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Dendritic cell-based immunotherapy – implications for novel vaccines from transgenic hSIGN and HUT9 mice

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1 Abbreviations

Ab	Antibody
APC	Antigen presenting cell
APC	Allophycocyanin (conjugated to Ab)
ATP	Adenosine tri-phosphate
BAC	Bacterial artificial chromosome
BMDC	Bone marrow derived dendritic cell
C. albicans	Candida albicans
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDNA	Complementary DNA
CFSE	5-(6)-carboxyfluorescein diacetate succinimidyl ester
CFU	Colony forming unit
CpG	Cytosine-guanosine oligonucleotide
CRD	Carbohydrate recognition domain
DC	Dendritic cell
DC-SIGN	Dendritic cell specific ICAM-3-grabbing nonintegrin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
Erk	Extracellular signal-regulated protein kinase
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallisable
FCS	Fetal calf serum
FITC	Fluorescein-5-isothiocyanate
FLT3(-L)	FMS-like tyrosine kinase (-ligand)
Foxp3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony stimulating factor
h	Hours
H. pylori	Helicobacter pylori
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase

hSIGN	Human DC-SIGN transgenic mouse
hu	human
HUT9	huTLR9 transgenic mouse
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobuline
IκBα	Inhibitor of kappaB alpha
IL	Interleukin
IRAK	IL-1-R-associated kinase 4
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
kb	Kilo-base
kDA	Kilo-Dalton
LAM	Lipoarabinomannan
l	Liter
LRR	Leucine-rich repeats
M	Molar
ManLAM	Mannosylated LAM
MAPK	Mitogen-activated protein kinase
MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter
MOI	Multiplicity of infection
M. bovis BCG	Mycobacterium bovis Bacillus Calmette-Guérin
mRNA	Messenger RNA
Mtb	Mycobacterium tuberculosis
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor-kappaB
NAIP	Neuronal apoptosis inhibitory protein
NALP	Pyrin domain–containing protein
NOD	Nucleotide oligomerization domain
ODN	Oligonucleotide
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PD	Phosphodiester
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
Pen	Penicillin
PRR	Pattern-recognition receptor
PS	Phosphorothioate
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
Strep	Streptomycin
Syk	Spleen tyrosine kinase
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor associated factor 6
TRIF	TIR domain-containing adaptor inducing IFN-ß
V	Volt
WT	Wild-type

2 Introduction

2.1 Dendritic cells – a promising target for novel vaccines

Since E. Jenner established the first successful vaccination against pox virus in 1796, a lot of efficient vaccines have been developed protecting us today against lethal infections such as diphtheria and polio (Roush and Murphy 2007). However, despite a declining incidence of infectious diseases, one third of the world population is still infected with tuberculosis (Tbc). Moreover, in 2007 approximately two million people died from human immunodeficiency virus (HIV) and more than one million deaths were caused by malaria (Fauci 2008). In addition, especially industrial countries are faced with increasing numbers of nosocomial infections due to multidrug-resistant strains such as methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant enterococci (VRE) (NNIS 2003).

Apart from infectious diseases, vaccinology is challenged by epidemiological data demonstrating a dramatic increase of allergic and autoimmune diseases such as asthma and multiple sclerosis (Bach 2002). Furthermore, today every tenth woman develops breast cancer and five percent of all humans will be diagnosed with large bowel cancer during their lifetime (Ries 2008).

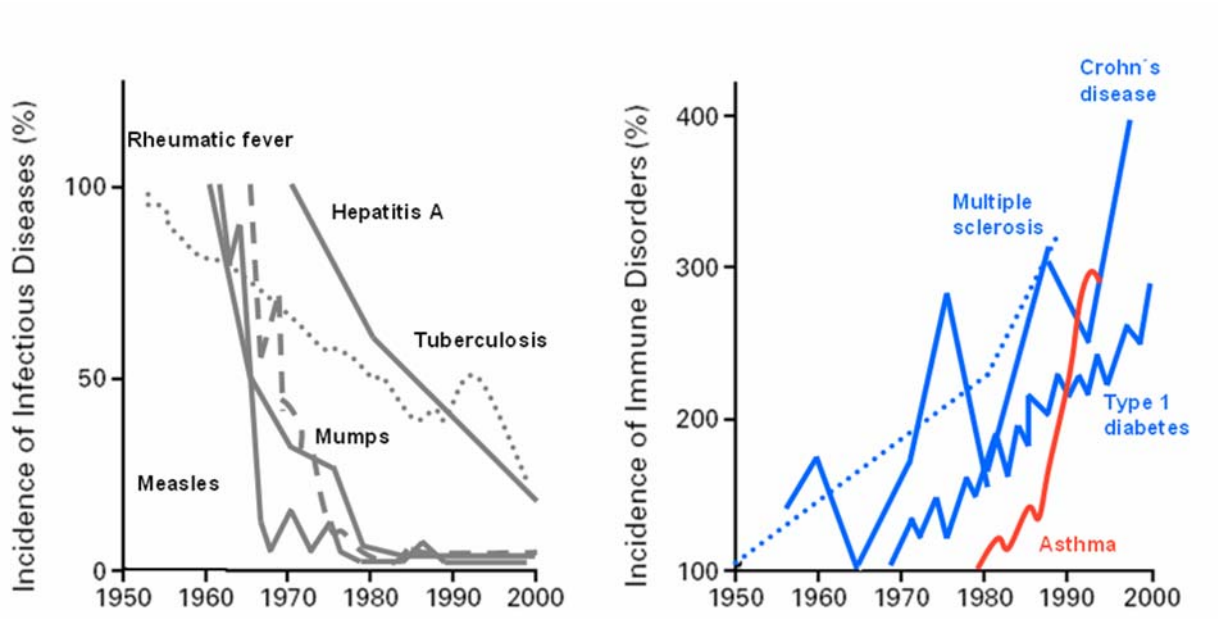


Figure 1: Inverse Relation between the Incidence of Prototypical Infectious Diseases (left Panel) and the Incidence of Immune Disorders (right Panel) from 1950 to 2000 (Bach 2002).

On the one hand, these data reflect a requirement of vaccines against pathogens and tumours while on the other hand, they also demonstrate a demand for vaccines to prevent and treat allergic and autoimmune diseases. Independent from its final purpose, a vaccine has to induce a defined and stable immune response carried out by antigen specific T cells and/or antibodies (Abs).

In this context, dendritic cells (DCs) play a pivotal role. The first time described as a new cell type in mouse spleen by R.M. Steinman in 1973, DCs develop as a subset of bone marrow (BM)-derived leukocytes in a multilinear process from haematopoietic stem cells. With a cellular proportion of 0.5 - 2 %, DCs obtain a sparse but ubiquitous distribution among lymphoid and non-lymphoid tissues (Steinman and Cohn 1973). Here, they act together with B-cells and macrophages as professional antigen presenting cells (APCs) scouring their environment for self and non-self antigens. By various receptors DCs constantly take up, process and present antigens via major histocompatibility complexes (MHC) to α/β T cells and thereby link the innate and the adaptive immune system (Banchereau and Steinman 1998).

If this process occurs in the absence of inflammatory stimuli, DCs stay immature (iDCs), express low levels of MHC class II complexes and downregulate co-stimulatory molecules such as CD86/B7.2. In the presence of transforming growth factor ß (TGF-ß), iDCs promote tolerogenic immune responses including expansion of $CD4^+$ $Foxp3^+$ regulatory T cells (Treg cells) (Chen, Jin et al. 2003).

By contrast, activated mature DCs upregulate expression of MHC class II complexes as well as co-stimulatory molecules and produce pro-inflammatory cytokines such as interleukin 6 (IL-6), IL-12 or IL-4 inducing distinct effector T cell subsets. The release of IL-12 from mature DCs favours differentiation of IFN-γ producing $CD4^+$ Th1 cells, Th1 isotypes and cytotoxic $CD8^+$ T cells (Murphy, Terres et al. 1994; Sparwasser, Vabulas et al. 2000). By secreting TGF-ß and pro-inflammatory cytokines such as IL-6, IL-21 or IL-23 DCs promote development of $CD4^+$ Th17 cells and IL-17 producing $CD8^+$ T cells, respectively (He, Wu et al. 2006; Veldhoen, Hocking et al. 2006). The priming of Th1 as well as Th17 cells needs to be highly controlled since both subsets are essential for clearance of pathogens but can also cause autoimmunity (Fritz, Le Bourhis et al. 2007; Luger, Silver et al. 2008). $CD4^+$ Th2 cells represent a subset that becomes induced by IL-4 producing thymic stromal lymphopoeitin (TSLP) -activated DCs or by IL-4 secreting basophils (Wang, Ito et al. 2006; Sokol, Barton et al. 2008). Th2 cells release IL-4, IL-5 and IL-13 and promote production of Th2 isotypes.

On the one hand, Th2 responses provide protection against parasite infections whereas on the other hand, an inadequate activation causes immediate hypersensitivity such as allergic asthma (Lambrecht, Salomon et al. 1998; MacDonald, Patton et al. 2002).

