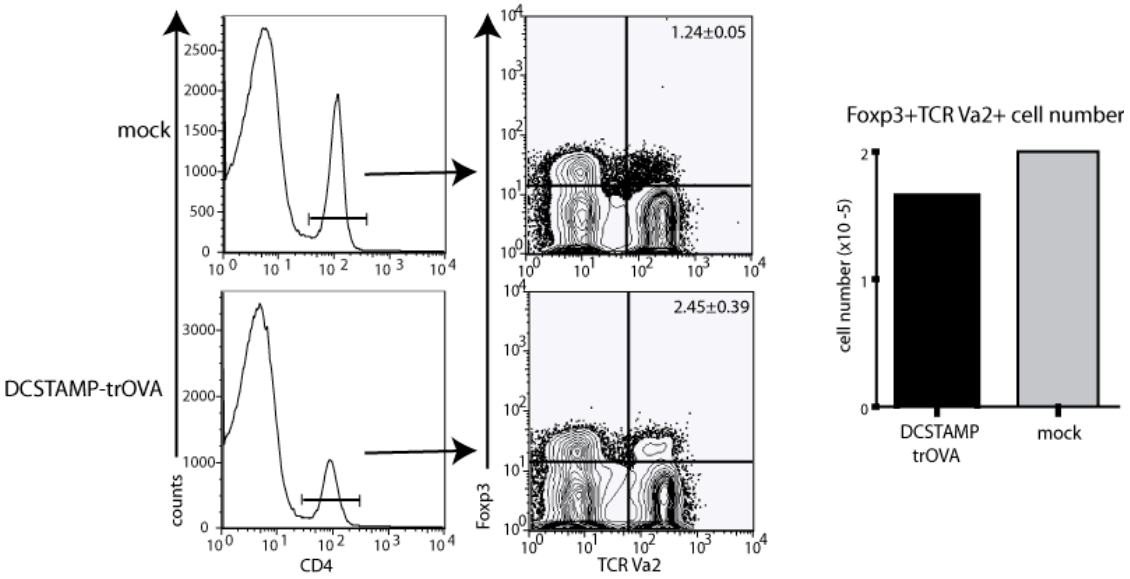

Figure 11: DC-STAMP promoter-regulated expression of OVA in dendritic cells does not lead to differentiation of Tregs in TCR-transgenic $CD4^+$ T cells. Bone marrow HSCs from OT-II mice were transduced with DCSTAMP-trOVA or DCSTAMP-mock lentivirus vector and bone marrow chimeras were

generated. After at least 5 weeks of transplantation, chimeras were sacrificed, and cells were analyzed by flow cytometry. Splenocytes were gated on $CD4^+$ T cells and $CD4^+TCRVa2^+$ T cells were analyzed according to their intracellular expression of Foxp3. Total numbers of $Foxp3^+CD4^+TCRVa2^+$ cells in both types of chimeras were compared. At least 3 mice per group were analyzed.

5.4 Transgene expression controlled by the DC-STAMP promoter leads to tolerance of autoreactive antigen-specific $CD8^+$ T cells.

The study showing that DCs are the main APCs able to delete autoreactive $CD4^+$ T cells in the thymus also showed that DCs partake in the process of autoreactive $CD8^+$ T cell-deletion (Gallegos and Bevan 2004). To find out whether our system would also lead to tolerance of $CD8^+$ T cells, we repeated the same procedure described in 5.3 using the OT-I mouse strain. This model is similar to the OT-II, except that the $CD8^+$ T cells recognize OVA in the context of the MHC-I. As described before in the OT-II system, we generated bone marrow chimeras that received HSC transduced with DCSTAMP-trOVA- or DCSTAMP-mock-vector. To our surprise, in contrast to what has been observed in the OT-II system, we detected no decrease in the frequency of OT-I cells in the thymus of our chimeras (Fig. 12a), despite the presence of OVA expression in DCs present in the thymus as confirmed by RT-PCR (Fig. 12b). However, after analyzing more carefully the CD8 single positive cells in this organ, we detected a slight increase in the expression of CD24 among the TCR $Vb5^+$ cells (Fig. 12a, right). As CD24 expression is an indicator of T cell maturity, we speculate that despite no reduction in the number of OT-I cells, there is perhaps a qualitative difference in these cells already in the thymus, predisposing them to the deletion process in the periphery. One possible explanation for the observed lack in central deletion of OT-I cells in the OVA-expressing chimeras could be that due to the experimental procedure of generating BM chimeras, contaminating mature OT-I cells were transferred together with the reconstituting BM. OT-I cells might recognize OVA-expressing DCs and kill them before they could induce tolerance. It is known that T cells transferred together with BM can recognize and mount an immune response against tissue/cells of the BM recipients (Sprangers, Van Wijmeersch et al. 2007). In this case, the expression of OVA could reflect DCs that didn't interact with mature OT-I T cells. To pursue the hypothesis that absence of central tolerance was due to the presence of such mature T cells, we repeated the same procedure as described above, but with $CD8^+$ T cell-depleted OT-I

donor BM. In this case, we detected a significant reduction in the frequency of mature $CD8^+$ thymocytes (Fig. 12c left, p=0.021, Students t-test). Further analysis revealed that significantly fewer CD8 thymocytes were of the OT-I phenotype $TCRVa2^+V\beta5^+$ in DC-STAMP-trOVA-chimeras as compared to mock-chimeras (Fig. 12c, p=0.004, Students t-test). This resulted in a nearly 4-fold reduction of total OT-I thymocyte numbers (Fig. 12c, right). Compared to the results obtained with the OT-II chimeras (Fig. 9), central deletion of $CD8^+$ T cells was less efficient, although all chimeras were generated with identical viral titers.