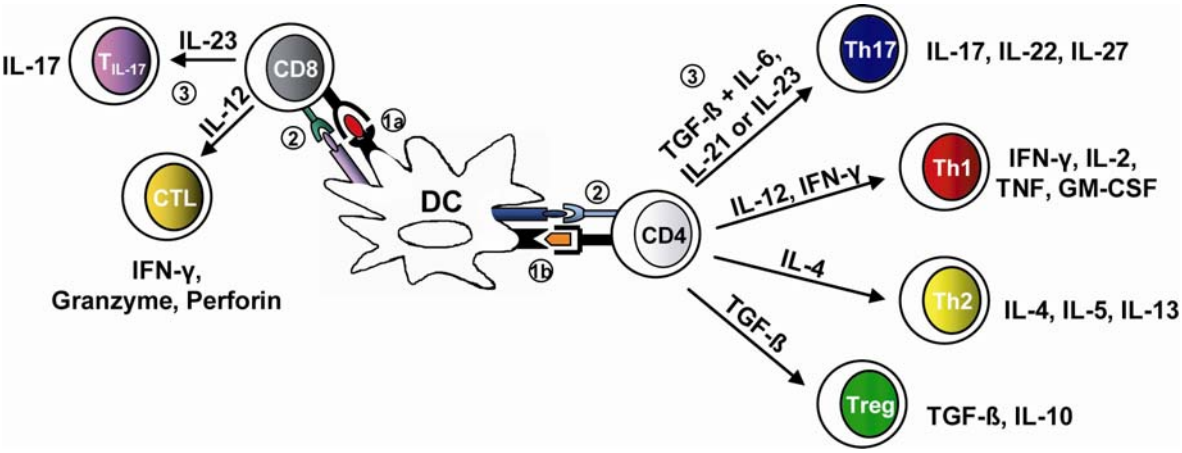


Figure 2: Induction of distinct T cell subsets by DC: By at least three signals DCs prime distinct $CD4^+$ T cell subsets including Th17, Th1, Th2 and Treg cells and promote development of $CD8^+$ T cells into CTLs and T_{IL-17} cells. The first signal is provided by MHC-I/TCR (1a) or MHC-II/TCR (1b) contact, the second is determined by co-stimulatory molecules (2) and the third depends on the cytokine secretion profile of DCs (3) (Kaufmann 2007).

2.2 Dendritic cell receptors

In contrast to T- and B-lymphocytes of the adaptive immune system, DCs are not equipped with antigen specific receptors but possess highly conserved non-rearranging pathogen recognition receptors (PRRs) detecting microbial-derived molecules, also referred to as pathogen associated molecular pattern (PAMPs).

The TREM family (triggering receptors expressed on myeloid cells) consists of the transmembrane receptors TREM-1, TREM-2 and the TREM-like transcripts, TLT-1 and TLT-2 regulating activation and differentiation of myeloid cells. It has been reported that TREM-2 upregulates CCR7 chemokine receptor expression on DCs and thereby promotes DC migration whereas TREM-1 amplifies inflammatory signals and its blockade can rescue mice from septic shock (Bouchon, Facchetti et al. 2001; Bouchon, Hernandez-Munain et al. 2001).

In addition, DCs are equipped with cytoplasmic PRRs such as Nod-like receptors (NLRs) including the NALP-, NAIP- and NOD-subfamilies which constitute a cytoplasmic receptor network, also referred to as inflammasome. Here, DCs detect bacterial compounds such as peptidoglycan and bacterial DNA as well as endogenous danger associated molecular pattern (DAMPs) like uric acid (Meylan, Tschopp et al. 2006). Notably, signalling of NLRs involves pro-inflammatory caspases cleaving inactive precursors of IL1-ß, IL-18 and IL-33. In humans this process includes caspases 1, 4 and 5 whereas in mice caspases 1, 11 and 12 are involved. In addition, retinoid-acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) sense cytoplasmic RNA. RIG-I detects viral 5´triphosphate single stranded (ss) RNA and MDA5 senses viral double stranded (ds) RNA (Takeuchi and Akira 2008). DNA viruses that reside in the cytoplasm are recognized by the DNA-dependent activator of interferon-regulatory factors (DAI) (Takaoka, Wang et al. 2007).

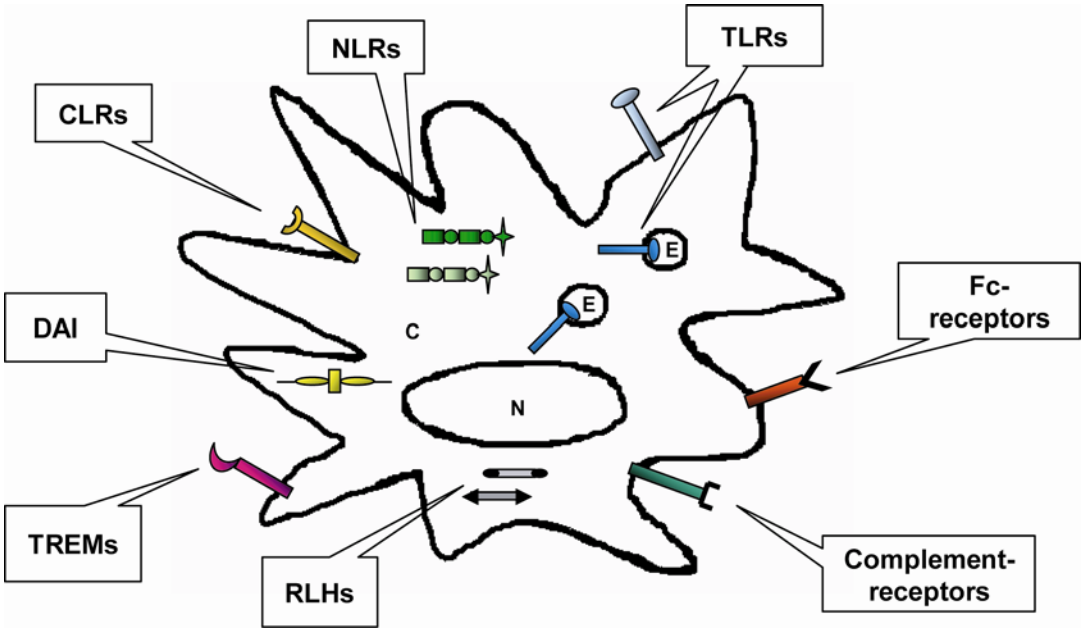


Figure 3: DC receptor repertoire for antigen sensing and uptake: On the one hand, DCs express receptors for antigen uptake such as Fc-receptors, Complement-receptors and C-type lectins (CLRs) located on the cell surface and on the other hand, DCs possess receptors that sense PAMPs and thereby activate DCs like triggering receptors expressed on myeloid cells (TREMs) on the cell surface and Nod-like receptors (NLRs), RIG like helicases (RLHs), DNA-dependent activator of IFN-regulatory factors (DAI) in the cytoplasm (C) as well as Toll-like receptors on the cell surface and at endosomal membranes (E). Nucleus (N) (Guy 2007).

2.2.1 TLR9 – a member of the Toll-like receptor family

Toll-like receptors (TLRs) represent the best characterized PRR family in mammalian species and currently comprise 10 receptors (TLR 1-10) in humans and 12 (TLR 1-9, 11-13) in mice (Takeda, Kaisho et al. 2003). As transmembrane receptors TLRs are expressed on the cell surface (TLR 1, 2, 4, 5, 6, 10-13) and at endosomal membranes (TLR 3, 7, 8, 9). The N-terminal part of TLRs contains a cystein-rich domain and a leucin rich repeats (LRRs) domain that are linked by a transmembrane region to the C-terminal cytoplasmic Toll/Interleukin-1 (TIR) homologous domain. Through the N-terminal domain TLRs sense PAMPs like lipopolysaccharide, flagellin and nucleic acids. Upon ligation TLRs induce inflammatory cytokines, chemokines and enhance expression of co-stimulatory molecules.

Already before Hemmi et al. discovered TLR9 as receptor for unmethylated CpG motifs containing prokaryotic DNA, Sparwasser et al. had shown that CpG oligodeoxynucleotides (CpG-ODNs) trigger activation and maturation of DCs (Sparwasser, Koch et al. 1998; Hemmi, Takeuchi et al. 2000). The gene encoding human TLR9 (huTLR9) is located on chromosome 3p21.3 and is exclusively expressed on B-cells and pDCs whereas mice additionally express TLR9 on cDCs (Hemmi, Takeuchi et al. 2000; Kadowaki, Ho et al. 2001). Released into the endoplasmic reticulum, TLR9 interacts with the polytopic membrane protein UNC93B1 that directs TLR9 to endolysosomal compartments (Kim, Brinkmann et al. 2008). By using distinct CpG-ODNs, studies elucidated cellular and species specificities as well as sequence requirements of TLR9 ligands. Regarding cell specific effects, three types of CpG-ODNs are defined. A-type CpG-ODNs, containing a natural phosphodiester (PD) backbone, strongly induce type I IFN production in pDCs but only moderately promote pDC maturation and poorly activate B-cells. B-type CpG-ODNs and C-type CpG-ODNs carry a phosphorothioate (PS) backbone that improves cellular uptake and nuclease resistance. While the B-type promotes B-cell proliferation and pDC maturation, C-type CpG-ODNs form higher-order tertiary structures through G tetrade formation and thereby comprise A- and B-type functions but both to a lesser extent (Krieg 2006).