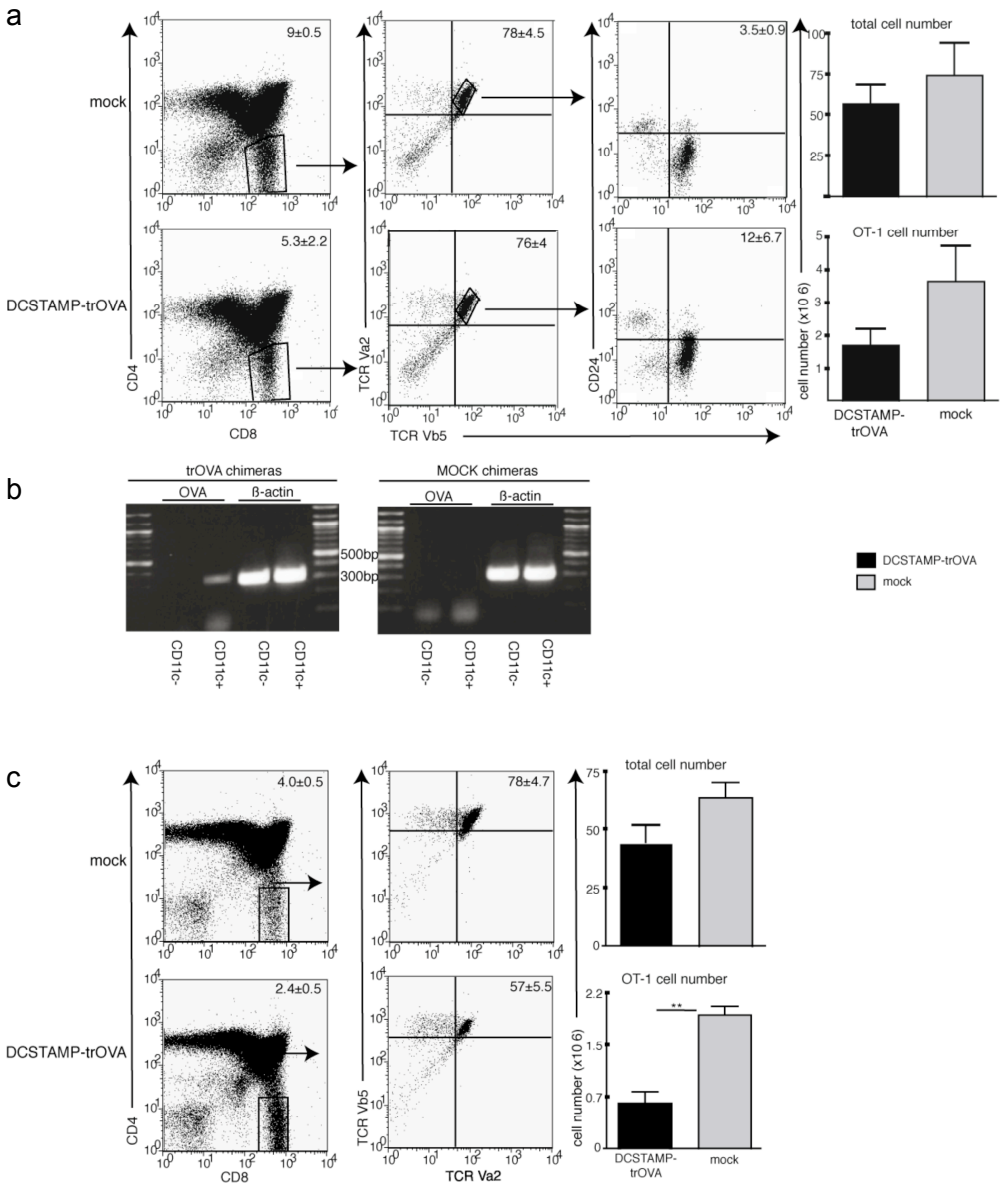

Figure 12: OVA-expressing dendritic cells induce antigen-specific central deletion of TCR-transgenic $CD8^+$ T cells. $CD8^+$ T cell non-depleted (a and b) and depleted (c) bone marrow HSCs from OT-I mice were transduced with DCSTAMP-trOVA or DCSTAMP-mock lentivirus vector and

bone marrow chimeras were generated. After at least 5 weeks of transplantation, mice were sacrificed and cells analyzed by flow cytometry. (a and c) Identification of OT-I cells was performed according to expression of CD8, TCRVα2 and TCRVß5 as indicated by gates and quadrants. Total numbers of thymocytes and total numbers of OT-I T cells were determined (**p=0.004, Student's *t* test). (b) CD11c-positive and -negative thymic cells were purified by magnetic bead sorting and RNA was isolated. RT-PCR analysis from these samples shows the presence of OVA-mRNA only in the CD11c-positive fraction, as identified by amplification of a 317 bp-fragment for trOVA. A 302bp-fragment for ß-actin served as a control.

When the peripheral lymphoid organs of both $CD8^+$ T cell-depleted or non-depleted BM recipient mice were analyzed, we consistently detected a reduction in the OT-I cell compartment, although the reduction was more accentuated in the $CD8^+$ T cell depleted chimeras, probably reflecting the combined central and peripheral tolerance induced in this group (Fig. 13). In the chimeras that received non-depleted DCSTAMP-trOVA transduced BM we observed approximately a 3-fold reduction of $CD8^+$ T cell frequencies in the spleen (Fig. 13a, *p*=0.01, Students t-test). Of those, circa 26% were of the OT-I phenotype ($TCRVa2^+Vß5^+$), compared to 76% in mock chimeras (Fig.13a, *p*=0.0001, Students t-test). Regarding the chimeras that received CD8 T cells depleted BM, a nearly 6-fold reduction of $CD8^+$ T cell frequencies in the spleen was observed (Fig. 13b left, *p*=0.009, Students t-test). Of those, only 14%, as compared to >80% in mock chimeras, were of the OT-I phenotype (Fig. 13b, *p*=0.0001, Students t-test). Together, this resulted in a 50-fold reduction of absolute OT-I T cell numbers as compared to control chimeras (Fig. 13b, right).

The remaining peripheral OT-I T cells were phenotypically equivalent in mice receiving either $CD8^+$ T cells depleted or not-depleted BM. Here they displayed elevated levels of CD69, CD44, and reduced CD62L expression as evidence of T cell activation or Ag-experience (Fig. 13c). However, in contrast to OT-II T cells (Fig. 10b), CD25 expression was not modulated (Fig. 13c). This data suggest that tolerance induced by Ag-expressing DCs leads to a strong reduction of Ag-specific $CD8^+$ T cells.