Bauer et al. demonstrated that ODNs containing GT*CG*TT motifs specifically bind to huTLR9 whereas GA*CG*TT motifs preferentially activate murine TLR9 (muTLR9) (Bauer, Kirschning et al. 2001). Furthermore, it has been shown that both cytosine methylation and the absence of CpG motifs abrogate immune stimulatory effects of PS ODNs indicating that these features are decisive for TLR9 activation (Krieg, Yi et al. 1995). However, Yasuda et al.

demonstrated that even methylated eukaryotic DNA with sparse CpG motifs activates TLR9 if it gains access to endosomal compartments (Yasuda, Rutz et al. 2006). This has been confirmed by the finding that murine pDCs still produce 10 % of IL-6 if stimulated with base-free PD 2´deoxyribose homopolymers and even 60 % if stimulated with PD non-CpG ODNs compared to 100 % induced by PD CpG B-type ODNs (Haas, Metzger et al. 2008). Consequently, even the natural PD but not the PS backbone activates TLR9. In line, Barton et al. observed that genetically manipulated mice, expressing the extracellular domain of TLR9 coupled to the transmembrane and cytoplasmic parts of TLR4 (TLR9N4C) at the cell surface, are capable of recognizing mammalian DNA (Barton, Kagan et al. 2006). Taken together, these results clearly underline that discrimination of self versus non-self-DNA does not depend on distinct structures but is determined by the endosomal localization of TLR9.

Upon ligand binding, TLR9 forms homodimers, recruits adaptor molecule myeloid differentiation factor 88 (MyD88) and initiates two distinct downstream signalling pathways. In one, MyD88 associates with IFN regulatory factor 5 (IRF5), IL-1R-associated kinase 4 (IRAK4) and tumour necrosis factor receptor associated factor 6 (TRAF6) activating NFκB for induction of co-stimulatory molecules, chemokines and inflammatory cytokines such as monocyte inflammatory protein 1 (MIP-1), IFNγ-inducible 10kDa protein (IP10), TNFα and IL-12. In a second pathway, MyD88 forms a complex with IRAK4, IRAK1, TRAF6 and IRF7. Upon phosphorylation through IRAK1, IRF7 translocates to the nucleus and promotes transcription of type I interferons (Ishii and Akira 2006). The cytokine profile released upon activation of TLR9 promotes Th1 type responses (Lipford, Sparwasser et al. 2000). Therefore, TLR9 ligands represent attractive adjuvants to enhance immune responses against tumours and intracellular pathogens.

2.2.2 DC-SIGN – a member of the C-type lectin family

C-type lectin receptors (CLRs) consist of 17 subgroups including transmembrane receptors, soluble plasma proteins and compounds of the extracellular matrix that bind to self and non-self glycoproteins in the presence of Ca^{2+} ions (van Kooyk and Rabinovich 2008). Type I Mannose receptor (MR/CD206) and DEC205 receptor (CD205) are both specialized for antigen uptake. While the MR detects PAMPs of fungi such as Cryptococcus neoformans, pathogen-derived ligands of DEC205/CD205 are still unknown (Syme, Spurrell et al. 2002).

Dectin-1 and DC-specific ICAM-grabbing non-integrin receptor/human CD209 (DC-SIGN/huCD209) belong to the type II subfamily. Dectin-1 recognizes zymosan, a cell wall compound of S. cerevisae. Upon ligation, Dectin-1 recruits the tyrosin kinase SYK to its immunoreceptor tyrosin-based activation motif (ITAM) and induces via caspase recruitment domain protein 9 (CARD9) and NFκB the release of cytokines such as TGF-ß, IL-6 and IL-23 priming and maintaining Th17 cells (Brown and Gordon 2001; LeibundGut-Landmann, Gross et al. 2007). DC-SIGN is a 41 kD type II transmembrane receptor (Geijtenbeek, Torensma et al. 2000). Together with genes encoding L-SIGN, L-SECtin and CD23, the sequence of DC-SIGN forms a tight cluster of 105 kb on chromosome 19p13.3. DC-SIGN is expressed on $CD11c^+$ cDCs and on subpopulations of macrophages in lung, uterus and placenta (Hofbauer cells) (Soilleux, Morris et al. 2002). It is neither expressed on $CD1a^+$ Langerhans cells nor on pDCs (Geijtenbeek, Torensma et al. 2000).

The extracellular domain of huCD209 consists of a c-terminal tetrameric carbohydrate recognition domain (CRD) that is stabilized by a coiled-coil α-helical neck region containing seven complete and one incomplete tandem repeats (Mitchell, Fadden et al. 2001). The N-terminal cytoplasmic domain comprises one incomplete ITAM motif and two putative internalization motifs.

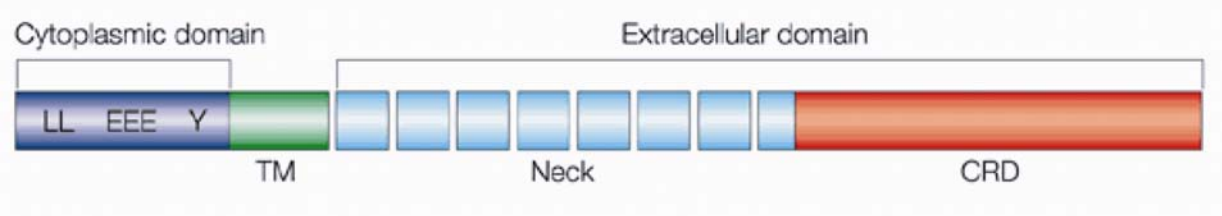


Figure 4: DC-SIGN structure: The extracellular part of DC-SIGN comprises a carbohydrate recognition domain (CRD) and a neck domain with seven complete and one incomplete tandem repeats. The transmembrane region (TM) connects it with the cytoplasmic domain that contains internalization motifs such as the di-leucine motif (LL) and the tri-acidic cluster (EEE) and an incomplete tyrosine-based activation motif (ITAM/Y) (van Kooyk and Geijtenbeek 2003).

A replacement of the di-leucin motif by alanines impairs antigen uptake (Engering, Geijtenbeek et al. 2002). Regarding the tri-acidic EEE motif, it has been reported that mutations in the corresponding EDE motif of DEC205 abrogate lysosomal targeting (Mahnke, Guo et al. 2000). Through its CRD DC-SIGN binds various self and non-self glycoproteins. In 2000, van Kooyk et al. observed that DC-induced T cell proliferation can be blocked by EDTA, mannan or anti-huCD209 antibodies (Abs) and thereby identified

DC-SIGN as a co-stimulatory molecule interacting with ICAM-3 on T cells (Geijtenbeek, Torensma et al. 2000). Further studies showed that treatment with peptide-N-glycosidases abrogates this interaction indicating that N-linked high-mannose oligosaccharides represent DC-SIGN specific ligands (Geijtenbeek, van Duijnhoven et al. 2002). However, even L-SIGN sharing 77 % amino acid identity with DC-SIGN interacts with ICAM-3. According to glycan-binding assays, Lewis antigens with terminal fucose residues bind exclusively to DC-SIGN but not to L-SIGN (Guo, Feinberg et al. 2004). Notably, only one amino acid is responsible for this effect. Mutation of Val351 into Gly in the CRD of DC-SIGN inhibits van der Waals interaction with a 2-OH group of fucose whereas L-SIGN gains this function if Ser363 is converted into valine or glycin (Van Liempt, Imberty et al. 2004). Furthermore, DC-SIGN ligates ICAM-2 on endothelial cells and thereby facilitates DC trafficking to sites of inflamed tissue. By ligating MAC-1 and CEACAM1 on neutrophils, huCD209 promotes DC maturation and prolongs neutrophil survival (van Gisbergen, Ludwig et al. 2005). Recently, Malcherek et al. identified the B7 homolog butyrophilin BTN2A1 as a further endogenous ligand of DC-SIGN (Malcherek, Mayr et al. 2007).

In 1992, Curtis et al. cloned the `HIV-1 envelope glycoprotein gp120 binding C-type lectin` which later turned out to be identical to DC-SIGN (Curtis, Scharnowske et al. 1992). This was the first time that huCD209 was assigned as a PRR. Except HIV-1, HIV-2 and simian deficiency virus 2 (SIV2), DC-SIGN interacts with Ebola virus (EBV), Cytomegalovirus (CMV), Hepatitis C virus (HCV) and Dengue virus. Moreover, huCD209 captures Lewis antigens and mannosylated proteins of H. pylori, K. pneumonia and strains of Yersinia and the mannose-caped glycolipid lipoarabinomannan (ManLam) facilitates uptake of M. tuberculosis via DC-SIGN. Through fucosylated GalNac-ß (1-4)-GalNac sequences and pseudo-Lewis-Y or Lewis-X antigens, DC-SIGN senses eggs and cercaria forms of S. mansoni whereas L. amastigotes interacts through lipophosphoglycans with DC-SIGN. Finally, even not yet identified structures of C. albicans are recognized by DC-SIGN (van Kooyk and Geijtenbeek 2003; Meyer, van Liempt et al. 2005).

Dependent on its ligand, DC-SIGN recruits distinct signalling pathways. Capparrós et al. observed that anti-huCD209 Ab MR-1 activates ERK and PI3K without promoting DC maturation (Caparros, Munoz et al. 2006). HIV in turn recruits RhoGTPases to DC-SIGN which play an essential role for transinfection of T cells (Hodges, Sharrocks et al. 2007). Moreover, the leukocyte-specific protein 1 (lsp1) specifically interacts with DC-SIGN and routes HIV to the proteasome (Smith, Ganesh et al. 2007). Interestingly, BMDCs from lsp1

deficient mice show an enhanced transfer of HIV to human T cells indicating that DC-SIGN limits distribution of the virus. Mycobacteria-derived ManLam engages the Ras/Raf-1 kinase pathway and thereby interferes with TLR-dependent NFκB activation (Gringhuis, den Dunnen et al. 2007). Regarding T cell responses towards DC-SIGN-ligating microbes, *in vitro* experiments could demonstrate that both H. pylori and eggs of S. mansoni promote Th2 priming (Bergman, Engering et al. 2004; van Liempt, van Vliet et al. 2007). In line, ManLam prolongs transcription of IL-10 and thereby probably inhibits Th1 priming. In respect to antigen processing and presentation, it has been demonstrated that DC-SIGN routes ligands onto MHC class I and MHC class II molecules (Tacken, de Vries et al. 2005). Based on these properties, targeting vaccines to DC-SIGN represents a promising strategy to enhance vaccine efficacy.

Besides CLRs, complement receptors and Fc receptors contribute to antigen uptake and target opsonized molecules to MHC presenting pathways. Moreover, cellular contacts influence DC function. While neutrophils promote DC maturation, erythrocytes bind through CD47 to the signal regulatory protein-α (Sirp-α) on DCs and thereby inhibit IL-12 and TNF-α production (van Gisbergen, Ludwig et al. 2005; Schakel, von Kietzell et al. 2006). Dependent on the interaction between macrophage galactose-type lectin (MGL) and CD45, immature DCs negatively regulate T cell receptor signalling and decrease T cell proliferation (van Vliet, Gringhuis et al. 2006). Interestingly, even T cells give negative feedback to DCs by limiting cytokine responses (Kim, Zhao et al. 2007).

2.3 Dendritic cell subsets

If only one DC type existed, it would have to express the entire set of PRRs to elicit appropriate immune responses against viruses, bacteria, fungi and parasites. In addition, depending on the localization DCs would have to induce tolerogenic responses towards commensal microbes in the gut or on the skin as well as immunogenic responses as soon as microbes invade organs such as spleen or liver. Consequently, the immune system has evolved distinct DC subsets to coordinate such opposing roles. For vaccinology it is fundamental to know about characteristics and distribution of DC subsets in order to target in an optimal way the appropriate DC compartment.

In mice, at least two major DC populations have been identified. One includes $CD11c^{+}B220^{+}CD45RA^{+}PDCA1^{+}$ plasmacytoid dendritic cells (pDCs), also known as natural type I interferon-producing cells, and a second contains $CD11c^{+}B220^{-}CD45RA^{-}PDCA1^{-}$ conventional dendritic cells (cDCs) (Shortman and Liu 2002). In the presence of FMS-related tyrosine kinase 3 ligand (FLT3-L) mouse BM-derived common myeloid precursor (CML) and common lymphoid precursor (CLP) differentiate into both DC subsets. Notably, FLT3-L deficient mice possess only about 10 % the DCs of WT animals (McKenna, Stocking et al. 2000). Recently, FLT3 has been identified as a marker of DC-restricted progenitor cells ($Lyn^{-}c\text{-}Kit^{int}Flt3^{+}M\text{-}CSFR^{+}$) (Onai, Obata-Onai et al. 2007). By using GM-CSF instead of FLT3-L, mainly cDCs arise from macrophage-dendritic cell precursor cells (Gilliet, Boonstra et al. 2002). Interestingly, even transplantation of thymus-derived common lymphoid precursors (CLP) into irradiated animals results in development of pDCs as well as cDCs (Ardavin, Wu et al. 1993).

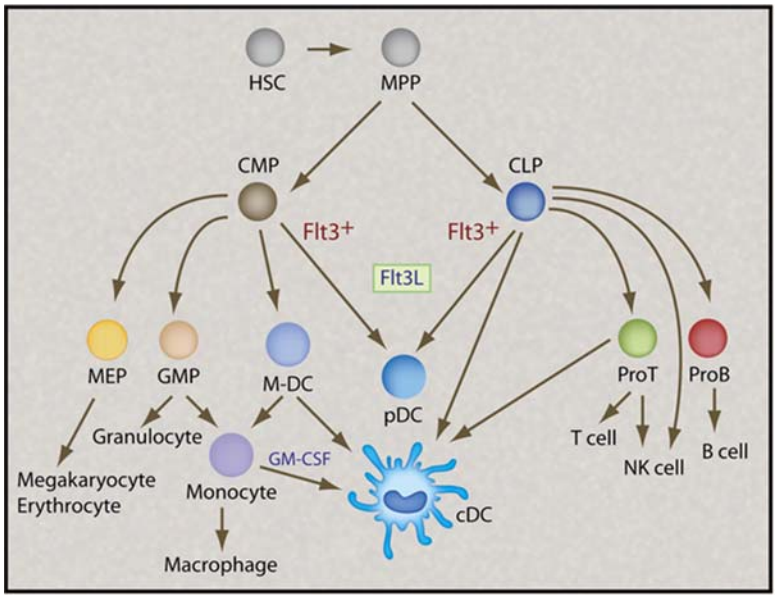


Figure 5: Developmental Scheme of Mouse DC Development from BM Haematopoietic Stem Cells and Lineage-Committed Progenitors: Both cDC and pDC can be generated from the Flt3 expressing early myeloid or lymphoid progenitors. FLT3-L is essential for the development of steady-state DC populations. Under inflammatory conditions, i.e., in the presence of GM-CSF, monocytes can differentiate into cDC. Abbreviations: HSC, haematopoietic stem cell; MPP, multipotent progenitors; CMP, common myeloid precursors; CLP, common lymphoid precursors; GMP, granulocyte and macrophage precursors; MEP, megakaryocyte and erythrocyte precursors; M-DC, macrophage and DC precursors. (Wu and Liu 2007)

Similar to mice, the human immune system has evolved $CD11c^{+}CD123^{low}BDCA\text{-}2^{-}$ cDCs and $CD11c^{-}CD123^{high}BDCA\text{-}2^{+}$ pDCs (Ito, Liu et al. 2005). *In vitro* experiments showed that

human DCs develop from $CD34^+$ haematopoietic stem cells in the presence of GM-CSF and TNF-α, from macrophages stimulated with M-CSF (Szabolcs, Avigan et al. 1996) and from monocytes supplied by IL-4 and GM-CSF (Romani, Gruner et al. 1994). Furthermore, injection of FLT3-L enhances DC frequencies in peripheral blood of humans (Maraskovsky, Daro et al. 2000).

Refering to phenotype and functional character, pDCs possess an immature shape but hardly need any cell division to become fully activated. High levels of TLR7 and TLR9 specialize this subset for clearance of viral infections, a process that substantially relies on type I interferon production (Krug, Luker et al. 2004). By contrast, cDCs are characterized by various cytoplasmic pseudopods, similar to the shape of a tree (Greek: dendron) and express a broad spectrum of PRRs. Moreover, cDCs can be subdivided into tissue resident DCs (e.g. thymic and splenic DCs) and migratory DCs including dermal Langerhans cells (LCs) and $CD103^+$ DCs in lung and gut (Johansson-Lindbom, Svensson et al. 2005; Sung, Fu et al. 2006; Shortman and Naik 2007). According to the surface markers CD8 and CD4, splenic DCs can be further categorized into $CD8^+CD4^-$, $CD8^-CD4^+$ and $CD8^-CD4^-$ double negative DCs (Vremec, Pooley et al. 2000). $CD8^+CD4^-$ DCs express CD205 whereas $CD8^-CD4^+$ and $CD8^-CD4^-$ DCs express Sirp-α (Lahoud, Proietto et al. 2006). Data from Dudziak et al. show that $CD8^+CD205^+$ cDCs are specialized for loading antigen on MHC class I complexes and promote CD8 T cell responses whereas $CD8^-CD205^-$ cDCs preferentially process MHC class II peptides and induce CD4 T cell responses (Dudziak, Kamphorst et al. 2007).

Intraepithelial $CD103^+CD11c^+Gr1^{low}MHCII^+$ DCs of the gut stay in a close contact with commensal microbes providing essential metabolites for the host organism. Sun et al. demonstrated that this subset is able to convert vitamin A into retinoid acid and thereby induces $CD4^+$ Treg cells (Sun, Hall et al. 2007). As a complementary subset, $CD103^-$ DCs reside in the lamina propria and release pro-inflammatory cytokines as soon as microbes invade this compartment. A similar tolerogenic function has been described for $Langerin^+$ intradermal DCs metabolizing vitamin D into 1,25-$(OH)_2$ vitamin D3 (Gorman, Kuritzky et al. 2007).

To what extent these recent findings reflect differentiation pattern and functionality of human DC subsets needs to be addressed in future studies.

2.4 Dendritic cell-targeted vaccines

In 1984, Celis et al. observed that antibodies to hepatitis B surface antigen potentiate T cell responses (Celis, Zurawski et al. 1984). Further studies confirmed that this phenomenon was based on an enhanced antigen uptake and presentation mediated through Fc receptors (FcRs) on APCs such as DCs (Snider, Kaubisch et al. 1990). Hence, it has been proposed that targeting antigen to DCs provides an appropriate strategy to improve vaccine efficacy. Currently, two different approaches of DC-based immunotherapy are under investigation. In one, termed *ex vivo* loading, DCs are isolated from peripheral blood of the patient, expanded *ex vivo*, loaded with antigen and re-injected into the patient. Here, first studies showed that DCs loaded with tumour antigens or tumour-derived MHC-I peptides are able to induce anti-tumour immune responses *in vivo*. However, so far this treatment could not reach a clinical response (Kavanagh, Ko et al. 2007; Redman, Chang et al. 2008). Similar results were obtained from patients receiving DCs transfected with tumour-derived RNA or DC-tumour cell hybrids (Krause, Neumann et al. 2002; Nair, Morse et al. 2002). Nevertheless, it has to be considered that patients are usually immune compromised because of a large tumour burden and prior radiation- and/or chemotherapy. The advantages of using *ex vivo* loaded DCs include a high specificity of cell compatibility as well as a high control of DC maturation. However, an extreme laborious procedure for DC generation causes very high costs and makes this method hardly available for all patients. Furthermore, the therapeutic variety is limited since not all DC subsets can be isolated from peripheral blood.