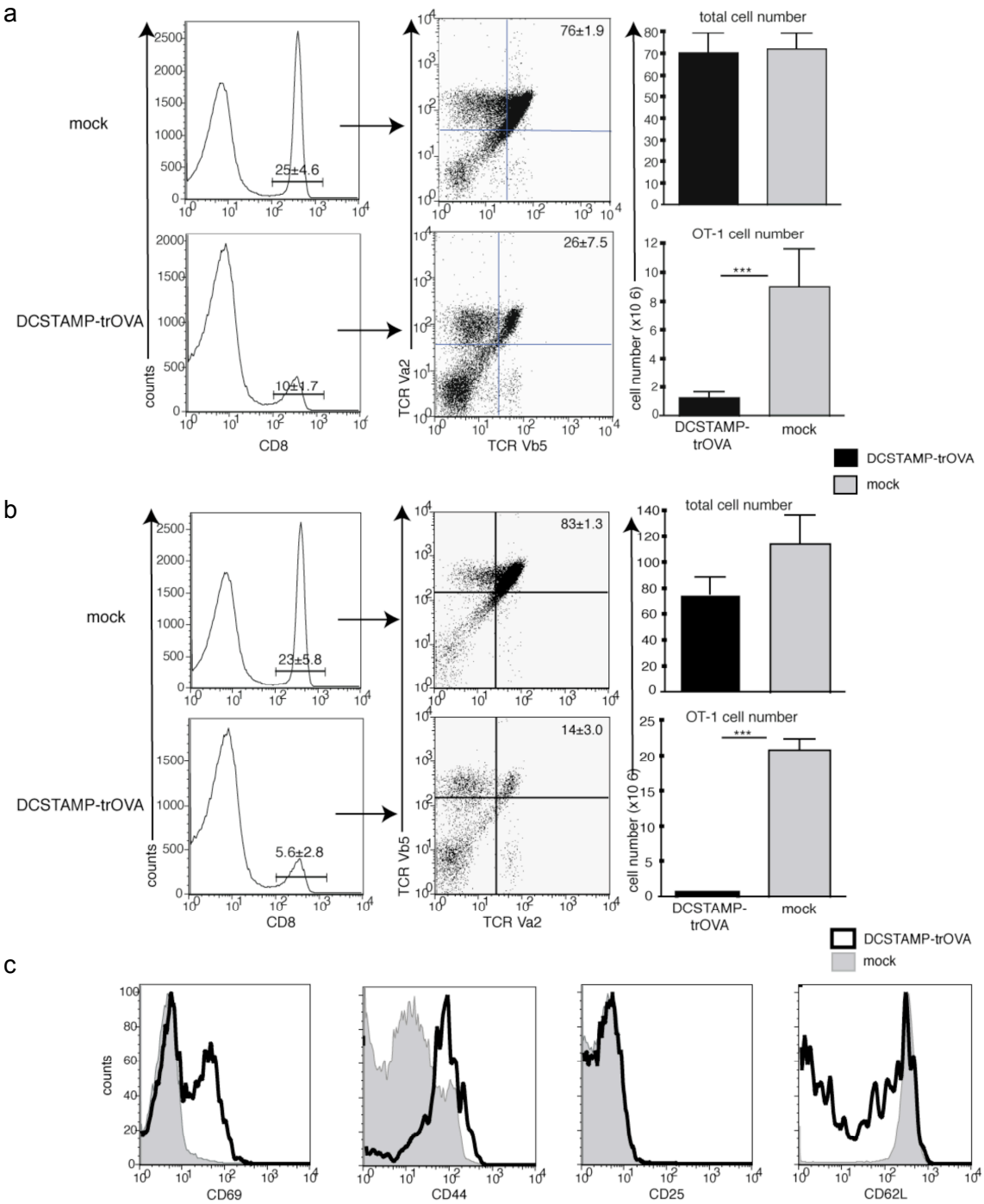

Figure 13: OVA-expressing dendritic cells induce/maintain tolerance of TCR-transgenic CD8$^+$ T cells. CD8$^+$ T cell non-depleted (a) or depleted (b) bone marrow HSCs from OT-I mice were transduced with DCSTAMP-trOVA or DCSTAMP-mock lentivirus vector, and bone marrow chimeras were generated. After at least 5 weeks post-transplantation, mice were sacrificed and cells analyzed by flow cytometry. (a and b) Identification of OT-I cells according to expression of CD8, TCRVα2 and TCRVß5 as indicated by gates and quadrants. Total numbers of splenocytes and total numbers of OT-I T cells of both types of chimeras were compared in CD8$^+$ T cell non-depleted and depleted BM recipients (***p≤0.0002; Student's *t* test). (c) OT-I T cells from spleens of chimeras that were reconstituted with CD8$^+$ T cells depleted and non-depleted BM were identified as shown in (a and b) and expression of the indicated surface molecules was analyzed in DCSTAMP-trOVA- (open histogram) and mock (grey histogram) - chimeras. At least 3 mice per group were used.

As more OT-I T cells were persisting in peripheral organs (Fig. 13) as compared to OT-II T cells (Fig. 10), we were able to isolate sufficient numbers of cells to analyze their function. To determine whether these non-deleted OT-I T cells from OVA-expressing chimeras could differentiate into effector T cells and exert autoimmune aggression *in vivo*, the RIP-Ova^{lo} mouse model was used. In this strain, transgenic OVA-expression in the pancreas is controlled by the rat insulin promoter (RIP) and serves as a model self-Ag (Kurts, Sutherland et al. 1999). When OT-I T cells are transferred into RIP-Ova^{lo} mice, they are ignorant due to low expression levels of OVA. However, upon Ag-specific immunization, transferred OT-I T cells may become activated, destroy the OVA^{+} pancreatic ß-islet cells and the mice develop diabetes. Upon transfer into these recipients, RIP-OVA^{lo} mice were immunized with OVA and all mice that received OT-I T cells from mock-chimeras or wild type OT-I donors developed diabetes with a similar kinetics (Fig. 14). In marked contrast, none of the mice receiving OT-I cells from the CD8^{+} T cell non-depleted DCSTAMP-trOVA-chimeras developed disease (Fig. 14). These results indicate that lentiviral vector-mediated expression of OVA in DCs was able to functionally and efficiently inactivate Ag-specific CD8^{+} T cells, even if only peripheral tolerance was induced.

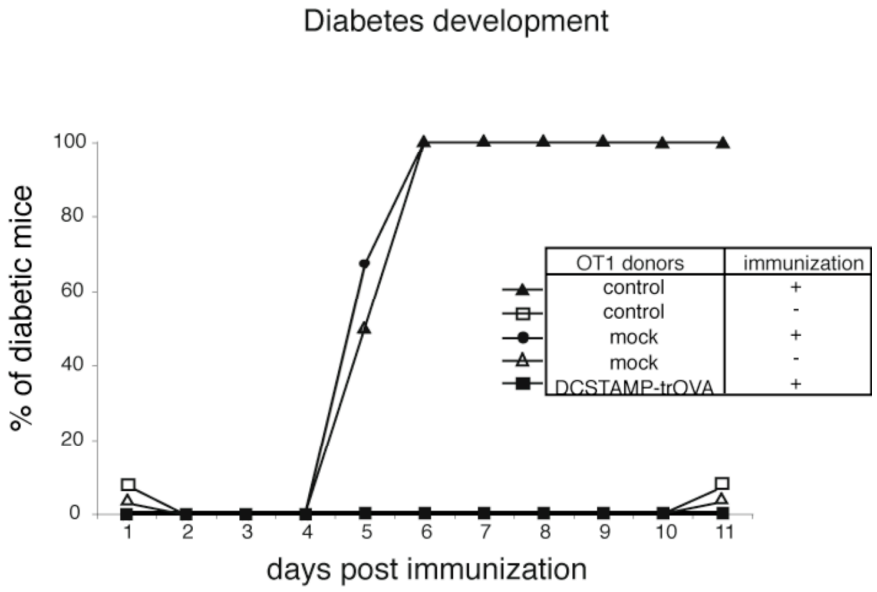

Figure 14: Lentiviral vector mediated targeting of Ag-expression to dendritic cells generates functionally inactivated CD8^{+} T cells. RIP-OVA^{lo} mice received 1x10^{6} OT-I T cells from DCSTAMP-trOVA chimeras, mock chimeras or normal OT-I mice. One day later, mice were immunized with either OVA-IgG antibody immune-complexes and CpG nucleotides (filled symbols) or CpG nucleotides alone

(open symbols). Diabetes induction was monitored and mice with >5,6 nmol/l glucose in their urine were considered diabetic. The percentage of diabetic mice over time is shown, n=3-5 mice per group.

5.5 Transgene expression controlled by the DC-STAMP promoter leads to tolerance of auto-reactive polyclonal antigen-specific $CD8^+$ T cells.

In a non-transgenic system with a polyclonal T cell repertoire, only few cells express TCRs that recognize a specific antigen, while it is estimated that potentially 10^{16} different TCRs can be generated (Abbas and Lichtman, 2003). In addition to these numerical differences, in TCR transgenic mice the TCR can be expressed at an earlier stage of thymocyte development influencing T cell selection (Baldwin, Sandau et al. 2005).

To determine if lentiviral-mediated transgene expression in DCs is able to induce tolerance in normal non-transgenic mice, we repeated the described procedure using C57BL/6 mice as BM donors and recipients. To evaluate if the polyclonal $CD8^+$ T cell repertoire was tolerant to OVA we immunized chimeric mice with a recombinant herpes simplex virus vector (HSV) encoding OVA (HSV-OVA), previously reported to induce strong OVA-specific $CD8^+$ T cell response (Lauterbach, Kerksiek et al. 2004). The frequency of $OVA_{257-264}$ specific $CD8^+$ T cells and HSV glycoprotein B (HSVgB) specific $CD8^+$ T cells was determined by H-$2K^b$/$OVA_{257-264}$ and H-$2K^b$/$HSVgB_{498-505}$ tetramer-staining respectively, at day 7 after immunization (Fig.15). While we saw significantly higher frequencies of OVA-tetramer positive CD8 T cells in mock chimeras than in DCSTAMP-trOVA chimeras, no reduction was found in the frequency of control HSVgB-tetramer positive CD8 T cells. The increased frequencies of HSVgB-tetramer positive CD8 T cells in DCSTAMP-trOVA chimeras could possibly reflect compensatory expansion resulting from the absence (or decrease) of interclonal competition with OVA-specific T cells. These results indicate that as observed in the transgenic OT-I system, also in a normal polyclonal repertoire there was a decrease in the amount of peripheral OVA reactive $CD8^+$ T cells. Moreover, in this system it was also possible to address directly the specificity of the tolerance induction, which is not feasible in a TCR-transgenic model.