In a second approach, DCs are targeted *in vivo*. Here, the molecular modification of antigens with receptor specific ligands allows targeting of distinct DC receptors. Based on a high endocytic activity, especially CLRs such as the MR and DEC205 receptor represent appropriate targets for this method.

Several studies have employed mannan, a mannose polymer and fungal cell wall compound, to target vaccines to the MR. In a phase I trial, patients suffering from advanced cancer of breast, colon, stomach or rectum received mannan conjugated to the tumour-associated antigen mucin 1 (MUC-1) (Karanikas, Hwang et al. 1997). About 50 % of all patients developed antigen specific humoral responses and even cytotoxic $CD8^+$ T cells were induced within a minority. In a further study, stage II breast cancer patients were treated with oxidized mannan conjugated to MUC-1. Five years after treatment, patients of the placebo group showed a tumour recurrence rate of 27 % whereas no tumour was detectable in patients

treated with oxidized mannan (Apostolopoulos, Pietersz et al. 2006). However, since mannan molecules bind also to other CLRs such as DC-SIGN, it is possible that rather a synergism of several receptors than the unique function of the MR induced these effects. In a more specific approach, human MR transgenic (hMR Tg) mice were immunized with anti-MR antibody (B11) coupled to the model antigen ovalbumin (OVA) and the TLR9 ligand CpG-DNA was applied as adjuvant. Vaccinated hMR Tg mice induce robust OVA specific CD4 and CD8 T cell responses and significantly reduce tumour growth. In the presence of Ribi, a classical oil-in-water adjuvant containing the TLR4 agonist monophospholipid A (MPL), transgenic mice produce significant IgG2a titer (He, Crocker et al. 2007).

With respect to DEC205, Bonifaz et al. observed that mice treated with anti-DEC205-OVA and anti-CD40 develop significant frequencies of antigen specific IFN-γ producing $CD4^+$ and $CD8^+$ T cells as well as corresponding isotypes (Bonifaz, Bonnyay et al. 2004). Mice inoculated with OVA-expressing Bl6 melanoma cells show significantly reduced tumour growth upon application of anti-DEC205-OVA and anti-CD40. In a similar approach, Mahnke and colleagues demonstrated that immunization with CpG-ODNs and anti-DEC205 coupled to the melanoma antigen tyrosinase related protein (TRP)-2 protects mice against B16 melanomas. This response is reduced in CD8 deficient mice and even totally abolished in CD4 deficient animals (Mahnke, Qian et al. 2005). Notably, upon treatment with anti-DEC205 conjugated to TRP-2 and gp100, approximately 70 % of all tumour bearing mice reject B16 melanoma cells.

Considering other CLRs, it has been shown that application of anti-dectin-1-OVA conjugate and polyI:C enhances antigen specific cellular and humoral responses in mice (Carter, Thompson et al. 2006). Interestingly, frequencies of $CD8^+$ T cells are lower compared to immunization with anti-DEC205-OVA whereas i.v. but not s.c. injection of anti-Dectin-1-OVA induces higher frequencies of $CD4^+$ T cells and higher antibody titer. Mainly antigen specific $CD8^+$ T cells become induced upon application of anti-Dectin-2-OVA conjugate (Carter, Thompson et al. 2006).

Taken together, these results demonstrate that *in vivo* targeting of CLRs has a strong potential to improve vaccine efficacy. Although this method can not fully compete with specificity of *ex vivo* loaded autologous DCs, it still comprises several advantages. First of all, the clinical intervention is minimized to injection and patients are not stressed by cytapheresis. The fact that only one product is generated reduces financial efforts and makes it available for the majority of patients. Since vaccines possess equal qualities, results are comparable and high

standards can be established. Finally, this approach allows targeting of distinct DC-subsets in various compartments and it activates DCs in their natural environment.

2.5 Aims of the work

According to *in vitro* studies, DC-SIGN-targeted vaccines are capable of enhancing antigen specific T cell responses indicating that DC-SIGN provides an appropriate receptor for DC-targeting strategies (Engering, Geijtenbeek et al. 2002). However, this issue has so far not been investigated *in vivo*. One reason is based on the fact that mice, as model organism of choice for immunologists, express eight homologues of DC-SIGN, termed SIGNR1-SIGNR8. However, none of these homologues fulfils expression pattern as well as glycan specificities of DC-SIGN (Powlesland, Ward et al. 2006). Thus, as it is not possible to infer *in vivo* function of DC-SIGN from wildtype (WT) mice, a conventional transgenic mouse model for human DC-SIGN has been generated, termed hSIGN mouse. Controlled by the murine CD11c promoter, DC-SIGN attains a DC-restricted expression in hSIGN mice mimicking human specific expression pattern (Schaefer, Reiling et al. 2008).
In the first part of this work, hSIGN mice will be employed to evaluate the potential of DC-SIGN-targeted vaccines *in vivo*. While most previous studies used antibody conjugates to target antigen to CLRs, this work attempts to use glycans coupled to the model antigen OVA to target DC-SIGN. Therfore, vaccination of hSIGN mice with glycoconjugates should inform about the capacity of DC-SIGN-targeted vaccines to modulate adaptive immune responses *in vivo*.

Since infection biology provides important information for development of vaccines, the second part of this work will study the role of DC-SIGN during mycobacterial infection. DC-SIGN has been described as the major receptor of M. tuberculosis on human DCs (Geijtenbeek, Van Vliet et al. 2003). Moreover, it has been shown that DC-SIGN induces a prolonged transcription of IL-10 if ligated by ManLam, a process which depends on the recruitment of the Ras/Raf-1 kinase pathway to DC-SIGN (Gringhuis, den Dunnen et al. 2007). To verify if signalling pathways are conserved in hSIGN mice, this work will analyze activation pattern of Raf-1 kinase in mycobacteria-infected DC-SIGN transgenic DCs. Furthermore, it has been demonstrated that hSIGN mice show a decreased histopathology and a prolonged survival during M. tuberculosis infection indicating that

altered cytokine pattern of hSIGN DCs modulate harmful T cell responses (Schaefer, Reiling et al. 2008). On this background, we will study the priming of naïve T cells in the presence of M. bovis BCG-infected hSIGN DCs. These results will shed new light on the *in vivo* function of DC-SIGN and will help to improve development of DC-SIGN-targeted vaccines.

Finally, the third part of this work will focus on the generation of transgenic mice for huTLR9, termed HUT9 mice. Results from mice clearly demonstrate that TLR9 ligands are very potent adjuvants enhancing protective immune responses against tumours and infectious diseases (Weiner, Liu et al. 1997; Vabulas, Pircher et al. 2000). However, first human studies could not establish clinical indications for TLR9-targeted adjuvants (Krieg 2006). One explanation for these divergent results is the fact that expression of huTLR9 is restricted to B-cells and pDCs whereas muTLR9 is also present on cDCs (Wagner 2004). Consequently, TLR9 ligands induce distinct cytokine profiles in mice and men regulating immune responses in a different manner.

By employing bacterial artificial chromosome (BAC) technology, we expect that HUT9 mice express huTLR9 exclusively in B-cells and pDCs. In this way, HUT9 mice would mimic human specific expression pattern and would allow us to investigate detailed *in vivo* functions of huTLR9. By studying infection and tumour models as well as models for autoimmunity, allergy and transplantation we could then define appropriate and safe indications for the employment of TLR9 ligands in human immunotherapy.

3 Materials and Methods

3.1 Materials

3.1.1 Equipment

Analytical balance	Sartorius Göttingen
Balance	Sartorius Göttingen
Biofuge Pico/Fresco	Heraeus, Hanau
Centrifuge, Multifuge 3	Heraeus, Hanau
FACS Calibur	Becton Dickinson, Heidelberg
Freezer -20°C	Siemens, München
Fridge 4°C	Liebherr, Switzerland
Heat block 2Q	VLM, Leopoldshöhe
Incubator BBD 6220	Heraeus, Hanau
Microscope, Axiovert 25	Zeiss, Jena
Multichannel pipettes	ThermoLabsystems, USA
Multipipette plus	Eppendorf, Hamburg
Nanodrop®ND-1000 Spectrophotometer	Nanodrop, Steinfurt
Neubauer counting chamber	Roth, Karlsruhe
Orion Microplate Luminometer	Berthold Detection Systems, Pforzheim
Pari-Master nebulizer aerosolisation	PARI GmbH, Starnberg
pH-meter Multical	WTW, Weilheim
Pipettes	Gilson, USA
Plastic ware	NUNC, Wiesbaden; Falcon, USA
Sterile bench	Heraeus, Hanau
Table centrifuge, Biofuge pico	Heraeus, Hanau
Table centrifuge, Biofuge fresco	Heraeus, Hanau
Vortexer Velp	Scientifica, Italy
Waterbath	Julabo, USA

3.1.2 Chemicals

1 kb ladder GeneRuler TM	Fermentas, St. Leonroth
6x loading buffer	Fermentas, St. Leonroth