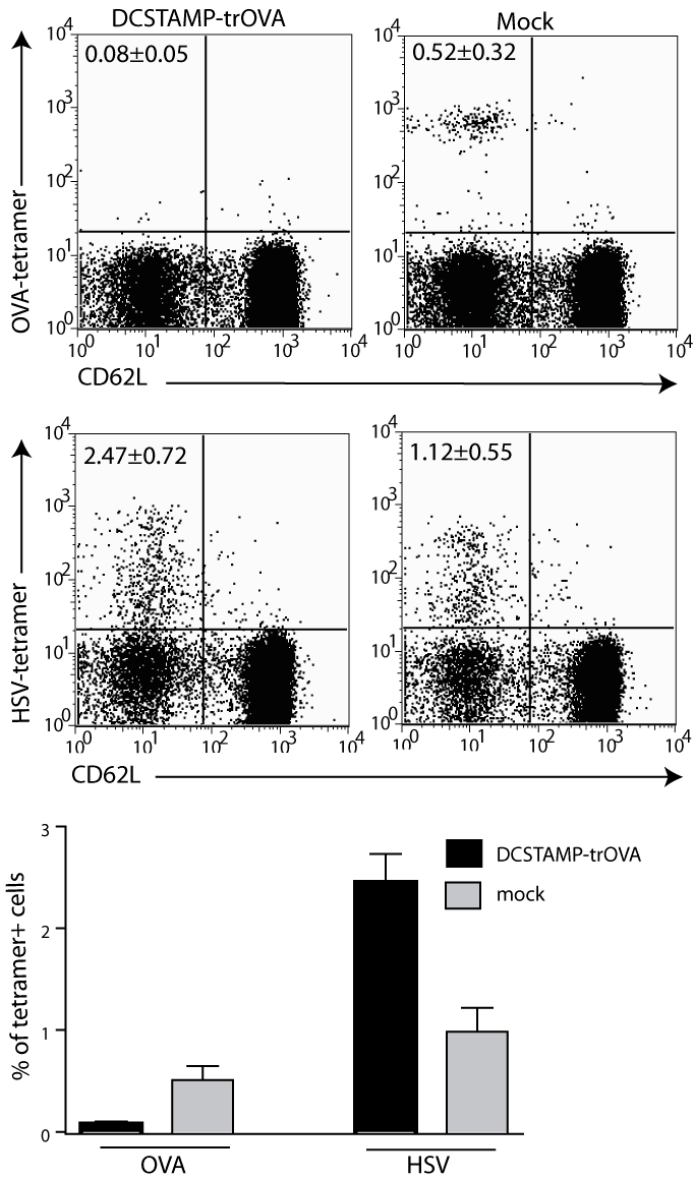

Figure 15: Antigen expression in dendritic cells induces Ag-specific depletion of CD8$^+$ T cells in a polyclonal repertoire. Bone marrow HSCs from C57Bl/6 mice were transduced with DCSTAMP-trOVA or DCSTAMP-mock lentivirus vectors, and bone marrow chimeras were generated. After at least 10 weeks following transplantation, chimeras were immunized by i.v. injection of 4x10^6 pfu of recombinant HSV expressing OVA. 7 days after immunization, leukocytes were isolated from peripheral blood of DCSTAMP-trOVA or mock chimeras and stained with antibodies specific for CD8 (not shown), CD62L as well as H2K^b-OVA- or H2K^b-HSVgB-tetramers, and the frequencies of H-2Kb/OVA or H-2Kb/HSV-specific cells among all CD8$^+$ T cells were analyzed by flow cytometry. N=2-4 mice per group.

To address the question whether or not OVA-specific CTL were present but below the detection limit of MHC-tetramer staining, we performed a highly sensitive *in vivo* killer assay and determined the percentage of specific cytolysis. This technique is based on the ratio of specific elimination of peptide-loaded spleen cells labeled with a

high concentration of CFSE over unloaded-control cells labeled with low concentration of CFSE. Consistent with the results obtained by the tetramer staining, DCSTAMP-trOVA chimeras were not able to perform significant lysis of $OVA_{257-264}$ loaded target cells, while the mock chimeras were able to kill approximately 90% of all $OVA_{257-264}$ loaded target cells (Fig. 16). As both mock and DCSTAMP-trOVA chimeras were equally efficient in killing of $HSVgB_{498-505}$ loaded cells (Fig. 16), we conclude that tolerance induction was Ag-specific. The results obtained with these experiments strongly suggest that transcriptional targeting of DCs with this lentivirus vector induces Ag-specific tolerance of $CD8^+$ T cells also in a normal non-transgenic polyclonal system.

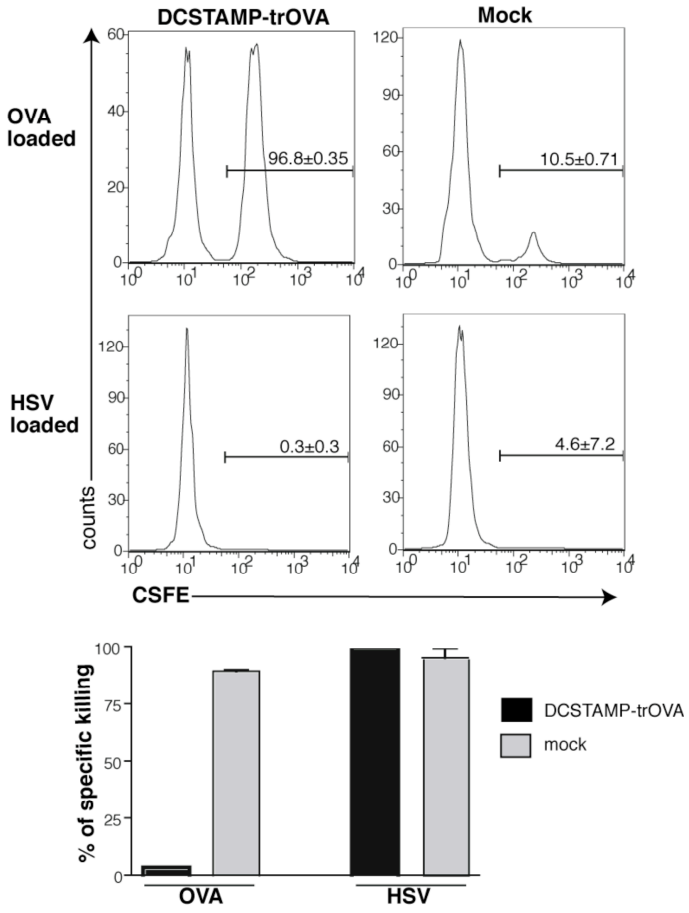

Figure 16: Antigen expression in dendritic cells induces Ag-specific functional tolerance of $CD8^+$ T cells in a polyclonal repertoire. Bone marrow HSCs from C57Bl/6 mice were transduced with DCSTAMP-trOVA or DCSTAMP-mock lentivirus vectors and bone marrow chimeras were generated. After at least 10 weeks following transplantation, chimeras were immunized by i.v. injection of $4x10^6$ pfu of recombinant HSV vector expressing OVA. On day 7 after immunization, a CFSE- based *in vivo* cytotoxic T cell assay was performed, and the specific lysis of OVA-SIINFEKL or HSVgB-SSIEFARL peptide loaded, CFSE-labeled target cells was determined by flow cytometry. N=2-4 mice per group.

5.6 The murine DC-STAMP promoter directs transgene expression in human DCs *in vitro*.

As it was described previously that DC-STAMP protein is highly conserved between human and mice and that its expression is basically restricted to DCs in both species, we hypothesized that the murine DC-STAMP promoter could also direct transgene expression specifically in human DCs. To answer this question, we used the Genomatix software to perform a computational comparison of the 3' UTR of the DC-STAMP promoter from mouse, human and dog, looking for conserved binding site modules in the three species. It is difficult to discriminate significant single transcription binding site elements important for promoter specificity. Common framework elements where more than one element is found in the same order and distance range in inter-species analysis are much more indicative, since it is known that there may exist a synergistic effect among transcription binding sites, and that the conservation of their sequential order is important to keep transcription specific to a certain cell type (Fig. 17; Werner 1999). Furthermore, since several proteins must interact with any given promoter to activate transcription (Thanos and Maniatis 1995), a set of binding sites instead of individual ones should be analyzed. As shown in Fig. 17, we were able to identify a conserved region (p-value of 4.79e-11, where the p-value is the probability to obtain the same model in a randomly drawn sample of human promoters), comprising 4 different elements in the same order of sequence around -1.5Kb in mouse and dog and around -2 kb in human DC-STAMP promoter, indicating a potential group of elements that could partake in the control of specificity of gene expression.

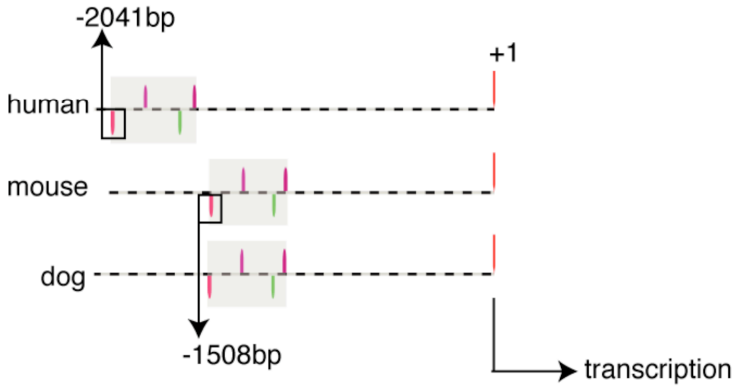

Figure 17: The DC-STAMP promoter is conserved among different species. Around 2 kb (human) and 1.5 kb (mouse and dog) of 5' UTR from DC-STAMP promoter sequence was obtained from

GeneBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html), and computational comparison was performed using the Genomatix software (www.genomatix.de/). Schematic representation of regulatory element comparison of DC-STAMP promoter from human, mouse and dog, where +1 is the transcriptional start site.

To test if the murine DC-STAMP promoter would also drive transgene expression in human DCs, we transduced mono nuclear cells (MNC) isolated from human BM with our vector containing the eGFP driven by the murine DC-STAMP promoter or by the constitutively active human ubiquitin-C promoter (ubiquitin-eGFP). Transduction with DCSTAMP-GFP-SIN-lentivirus resulted in higher DC-specificity as compared to the ubiquitin-GFP-SIN-lentivirus (Fig. 18). A ratio of ~3 was obtained for GFP^+CD1a^+ DC and GFP^+CD1a^- non-DC for DC-STAMP-transduced DCs as compared to a ratio of 1 for DC transduced with the ubiquitin-C promoter containing lentiviral vector (Fig. 18). Accordingly, also fewer $CD14^+$ monocytes and $CD19^+$ B lymphocytes expressed eGFP in DCSTAMP-lentivirus transduced cultures (Fig. 18). This preliminary data indicates that the murine DC-STAMP promoter used in the SIN-lentivirus vector context supports expression of transgenes also in human DCs.

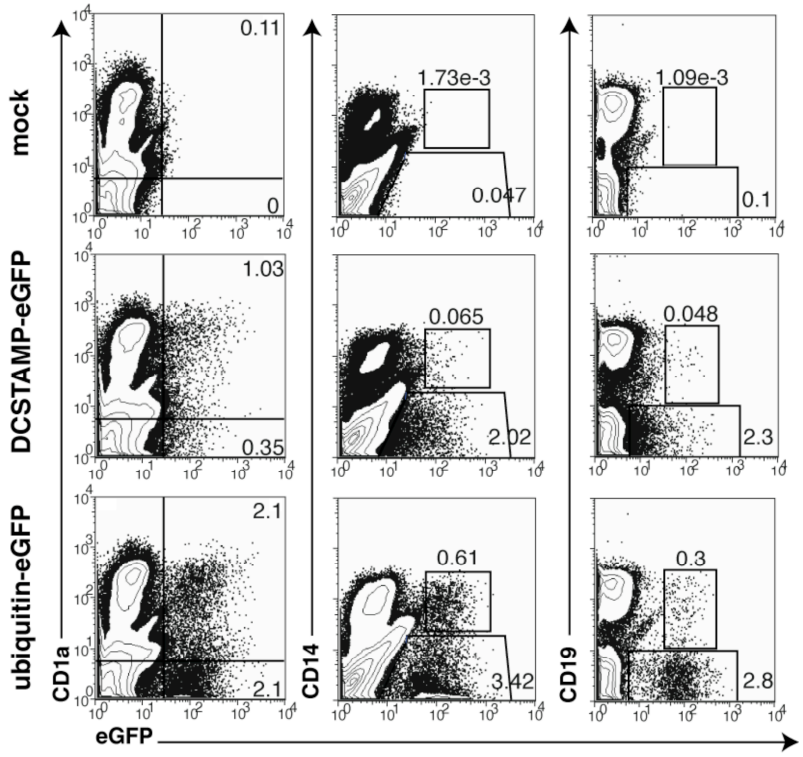

Figure 18: The murine DC-STAMP promoter targets expression to human dendritic cells *in vitro*. Human bone marrow was cultured with GM-CSF, IL-4 and TNF-α and, at day 2 of culture, cells were transduced with DCSTAMP-eGFP, ubiquitin-eGFP or DC-STAMP mock lentivirus vector. Flow cytometry was performed at day 11 of culture with the indicated antibodies.

6. Discussion

6.1 The murine DC-STAMP promoter targets transgene expression to DCs

There are several transgenic mice that express different molecules selectively in DCs. These mice have been very helpful in elucidating the functional differences among the APCs and especially the role of DCs in the immune system. All transgenic mice with DC-specific expression were generated using the murine CD11c promoter (Brocker, Riedinger et al. 1997). In principle, murine CD11c is expressed only in DCs and in all main sub-populations of DCs, although with different levels of expression depending on the type of DC. Generally, CD11c is considered a myeloid marker and possibly for this reason, its expression in pDCs can be very low. Indeed, it was described that in transgenic mice in which cDNA expression was driven by the CD11c promoter, little or no transgene expression could be detected in pDCs (Sapoznikov, Fischer et al. 2007). For this reason, we decided to test an alternative promoter that could potentially target all DC sub-populations and states of maturation. These characteristics, among others, led us to choose the DC-STAMP promoter. In addition, because the generation of transgenic mice is a time consuming and expensive process, our aim was to develop a viral system which could allow stable transgene expression in DCs without the need to produce transgenic mice. Moreover, when considering the targeting of DCs for future clinical applications, it is of fundamental importance to choose a system which can also be used in humans, such as retro or lentiviral vectors. In the present study, we developed a lentiviral vector that targets transgene expression mainly to DCs. In addition to confering murine DC-selective transgene expression, this system seems to be promissory for targeting human DCs.

6.1.1 A SIN-lentiviral but not retroviral vector allows specific transgene expression in DCs

In both basic research and clinical applications, there are several instances in which a methodology that allows stable instead of transient transgene

expression by DCs would be advantageous. Taking this prerequisite under consideration, we believe that either retro- or lentiviral vectors could lead to satisfactory results, since both vectors can integrate into the genome of the infected cell, leading to stable transgene expression. Our aim was to transduce BM-derived HSCs and repopulate irradiated recipient mice with these cells. This would result in reconstitution of the immune system with the transduced HSC derived cells, but with transgene expression only in DCs. When cell-specific promoters are placed into a retro- or lentiviral vector, it is essential to use SIN virus vector backbones in order to gain cell-specificity. It was shown in independent studies that the native viral promoter elements can interact with the internal promoter, reducing gene expression (Yee, Moores et al. 1987; Soriano, Friedrich et al. 1991). However, one important restriction of SIN retroviruses is the reduced virus titer obtained (Riviere, Brose et al. 1995; Vile, Diaz et al. 1995).

In principle, only lentivirus, but not retrovirus can efficiently infect HSCs, since these cells are known to be quiescent and only lentivirus have the ability to infect cells in a non-proliferative state. However, retroviral vectors were used successfully before in our laboratory to transduce BM derived HSCs with the objective of targeting transgene expression to B cells (Werner, Kraunus et al. 2004; Werner-Klein, Dresch et al. 2007). These previous results encouraged us to use the same retroviral vector backbone to transcriptionally target DCs. The first drawback we faced with the use of this retroviral system was the low viral titers obtained (Fig. 5). This was probably due to the fact that our vector was a SIN vector, and that the size of the promoter used to target DCs was considerably large. In fact, the small transgene capacity of retro- and lentiviral vectors (limit of 7-8 kb) is a major limitation for their use in gene therapy. In the work using the retroviral vector to drive transgene expression in B cells, the promoter used was approximately 700 bp shorter than the DC-STAMP promoter used in our work. This difference in size may have allowed the production of higher virus titers. Although the transgene capacity of retroviruses and lentiviruses is the same, the negative effect of the deletion of the regulatory sequences in the U3 region responsible for conferring the SIN characteristics is not the same for the two vectors. Low titers from SIN vectors are associated only with retro but not lentiviral vectors (Yee JK, 1987;

Zufferey R, 1998), which could explain the low titers obtained from the retrovirus vector production when compared to the lentivirus vector production (Fig. 5).