Aluminium hydroxide, $Al(OH)_3$	Sigma, Taufkirchen
ß-mercaptoethanol 50mM	Invitrogen, Karlsruhe
BSA (albumin from bovine serum)	Sigma,Taufkirchen
5-(6-)Carboxyfluorescein diacetate succinimidyl ester (CFSE)	Invitrogen, Karlsruhe
Chloroform	Baker, Griesheim
Cytofix/Cytoperm	BD Bioscience, Heidelberg
Dimethylsulfoxid (DMSO)	Sigma, Taufkirchen
dNTPs, Roti-Mix® PCR3	Roth, Karlsruhe
EDTA	Sigma, Taufkirchen
Embryo Water	Sigma, Taufkirchen
Ethanol	Merck, Darmstadt
Ethidiumbromide	Sigma, Taufkirchen
Ethidium monoazide (EMA)	Invitrogen, Karlsruhe
Fetal calf serum	Perbiol/HyClone, USA
Glycerol	Sigma, Taufkirchen
Hydrogen peroxide (H_2O_2)	Sigma, Taufkirchen
L-Glutamine 200 mM	Biochrom AG, Berlin
Methanol	Roth, Karlsruhe
Middlebrook 7H10 Agar	BD Bioscience, Heidelberg
Middlebrook 7H9 Broth	BD Bioscience, Heidelberg
Natriumchlorid (NaCl)	Roth, Karlsruhe
Paraformaldehyde	Sigma, Taufkirchen
PBS Dulbecco	Biochrom AG, Berlin
Penicillin/Streptomycin	Gibco, USA
Propidium iodide	Sigma, Taufkirchen
Red Blood Cell Lysing Buffer	Sigma, Taufkirchen
SIINFEKL peptide	PeproTec, UK
Taq polymerase	Roche Diagnostics, USA
Tween 20	Sigma, Taufkirchen

3.1.3 Expandable items

Cell-culture plates and petri-plates	Nunc, Wiesbaden

Insulin-syringe 1 ml Sub Q	Becton Dickinson, Heidelberg
MaxiSorp 96-well plates	Nunc, Wiesbaden
Parafilm	Roth, Karlsruhe
PCR tubes 8-strip	Kisker, Steinfurt
Sterile injection-needles, Microlane^{TM}3	Becton, Dickinson, Heidelberg
Plastique Material	Nunc, Wiesbaden

3.1.4 Primer for PCR

Gene	5´- 3´ sequence	Company
DC-SIGN	**- CGGGATCCGAGTGGGGTGACATGAGTGACT -** **- ACGCGTCGACAAAAGGGGGTGAAGTTCTGCTACG -**	**TIB MOLBIOL**
GFP	**- GCGAGGGCGATGCCACCTACGGCA -** **- GGGTGTTCTGCTGGTAGTGGTCGG -**	**TIB MOLBIOL**
DTR	**- CCCAGGTTACCATGGAGAGA -** **- GAACTTCAGGGTCAGCTTGC -**	**TIB MOLBIOL**
OT-I	**- AAGGTGGAGAGAGACAAAGGATTC -** **- TTGAGAGCTGTCTCC -**	**TIB MOLBIOL**
OT-II	**- GCTGCTGCACAGACCTACT -** **- CAGCTCACCTAACACGAGGA -**	**TIB MOLBIOL**
	- AAAGGGAGAAAAAGCTCTCC - **- ACACAGCAGGTTCTGGGTTC -**	
huTLR9 BAC CDS	**- GTGCCCCACTTCTCCATG -** **- GGCACAGTCATGATGTTGTTG -**	**TIB MOLBIOL**
huTLR9 BAC 5´	**- GTCAGGCTGGTCTCGAACTC -** **- CAGGAAAGGGAACATCCTCA -**	**TIB MOLBIOL**
huTLR9 BAC 3´	**- CGGACTCTCGCTCTGTTACC -** **- GGAGCTGTGTTTTCTCCTC -**	**TIB MOLBIOL**
muTLR9	**- GAAGGTTCTGGGCTCAATGGTCATGTG -** **- GCAATGGAAAGGACTGTCCACTTTGTG -**	**TIB MOLBIOL**
muTLR9 KO	**- GCAATGGAAAGGACTGTCCACTTTGTG -** **- ATCGCCTTCTATCGCCTTCTTGACGAG -**	**TIB MOLBIOL**

3.1.5 Oligonucleotides

CpG-ODN	5´ - 3´ sequence	**Company**
1826	**- TCCATGACGTTCCTGACGTT -**	**TIB MOLBIOL**
2006	**- TCGTCGTTTTGTCGTTTTGTCGTT -**	**TIB MOLBIOL**

3.1.6 Antibodies and Tetramers used for FACS

Antigen	recognized Species	Isotype	Conjugate	Dilutions for FACS	Company
CD4	mouse	rat, IgG2b,κ	APC	1: 200	NatuTec
CD8	mouse	rat, IgG2a,κ	APC	1: 200	NatuTec
CD11c (Integrin α-chain)	mouse	armenian hamster IgG1,λ	FITC	1: 100	BD Bioscience
CD11c (Integrin α-chain)	mouse	armenian hamster IgG1,λ	APC	1: 100	BD Bioscience
CD45R/B220	mouse	rat, $IgG_{2\alpha}$	FITC	1: 200	eBioscience
CD16/CD32	mouse	IgG2a,λ	pure	1: 100	self made
(MHCII) I-A/I-E	mouse	IgG2b,κ	APC	1: 200	eBioscience
CD62L	mouse	rat, IgG2a	APC-Alexa 750	1: 200	NatuTec
CD209	human	mouse, IgG2b	R-PE	1: 200	R&D Systems
CD45.1	mouse	rat, IgG2a,κ	PE	1: 200	eBioscience
phospho-Raf-1	mouse	rabbit, IgG	pure	1: 1000	calbiochem
IgG	rabbit	goat, IgG	FITC	1: 500	Molecular Probes
SIINFEKL-Tetramer	mouse	mouse	R-PE	1: 5	Busch Lab

3.2 Methods

3.2.1 Cell biology

3.2.1.1 Medium used for the culture of eukaryotic cells

500ml RPMI 1640

50 ml heat inactivated FCS

5 ml Penicillin/Streptomycin

5 ml L-Glutamine 200mM

500 µl ß-mercatoethanol 50 mM

3.2.1.2 Preparation of bone marrow derived mouse dendritic cells

The mice were sacrificed, hind legs were cut off and bones were separated. Before opening the bones they were briefly rinsed with 70 % ethanol for disinfection. Afterwards the bone marrow was extracted by flushing the bones with 1 x PBS using an injection needle. After centrifugation at 1300 rpm for 7 min, the supernatant was discarded and erythrocytes were lysed by adding 1 ml Erythrocyte Lysis buffer for 2 min. Next, 10 ml 1 x PBS was added and cells were again centrifuged at 1300 rpm for 7 min. The supernatant was discarded and the cells were resuspended in complete RPMI medium, supplemented with charge-dependent concentrations of GM-CSF or FLT3-L, plated in a total volume of 10 ml each on petri dishes and incubated at 37°C. FLT3-L stimulated cells were cultured for 10 days without medium exchange. Two days after preparation 10 ml RPMI medium containing GM-CSF were added to each dish of GM-CSF cells. On day 4, 10 ml per petri dish were centrifuged at 1300 rpm for 7 min and cells were resuspended in fresh medium. Afterwards, 10 ml of the cell suspension were added to each dish. On day 6, cells were treated in the same manner as on day 4. Cells were harvested on day 8 of cultivation.

3.2.2 Mice

All transgenic mice were backcrossed at least 10 generations onto a C57BL/6 or a BALB/c background. Mice were kept under pathogen-free conditions at the animal facility of the Institute of Medical Microbiology, Immunology and Hygiene (Technical University, Munich, Germany). Animal experiments were approved and authorized by the local government. Experiments were performed with 8-12 week old mice of both sexes, unless otherwise stated. Prior to the experiment, all the mice were genotyped by screening PCR according to standard laboratory protocol.

3.2.3 Culture of prokaryotic cells

3.2.3.1 Media and buffers for the culture of prokaryotic cells

Middlebrook 7H9 Broth	4,7	g Middlebrook 7H9 Broth
	2	ml Glycerol
	899.5	ml H_2O and autoclaved at 121°C for 10 min add aseptically 0.5 ml Tween 20 and 100 ml of Middlebrook ADC Enrichment after solution is cooled down to 45 °C

Middlebrook 7H10 Agar	19	g Difco™ Middlebrook 7H10 Agar
	5	ml Glycerol
	900	ml H_2O and autoclaved at 121 °C for 10 min
		add aseptically 100 ml of Middlebrook OADC Enrichment after solution is cooled down to 50-55°C

The medium was prepared by the media-kitchen of the institute. For agar-plates, 30 g of agar were dissolved in one litre of the solution. In each case, either for liquid medium as well as for agar-plates, the solution was autoclaved. Liquid medium without any antibiotics can be stored at room temperature, plates were stored at 4°C.

3.2.3.2 Bacteria and infection

M. bovis BCG was grown in Middlebrook 7H9 Broth supplemented with Middlebrook ADC-Enrichment. Midlog phase cultures (OD 0,3-0,6) were harvested, aliquoted and frozen at -80°C. After thawing, viable cell counts were determined by plating serial dilutions of the cultures on Middlebrook 7H10 agar plates followed by incubation at 37°C. Before infection with M. bovis BCG, stock solutions of mycobacteria were diluted in PBS and the preparation was passed six times through a 27 gauche needle to ensure proper dispersion of mycobacteria.