As there is no published work showing a DC-specific promoter in the context of a viral vector, we decided to test both retro and lentiviral vectors in an *in vivo* approach. Surprisingly, we obtained different results from the two vectors although they contained the same fragment of the DC-STAMP promoter. While the DC-STAMP promoter in the context of the SIN-retrovirus could render only weak basal transgene expression in the different cells of the immune system, the same promoter when in the context of the SIN-lentivirus vector resulted in transgene expression mainly in DCs (Fig. 6 and 7). Although to our knowledge there is no work where the same tissue-specific promoter was compared side by side in the context of a retro and a lentiviral vector, we suggest that the nature of the HSCs transduced with the viral vectors may be responsible for these results. HSCs are constantly choosing between a state of quiescence, self-renewal and lineage differentiation, where self-renewal can be only maintained in an appropriate microenvironment in distinct locations within the BM (Arai, Hirao et al. 2005). HSCs can be classified in three major categories according to functional hierarchies. The most undifferentiated would be the quiescent HSCs (0.00125% of BM cells), followed by activated or self-renewing HSCs (0.00425% of BM cells) and differentiating HSCs (0.3% of BM cells) (Wilson, Oser et al. 2007). Several groups have analyzed the potential of long-term reconstitution of HSCs at different states of “maturation”, and it is currently believed that the dormant and activated cells would retain the highest repopulating capacity (Kiel, Yilmaz et al. 2005). Since special BM niches are required to HSC self-renewal (Huang, Cho et al. 2007), and consequently it is not possible to keep these cells in culture, the quiescent HSCs would probably be the main subset of HSCs that reconstitute the hematopoietic system after BM transplantation. Therefore, it is possible that we were able to observe DC-specific transgene expression only when quiescent HSCs were modified. The fact that only lenti- but not retroviral vectors are able to infect quiescent cells would explain the different results obtained with the two vectors. The branching points at which hematopoietically derived cell lineage commitment occurs is still controversial (Akashi 2007; Iwasaki and Akashi 2007), and little is known about the early regulation of gene expression and silencing during the initial stages of cell differentiation. However, it is

known that pluripotency as well as lineage differentiation depend upon specific chromatin organization, which is required for establishing and maintaining gene expression programs (Teitell and Mikkola 2006). We therefore suggest that at a later differentiation point, the DC-STAMP promoter could be silenced, resulting in only a low background of transgene expression. However, such a phenomenon was not observed with the CD19 promoter, which supported transgene expression in B cells, even if HSCs from a late state of differentiation were infected with standard retrovirus vectors. While we can only hypothesize about the factors influencing transgene expression with different kinds of viral vectors, we clearly showed (Figs. 6 and 7) that the DC-STAMP promoter can efficiently target transgene expression to DCs *in vivo* in the context of a SIN-lentiviral but not SIN-retroviral vector.

6.1.2 The DC-STAMP promoter drives transgene expression mainly in DCs

Although DC-STAMP was originally isolated from a cDNA library of human monocyte–derived dendritic cells (Hartgers, Vissers et al. 2000), recent studies have clearly shown its expression and function in osteoclasts (in the bone) and giant cells [in different tissues; (Kukita, Wada et al. 2004; Vignery 2005)]. Osteoclasts and giant cells are multinucleated cells originating from the fusion of macrophages, although the mechanisms that govern this process are poorly understood. In fact, the monocyte/macrophage lineage is quite heterogeneous and it is believed that such heterogeneity is physiologically relevant, since it is conserved in human and mouse (Gordon and Taylor 2005).

While it is clear that monocytes can give rise to different specialized cells, further studies are necessary to understand for example how monocytes are recruited to particular sites of inflammation and what determines their differentiation into DCs or into macrophages. By using our lentiviral vector with transcriptional control by the DC-STAMP-promoter, we were able to transduce HSCs and obtain transgene expression mainly in DCs, but also in some monocytes (Fig. 6 and 7). In the present study we identified monocytes as $CD11b^{+}CD11c^{-}$ cells. Although it is known that monocytes are progenitors of at least some subpopulations of DCs, the differentiation

pathways have not yet been identified (Gordon and Taylor 2005), and it is difficult to differentiate between “real” monocytes and DC-precursors. But as DC-STAMP is a protein expressed in cells originating from monocytes, it is not surprising that some transgene expression driven by the DC-STAMP promoter was also detected in this type of cells. Nevertheless, since the monocyte lineage is closely related to DCs, there are few, if any, negative implications that could possibly arise from transgene expression in both monocytes and DCs. Interestingly, although transgene expression in DCs and monocytes points towards a preferencial activity of the DC-STAMP promoter in myeloid cells, we also observed a high expression of the reporter transgene in the $CD8^+$ DC population, which has been described as being of lymphoid origin (Ardavin 2003). In contrast, pDCs that are also considered as predominantly of lymphoid origin expressed DC-STAMP-transgene only weakly in most experiments (Fig. 7a). Therefore, the lentiviral DC-STAMP system may be a valuable tool also to study DC origin and development.

As DC-STAMP is a protein that was originally identified in human DCs (Hartgers, Vissers et al. 2000), it would be expected that its promoter would also be efficient in targeting DCs from human origin. When we compared transgene expression regulated by DC-STAMP promoter and an unspecific ubiquitous promoter, we showed that DC-STAMP was more efficient to target transgene expression in DCs (Fig. 18). Therefore, our preliminary results *in vitro* indicated that the DC-STAMP-lentivirus might be promising for targeting human DCs. However, further studies will be required to demonstrate its DC-specificity in human hematopoietic systems.

6.2 DC-STAMP-lentivirus mediated transgene expression induces antigen-specific tolerance in $CD4^+$ and $CD8^+$ T cells *in vivo*

Although T cells expressing a TCR with high affinity for self-antigens are mainly deleted in the thymus, it is unlikely that the deletion of all autoreactive T cells occurs exclusively there. To control autoreactive T cells that have escaped negative selection in the thymus, peripheral tolerance is an important mechanism to complement

central tolerance (Mathis and Benoist 2004). DCs play a central role in maintaining both central and peripheral tolerance by inducing clonal deletion or non-responsiveness. Antigen presentation by DCs was shown to be important in tolerance induction in transgenic murine models (Gallegos and Bevan 2004) and targeting DCs with specific antibodies (Gunzer, Weishaupt et al. 2004) or DC-specific promoters (Probst, Lagnel et al. 2003; Probst, McCoy et al. 2005) results in tolerance to the corresponding antigen. The activation state of the DCs that present self-antigens is pivotal to the outcome of T cell activation. When DCs are activated T cell priming occurs and when resting DCs present the antigen T cell tolerance takes place (Banchereau and Steinman 1998; Hawiger, Inaba et al. 2001; Steinman, Hawiger et al. 2003). Since DCs are implicated in induction and maintenance of T cell tolerance, we wanted to investigate if our lentiviral vector system could be applied for antigen-specific tolerance induction.

In this study we were able to show that lentiviral vector-mediated expression of a transgene by DCs induces central tolerance of antigen-specific $CD4^+$ T cells (Fig. 9). While the importance of DCs in inducing central tolerance of $CD4^+$ T cells is well accepted, the consensus about participation of DCs in generation of natural $Foxp3^+CD25^+$ Tregs is more conflicting. While it is widely accepted that natural Treg cells originate in the thymus as a functionally distinct and mature population, there is evidence that T cells with similar immune suppressive activity can be generated from naïve T cells in the periphery after, for example, chronic antigen stimulation *in vivo* (Chen, Jin et al. 2003; Apostolou and von Boehmer 2004). As we analyzed all BM chimeras only in steady state but not under infection or inflammatory conditions, the question if lentiviral vector-mediated expression of antigen by DCs would lead to peripheral Treg differentiation requires further analysis.

In addition, the exact nature of the antigen-expressing and/or antigen-presenting cells involved in natural Treg cell development within the thymus is unknown. An in vitro study with human thymus suggested that thymic DCs conditioned with thymic stromal lymphopoietin may promote the generation of Treg cells in the human thymus (Watanabe, Wang et al. 2005). More recently it was shown that antigen specific presentation by DCs led to deletion of autoreactive $CD4^+$ T cells in the thymus, while antigen presentation by thymic epithelial cells would lead to the differentiation of natural Treg (Aschenbrenner, D'Cruz et al. 2007). Moreover, in the same study it was

suggested that DCs are not able to delete $CD4^+$ T cells that were already differentiated into Treg. These findings are in accordance with our preliminary results, in which no difference could be observed in total numbers of $Foxp3^+CD25^+$ Treg cells between mock and SIN-DC-STAMP-trOVA chimeras (Fig.11). These results indicate that lentiviral vector-mediated expression of transgene by DCs leads to central tolerance of $CD4^+$ T cells by depleting autoreactive cells, but not by inducing differentiation of these cells into natural Treg cells.

Although we showed that lentiviral vector-mediated expression of the transgene by DCs was sufficient for inducing central tolerance of both autoreactive $CD4^+$ and $CD8^+$ T cells, central deletion of Ag-specific $CD8^+$ T cells was less efficient (Fig. 9 and 12). These results are in accordance with previous findings showing that DCs are preferentially involved in $CD4^+$ T cells central tolerance induction, but that mTECs can participate in the deletion of $CD8^+$ T cells (Gallegos and Bevan 2004). However, in Gallegos and Bevan's work not the DCs but the thymic epithelial cells were expressing the OVA protein and the DCs were acquiring and cross-presenting the peptide. However, the process of cross-presentation or even a possible interaction/cooperation between the TECs and DCs may be important for the deletion of the autoreactive $CD8^+$ T cells in the thymus. Consequently, the lack of transgene-expression by TECs in our BM chimeras may result in lower deleting-efficacies of $CD8^+$ thymocytes. Nevertheless, Ag-expression in DCs led to a tolerant $CD8^+$ T cell compartment. We showed that $CD8^+$ T cells escaping central thymic deletion were functionally inactivated, since none of the RIP-OVA^{lo} mice that received OT-I cells from our DC-STAMP-trOVA-chimeras developed diabetes (Fig. 14).

The OT-I and –II TCR-transgenic read-out systems used in this study demonstrated that lentiviral targeting of DCs imposed robust tolerance induction even in presence of artificially high precursor-frequencies of Ag-specific T cells (Fig. 9, 10, 12 and 13). However, transgenic TCRs can be expressed at earlier stages of thymocyte-development as compared to natural endogenous TCR-proteins and this may affect T cell selection (Baldwin, Sandau et al. 2005). To exclude the possibility that our system would be efficient only in TCR transgenic mice, we repeated the same procedure in wild type non-transgenic mice. We found that polyclonal "normal" T cells could also be tolerized, as complete absence of Ag-specific $CD8^+$ T cell immunity in peripheral organs

was demonstrated (Fig. 15 and 16). Therefore, lentiviral vector-mediated DC-targeting seems to induce tolerance also in wild-type T cell populations, although we were not able to distinguish in this case if tolerance was imposed centrally or in peripheral organs by deletion or anergy.

6.2.1 Effect of $CD8^+$ T cell depletion from the donor bone marrow on tolerance induction

One interesting observation from our results is the absence of central deletion of OT-I cells in the chimeric recipients of BM non-depleted of $CD8^+$ T cells. In contrast, in the same chimeras peripheral deletion and tolerance induction of OT-I cells occurred (Fig.12 and 13). However, when the $CD8^+$ T cells were depleted from the donor bone marrow the amount of OT-I cells was also reduced in thymus, although we observed a strong reduction in both percentage and total cell numbers only within spleen and LN (Fig. 12, 13 and data not shown). The fact that we could not detect any difference in the OT-I cell number or frequency in the thymus of $CD8^+$ T cell non-depleted BM recipients, suggests a possible donor T cell cytotoxic effect restricted to DCs resident in the thymus, but not in the peripheral lymphoid organs. This cytotoxity could lead to killing of DCs presenting OVA antigen, although OVA RNA could be detected in the DC^+ fraction of cells isolated from thymus (Fig. 12b).

In addition, it was possible to detect an increase in expression of CD24 in the OT-I cells in the thymus of SIN-DC-STAMP-trOVA chimeras compared to mock chimeras (Fig.10a), indicating an immature state of these cells. Together, these results suggest that even if killing of OVA presenting DCs occurred, OT-I cells might have had contact with at least some DCs. This interaction was not sufficient for depletion of autoreactive $CD8^+$ T cells, but may have been sufficient to lead these cells to a functional and/or qualitative change. Therefore, although no difference in total numbers of OT-I cells was detected, it remains possible that the OT-I cells were qualitatively different and left the thymus with a “predisposition” to be tolerized. Indeed it has previously been discussed that tolerance to host class I antigens is more marked in LN than in thymus, and that auto-reactive $CD8^+$ T cells could be rendered “semi-tolerant” in the thymus to have the complete process of tolerization taking place in peripheral

lymphoid organs (Sprent, Kosaka et al. 1993). In accordance with this idea, it was shown that $CD8^+$T cells isolated from the thymus of chimeras give significant proliferative responses to host-type Ags when stimulated *in vitro*, while cells isolated from LN and spleen gave extremely low responses under the same conditions (Kosaka and Sprent 1993). This would also be in consistent with our results regarding $CD8^+$T cell depleted BM recipients, where tolerance induction was much more efficient in the periphery than in the thymus.

It is also possible that the kind of interaction and signaling between DCs and T cells in the thymus is different as compared to T cell signaling in secondary lymphoid organs. Such differences could allow killing of thymic DCs. Indeed, thymic DCs have some particularities when compared with the peripheral counterparts. The majority (75% in B6 and 90% in Balb/c) of DCs in thymus are $CD8^+$ (Vremec, Pooley et al. 2000). Moreover, the great part of the thymic $CD8^+$ DCs are different as compared to $CD8^+$ DCs found in the periphery. A proportion of thymic $CD8^+$ DCs, but not of peripheral DCs, express mRNA and surface BP-1 [a glycoprotein mainly expressed by early B cells; (Wu, Vremec et al. 1995; Okada, Lian et al. 2003)]. These particularities are in agreement with the different origin of thymic and peripheral DCs. Currently it is believed that the major thymic DCs are generated within the thymus (Ardavin, Wu et al. 1993). Furthermore, many $CD8^+$ thymic DCs have a more “activated” state than their peripheral counterparts, since the co-stimulatory molecules CD80 and CD86 are expressed at higher levels (Wilson, El-Sukkari et al. 2003). This more activated state could also explain why mature donor OT-I cells could kill DCs in the thymus but not those found in the periphery. However, further studies are necessary to address these questions directly.