3.2.4 Molecular biology

3.2.4.1 Buffers and solutions for molecular biology

50 x TAE	42	g Tris
	500	ml H_2O_{dd}
	100	ml 0,5 M Na_2EDTA, pH 8,0 ad 1000 ml H_20_{dd}

Kill Juice	50	mM Tris-HCl pH 8.0
	100	mM NaCl
	25	mM EDTA
	0.9	% SDS

Phenol/Chloroform	Tris-buffered phenol (pH 8.0)
	Chloroform
	Isoamyl Alcohol
	Ratio 25 : 24 : 1

3.2.4.2 Isolation of genomic DNA from tissue samples

Pieces of 1-2 mm length were cut with a sterile scisor from tails of mice. For protein denaturation, 500 µl Kill Juice and 5 µl Proteinase K were added and samples were incubated in a thermo-shaker at 55°C overnight. After a short centrifugation, the soluble phase was taken into 600 µl phenol/chloroform solution and samples were centrifuged for 30´ at 13.000 rpm. The upper phase containing solved DNA was taken into 1 ml 100 % ethanol to precipitate DNA. Next, samples were again centrifuged for 15´ at 13.000 rpm, supernatants were discarded and 600 µl of 70 % ethanol were added for a final centrifugation step at 13.000 rpm for 8´. After discarding the supernatant, DNA was air dried for at least 30´ and DNA was solved in 50 µl embryo tested water.

3.2.4.3 Agarose gel electrophoresis

DNA fragments of variable size were separated by agarose gel electrophoresis using gels containing 1 % agarose and 0,01 % ethidiumbromide. For size determination of the fragments, 5 µl of 1 kb ladder were used. Electrophoresis took place at 70 V.

3.2.4.4 PCR

PCR mix for 25 µl:	0.5 µl 10mM dNTPs
	0.5 µl 10 µM primer Mix
	1 µl Taq polymerase
	2.5 µl buffer
	19.5 µl H_2O
	50 µl ng DNA in 1 µl
Primer mix: 5´ and 3´ primers	10 pMol

In general, amplification occured using the following protocol with modifications for annealing temperature (dependent on used primer combination) and extension time (dependent on the size of the amplified fragment):

94°C	5 min	
94°C	1 min	30 – 35 X
58°C	1 min	
72°C	1,5 min	
72°C	10 min	
16°C	∞	

3.2.5 Immunology

3.2.5.1 Enzyme linked immunosorbent assay (ELISA)

3.2.5.1.1 Principle of ELISA

A capture antibody is linked to a polymeric matrix. By adding cell extracts or supernatants, antibody-antigen complexes are formed. These complexes can be detected by adding a detection antibody which recognizes a different epitope of the antigen. The detection antibody is tagged with an enzyme (e.g. peroxidase) which transforms a colourless substrate into a coloured product. By measuring colour intensity, the amount of a certain protein in the sample can be determined.

To determine antigen specific isotypes, plates are coated with the appropriate antigen, e. g. Ovalbumin (OVA). By adding samples containing antigen specific antibodies, antibody-antigen complexes are formed. A second biotinylated antibody then detects specific isotype of the bound antibody. A complex containing streptavidin and an enzyme binds to the second antibody and the enzyme transforms a colourless substrate into a coloured product.

3.2.5.1.2 ELISA buffers and solutions

Coating solution:	1x PBS
Blocking buffer:	1x PBS
	1% BSA

Reagent Diluent:	1x PBS 1% BSA
Washing buffer:	1x PBS 0.05% Tween 20
Capture buffer:	55 µl of capture antibody (720 ng/ml) 10 ml 1x PBS
Detection buffer:	55 µl of detection antibody (36 µg/ml) 10 ml reagent diluent
Conjugation buffer 1:	55 µl HRP buffer 10 ml reagent buffer
Conjugation buffer 2:	10 µl streptavidin/alkaline phosphatase 10 ml reagent buffer
Substrate reagent 1:	1 tablet Tetramethylbezine (TMB) 10 ml 0.05 M Phosphate-Citrate buffer 2 µl H_2O_2 30%
Substrate reagent 2:	1 tablet phosphatase substrate 5 ml 0.1 diethanolamine
Phosphate-Citrate buffer:	25.7 ml 0.2 M NA_2HPO_4 24.3 ml 0.1 M Citric Acid 1-hydrate (pH 5.0) 50 ml H_2O_{dd} pH ad 5.0 using HCl
Stop solution 1:	2 N H_2SO_4
Stop solution 2:	10 N NaOH

3.2.5.1.3 Cytokine ELISA

Plates were coated with 50 µl per well of capture antibody (720 ng/ml) and incubated overnight at 4°C. The next day, the plate was washed, tapped dry and 200 µl per well of blocking buffer were added. The following incubation took place for 2 h at room temperature or at 4°C overnight. Afterwards, the plate was washed 3 x with washing buffer, tapped dry and 50 µl per well of the samples were added. Incubation took place at room temperature for 2 h or at 4°C overnight and was followed by 3 washing steps. Detection antibody (36 µg/ml) was added in a volume of 50 µl per well, the plate was incubated for 2 h and washed 3 x afterwards followed by 20 min of incubation with conjugation buffer 1 (50 µl/well). Finally, the plate was washed 3 x again and fresh substrate reagent 1 was added (100 µl/well). The plate was incubated in the dark because the substrate reagent contains light sensitive H_2O_2. The incubation time ranged from 20 - 60 min. To stop the reaction 50 µl/well of 2N H_2SO_4 were added and the plate was analyzed in the ELISA reader at 450 nm (Ref.: 570 nm).

3.2.5.1.4 Isotype ELISA

Plates were coated with 50 µl Ova (10 µg/ml) in PBS and incubated at 4 °C overnight. The next day, plates were washed 3 x with washing buffer, tapped dry, 200 µl per well blocking buffer were added and plates were incubated for 2 h at room temperature. After 3 washing steps 50 µl of the samples were added, plates were incubated for 1 h at 37°C and three washing steps followed. Next, plates were tapped dry, charged with 50 µl of the secondary antibody and incubated for 1 h at 37°C. After 3 washing steps, 50 µl of conjugation buffer 2 were added and plates were left for 30 - 60 min at room temperature before three final washing steps were performed. After plates were tapped dry, 50 µl of fresh substrate solution 2 were added and plates were incubated between 30 and 60 min in the dark. Finally, the reaction was stopped with 20 µl NaOH and analyzed in the ELISA reader at 450 nm (Ref.: 570).

3.2.5.2 Flow cytometry

3.2.5.2.1 Flow cytometry buffers

FACS buffer:	1	% BSA in 1 x PBS
Erythrocyte lysis buffer:	8,29	g NH_4Cl
	1	g $KHCO_3$
	37,2	mg Na_2EDTA
	800	ml H_2O (pH 7,2-7,4 with 1N HCl)
	ad 1000	ml H_2O_{dd}

3.2.5.2.2 Analysis of cell surface antigen expression by flow cytometry

Up to 1 x 10^6 cells per staining were centrifuged at 2000 rpm at 4°C for 1 min (Biofuge Fresco) in a 1,5 ml tube. The supernatant was discarded and the cells were washed twice with 150 µl FACS-buffer (centrifugation at 1300 rpm, 4°C, 3 min). To block cell surface Fc-receptors, cells were incubated with unlabeled anti-CD16/CD32 antibody for 10 min at 4 °C. Meanwhile, the appropriate staining solutions were prepared. After blocking Fc-receptors, the cells were centrifuged and resuspended in 50 µl of the appropriate staining solutions. Incubation lasted for 20 min in the dark at 4°C and was followed by two washing steps (150 µl/sample, centrifugation at 1300 rpm, 4°C, 3 min). Finally, the supernatant was discarded, cells were resuspended in 150 µl washing buffer containing the live/dead discriminating dye propidium iodide (2 µg/ml) and transferred to FACS tubes. Stained samples were aquired using FACSCalibur and the data were analyzed with the software FlowJo. When peripheral blood or splenic samples were analyzed, an erythrocyte lysis step was performed prior to general staining procedures.

3.2.5.2.3 Analysis of intracellular antigens by flow cytometry

For intracellular staining, cells were stained for 20 min with ethidium monoazide (EMA, 1 µg/ml) and simultanously loaded with Fc-block. By exposing the samples to direct light, EMA covalently binds to DNA in dead cells. Next, samples were washed three times. Prior to the incubation with staining solutions the samples were fixed with paraformaldehyd (3 %) and permeabilized with methanol (90 %). After each step, cells were washed two times.

3.2.5.3 *In vivo* kill assay

Sex and age matched WT mice were sacrificed, spleens were collected and smashed through a cell strainer. After centrifugation for 7 min at 1300 rpm, supernatant was discarded and erythrocyte lysis buffer was added (5 ml/spleen). Lysis took place at room temperature for 5 min and was stopped by adding 10 ml PBS. Next, cells were centrifuged, washed with endotoxinfree PBS and cell concentration was adjusted to 20 x 10^6 cells/ml. Cells were splitted into two fractions with equal cell numbers and differentially labelled with 10 µM or 1 µM CFSE. After an incubation for 10 min at 37°C, both fractions were quenched with cooled RPMI plus 10 % FCS and cells were incubated for 5 min on ice. Afterwards cells were washed two times and resuspended in 1 ml RPMI plus 10 % FCS. $CFSE^{low}$ labeled cells were loaded with 1 µM SIINFEKL peptide for 60 min at 37°C. After two washing steps cells from both fractions were counted and adjusted to 100 x 10^6 cells/ml in PBS. $CFSE^{low}$ and $CFSE^{high}$ cells were mixed at a ratio 1:1. Finally, 200 µl of this mixture were injected i.v. into immunized and control mice.