6.3 Therapeutic potential of gene therapy for tolerance induction by a DC-specific lentiviral vector

Gene-therapy is considered an efficient method to induce tolerance, when the identity of target-Ags in autoimmune diseases and transplant rejections are known, and several studies on this topic have been published (Ally, Hawley et al. 1995; Kang, Melo et al. 1999; Tsokos and Nepom 2000; Bagley, Tian et al. 2002; Kang and

Iacomini 2002; Tian, Bagley et al. 2003). However, in these studies tolerance induction was achieved with conventional retroviral vectors leading to transgene expression in multiple cell-types. This can be accompanied by potentially dangerous activities of viral enhancers (Li, Modlich et al. 2004), as vector integration in the proximity of a proto-oncogene promoter could induce uncontrolled exponential clonal cell proliferation. The development of T-cell leukemia in three out of 20 patients following gene-therapy for the treatment of X-linked severe combined immune deficiency (X-SCID) has highlighted the adverse effect of insertional mutagenesis and led to a re-evaluation of this approach (Hacein-Bey-Abina, Von Kalle et al. 2003; Marshall 2003; Check 2005). The gene IL2RG encodes the gamma-chain of the interleukin-2 receptor and is mutated in patients with X-SCID. It has been shown that retroviral integration of the corrective IL2RG occurred near the locus of the LMO2 oncogene5 and that this integration may have upregulated the expression of LMO2 and, eventually, led to leukemia in 3 gene therapy patients (Hacein-Bey-Abina, Von Kalle et al. 2003). The possibility that IL2RG might itself be a contributor to oncogenesis raised again the value of gene therapy. In a recent study, it was shown that 33% of C57BL6 X-SCID mice developed T-cell lymphomas after reconstitution with X-SCID or wild-type bone-marrow stem cells transduced with lentiviral vectors encoding IL2RG. However, X-SCID mice engrafted with bone-marrow cells treated by mock transduction, did not develop lymphomas. As there were no common genomic targets in the five mice with lymphoma and because the controls did not develop disease, the authors concluded that the lymphomas were not caused by insertional mutagenesis and that the therapeutic transgene itself is intrinsically oncogenic (Woods, Bottero et al. 2006). In accordance with the idea that not the virus per se is responsible for cancer development, is the fact that HIV^+ patients do not have an increased frequency of cancers that could be correlated with insertional mutagenesis. A higher incidence of cancer in AIDS patients has been reported, but this has been correlated rather with immunosupression than viral insertion (Grulich, van Leeuwen et al. 2007). These new findings are causing researchers to reconsider gene therapy with a more optimistic view.

Despite this, the random insertion of the viral vector into the genome and its potential risks are still a negative characteristic of gene therapy, and approaches to reduce these risks are necessary. For example, the use of lentiviral vectors equipped

with an appropriate eukaryotic promoter and without viral enhancers can minimize potential dangers. The approach presented in this work was to target transgene expression to DCs, a cell type present only in low numbers with a low propensity for proliferative disorders. In addition, DCs are the functionally most relevant and efficient cell-type for immune modulations such as tolerance induction. Several reports describe *in vitro* modifications of human DCs for boosting immune responses against cancer (Schuler, Schuler-Thurner et al. 2003) or induction of tolerance (Dhodapkar, Steinman et al. 2001; Dhodapkar and Steinman 2002). The major drawbacks of these studies included the difficulties to obtain sufficient amounts of DCs for application, functional DC-changes by their *ex vivo* manipulation, as well as influences of the route of DC-application on the experimental outcome (Fong, Brockstedt et al. 2001). Moreover, whether antigen-pulsed DCs induce autoimmunity or tolerance is still controversial. For example, DCs have been described to induce experimental autoimmune encephalomyelitis [EAE, (Weir, Nicolson et al. 2002)] and diabetes (Ludewig, Odermatt et al. 1998) in mice. However, DCs have also been reported to mediate protection against the same diseases (Huang, Yang et al. 2000; Papaccio, Nicoletti et al. 2000). Indeed, adoptive transfer of spleen-derived DCs from mobilized donor mice failed to confer protection from GVHD (MacDonald, Rowe et al. 2005), a result that has been attributed to an inadequate state of DC maturation. Approaches involving the genetic modifications of DCs are mostly based on the viral transduction of genes encoding immunosuppressive molecules, such as CD95 (Fas) ligand, IL-10 and CTLA4 (Takayama, Nishioka et al. 1998; Takayama, Morelli et al. 2000; Buonocore, Van Meirvenne et al. 2002; Buonocore, Paulart et al. 2003). Whereas the *in vitro* immunosuppressive efficacy of such modified DCs is unquestionable, its *in vivo* effect is not guaranteed. A possible explanation for this is that *ex vivo* manipulation and virus vector transduction induce DC maturation and activation (Miller, Lahrs et al. 2002). However, the maturation state of DCs does not seem to be the only factor influencing T cell responses. Recent studies show that fully mature DCs can be tolerogenic (Albert, Jegathesan et al. 2001). Cross-tolerance of Ag specific $CD8^+$ T cells requires DCs with a mature phenotype (Albert, Jegathesan et al. 2001). This raises the question which DC maturation process can induce tolerance.

Further, a general restriction of adoptive DC-transfer is the limited survival of cultured DCs *in vivo*, making several consecutive therapeutic interventions necessary to obtain measurable DC-induced immune modulation. Induction of $CD8^+$ T cell tolerance depends on long-term exposure of T cells to Ag-presenting DC *in vivo* (Redmond and Sherman 2005). Also *in vivo* imaging has shown that multiple brief DC-$CD8^+$ T cell contacts were required over prolonged periods of time for efficient tolerance induction (Hugues, Fetler et al. 2004). Therefore, the lentiviral system presented in our study would be advantageous as it allows the modification of autologous bone marrow for continuous output of genetically modified tolerogenic “steady-state” DCs without the need for multiple *ex vivo* manipulations.

Through computational analysis we compared the DC-STAMP promoter sequence of mouse, human and dog, and found highly conserved regulatory regions among the three sequences (Fig. 17). These findings suggest that a DC-STAMP promoter could also be used to target DCs in different species. Indeed our preliminary results *in vitro* indicated that the DC-STAMP-lentivirus might be promising for targeting human DCs (Fig. 18). However, further studies will be required to demonstrate its DC-specificity in human hematopoietic systems.

In the present work, we tested the efficiency of T cell tolerance induction by lentiviral vector-mediated transcriptional targeting of DCs. Tolerance induction occurred in Ag-specific $CD4^+$ and $CD8^+$ T cell compartments, in both transgenic and wild type mice. Taken together, our results provide strong evidence that DC-specific lentiviral approaches are a potent means to induce and maintain Ag-specific $CD8^+$ and $CD4^+$ T cell tolerance and may be of clinical relevance for therapeutic applications in a transplantation or autoimmune setting where T cell tolerance is required to limit tissue pathology.

7. Outlook

In the prophylaxis/treatment of transplantation and autoimmune diseases, standard immunosuppressive strategies consist of the inactivation of the whole or large part of the immune system. This nonspecific inhibition compromises the ability of the host to combat opportunistic infections and/or increase the risk of cancer development. Therefore, self Ag-specific tolerance strategies have the best therapeutic potential, since they maintain the capacity of the immune system to clear non-self Ags. Several clinical trials have been performed to induce antigen-specific tolerance in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and type I diabetes. To date, the efficacy of the employed immunotherapies is uncertain, in part because of factors such as route, dosage and frequency of antigen administration. Oral administration of Ag has been effective in inhibiting the induction of autoimmune disease in animal models, however, there is to date no indication of efficacy of this kind of therapy for the treatment of autoimmune diseases in humans. Even when the protein involved in the development of the autoimmunity is identified, as the myelin basic protein in the case of multiple sclerosis, the efficacy of the peptide administrated to induce tolerance can vary depending on the HLA of the patient. The diversity of the human HLA haplotype is probably one of the factors contributing to the variable results from the so far tested Ag-specific tolerogenic approaches. Besides being specific and long-lasting, another advantage of the lentivirus-mediated tolerance induction presented in this work is that the identification of the specific epitope(s) involved in the onset of the autoimmune disease (and the correlation of such peptide and specific HLA) is not necessary. The DC expresses the whole cDNA of interest, leading to presentation of several different epitopes. The same advantage would apply to tolerance induction in case of allergy and transplantation. Regarding basic research, the targeted transgene expression by DCs through the lentiviral vector presented in this work, will enable the study of questions related to the biology of DCs and perhaps the regulation/differences between central and peripheral tolerance induction.

8. Bibliography

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