3.2.5.4 Proliferation assay

Day 7 GM-CSF cultured BMDCs were irradiated with 3000 rd, adjusted to 1x10^6 cells/ml in complete RPMI and 100 µl/well were plated on 96 well plates. Next, 50 µl complete RPMI containing 300, 30 or 3 µg/ml OVA protein were added, respectively, and BMDCs were pulsed overnight at 37°C. The next day, para-aortic lymphnodes from immunized and control mice were prepared and $CD4^+$ T cells were purified by negative selection using magnetic beads according to the manufacturer´s instructions (Dynal; Invitrogen). Viable $CD4^+$ T cells were adjusted to a concentration of 1x10^6 cells/ml and 100 µl/well were added to OVA pulsed BMDCs in triplicates. Co-cultures were incubated for 3 days at 37°C. Proliferation was quantified using the Cell Titer-Glo® Luminescent Cell Viability Assay according to the manufacturer´s manual (Promega). Briefly, cells were resuspended, 100 µl cell suspension were added to wells of an opaque-walled 96 well plate and the contents was equilibrated to room temperature for approximately 30 min 100 µl of CellTiterGlo® Reagent were added and plates were mixed for 2 min on an orbital shaker to induce cell lysis. To stabilize the luminescent signal plates were incubated for 10 min at room temperature. Finally, relative luminescence was measured by luminometer.

3.2.5.5 Adoptive cell transfer

Sex and age matched CD45.1/OT-I and CD45.1/OT-II mice were sacrificed, lympnodes were collected and single cell suspensions were prepared as described before. $CD8^+$ and $CD4^+$ T cells were magnetically purified by negative selection according to the manufacturer´s protocol. After purification, T cells were counted and adjusted to $1x10^6$ cells/ml with endotoxinfree PBS. Finally, a volume of 200 µl containing $2x10^5$ CD45.1/OT-I or CD45.1/OT-II cells were injected i.v. into DC-SIGN transgenic and WT mice.

3.2.5.6 Vaccines

Vaccine	Concentration	Solved in
OVA-Lewis X	4.1 µg/µl	PBS
OVA-sulfo Lewis A	3.85 µg/µl	PBS
OVA	2.85 µg/µl	PBS

3.2.6 Statistics

Mean values, standard deviations and p-values (Student´s t-test) were calculated with Microsoft® Excel.

4 Results

4.1 Generation of conventional DC-SIGN transgenic hSIGN mice

DC-SIGN/huCD209 is predominantly expressed by $CD11c^+$ myeloid dendritic cells. Since the sequence of its promoter has not been completely identified, human specific expression pattern of DC-SIGN was mimicked by using the murine CD11c promoter (Brocker, Riedinger et al. 1997; Liu, Yu et al. 2003). Therefore, total cDNA from human peripheral blood-derived monocytes was used as a PCR template to amplify the 1.3 kb cDNA of DC-SIGN. The amplified product was cloned 3´ of the 5.5 kb murine CD11c promoter via Eco RI restriction sites. An appropriate polyadenylation signal was provided by the vector containing the CD11c promoter. Next, the DNA fragment for injection was obtained by a Not I/Cla I double digest and purified by agarose gel electrophoresis. Finally, the construct, named CD11c-DC-SIGN, was successfully transferred into pronuclei of fertilized B6D2/F1 hybrid oocytes by microinjection. DC-SIGN transgenic offspring were termed hSIGN mice.

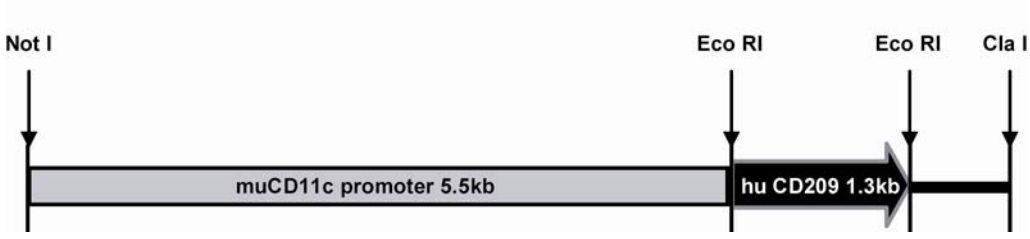


Figure 6: Scheme of the construct for generation of hSIGN mice. The cDNA of DC-SIGN (hu CD209) is inserted via Eco RI sites 3´ of the murine CD11c promoter. For microinjection the construct was linearized by a Not I/Cla I double digest (Schaefer, Reiling et al. 2008).

4.2 Characterization of hSIGN mice

The use of hSIGN mice for studying huCD209 *in vivo* required detailed information about transgene expression pattern. Based on the murine CD11c promoter, DC-SIGN was expected to be expressed by $CD11c^+$ DCs. *In vitro* stimulation of murine bone marrow cells with FLT3-L or GM-CSF allows generation of BMDCs. While GM-CSF promotes development of cDCs, FLT3-L induces differentiation of both pDCs and cDCs. On day eight, GM-CSF-cultured BMDCs were harvested and stained for murine CD11c and huCD209. Compared to WT BMDCs which lack DC-SIGN expression, 60-70 % GM-CSF-derived $CD11c^+$ hSIGN BMDCs stain positive for DC-SIGN. FLT3-L BMDCs were harvested on day 10 and expression of murine CD11c, CD45R (B220) and huCD209 was assessed. *In vitro*, DC-SIGN is weakly expressed on a subset of FLT3-L-derived transgenic $CD11c^+B220^+$ pDCs.

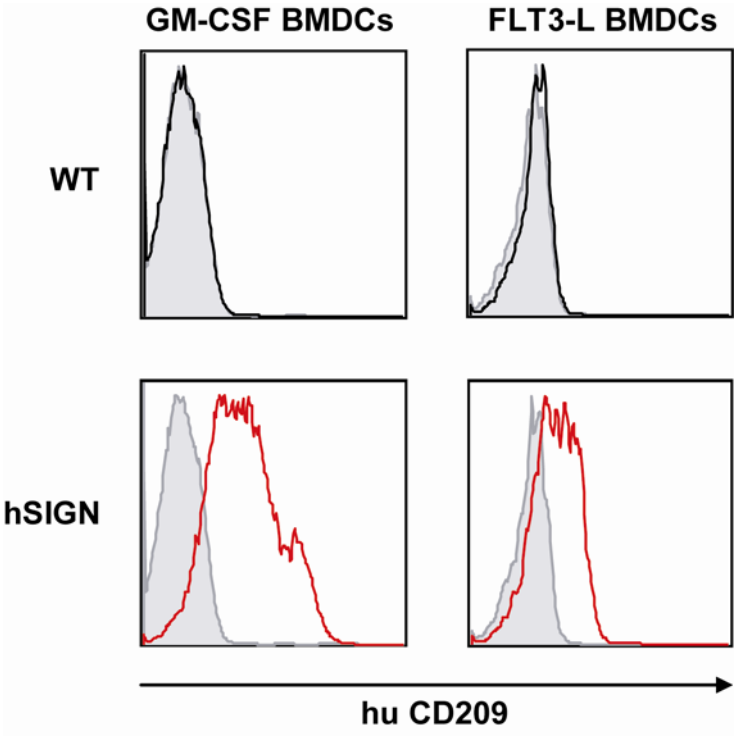


Figure 7: FACS analysis of DC-SIGN expression on *in vitro* generated BMDCs. BM cells were cultured in the presence of GM-CSF or FLT3-L. GM-CSF derived BMDCs were stained for CD11c/huCD209 and FLT3-L BMDCs were stained for CD11c/CD45R/huCD209. Histograms show DC-SIGN expression on electronically gated living CD11c (GM-CSF BMDCs) or CD11c/CD45R (FLT3-L BMDC) cells. Filled histograms: isotype control, solid lines: specific DC-SIGN staining (Schaefer, Reiling et al. 2008).

To verify whether DC-SIGN is expressed on *ex vivo* isolated DCs, spleens from sex and age matched WT and hSIGN mice were prepared. After digest with Collagenase/DNAse, splenic mononuclear cells were enriched by a density gradient and stained for CD11c, CD45R (B220) and huCD209. Based on expression levels of CD45R and CD11c, four populations can be distinguished (Fig. 8). $CD11c^-$ splenocytes mainly contain $CD45R^-$ T cells and macrophages (I) as well as $CD45R^+$ B-cells (II). $CD11c^+$ DCs can be subdivided into $CD45R^+$ pDCs (III) and $CD45R^-$ cDCs (IV). Staining for huCD209 revealed that almost 100 % of transgenic $CD11c^+CD45R^-$ cDCs express the transgene. In contrast, only a small fraction of *ex vivo* isolated pDCs is positive for huCD209. DC-SIGN is neither expressed on B-cells nor on $CD45R^-CD11c^-$ cells.

By RT-PCR analysis, it could further be demonstrated that huCD209 transcripts are present in thymus and lymphnodes as well as in extralymphatic tissues such as lung, liver and gut (data not shown).

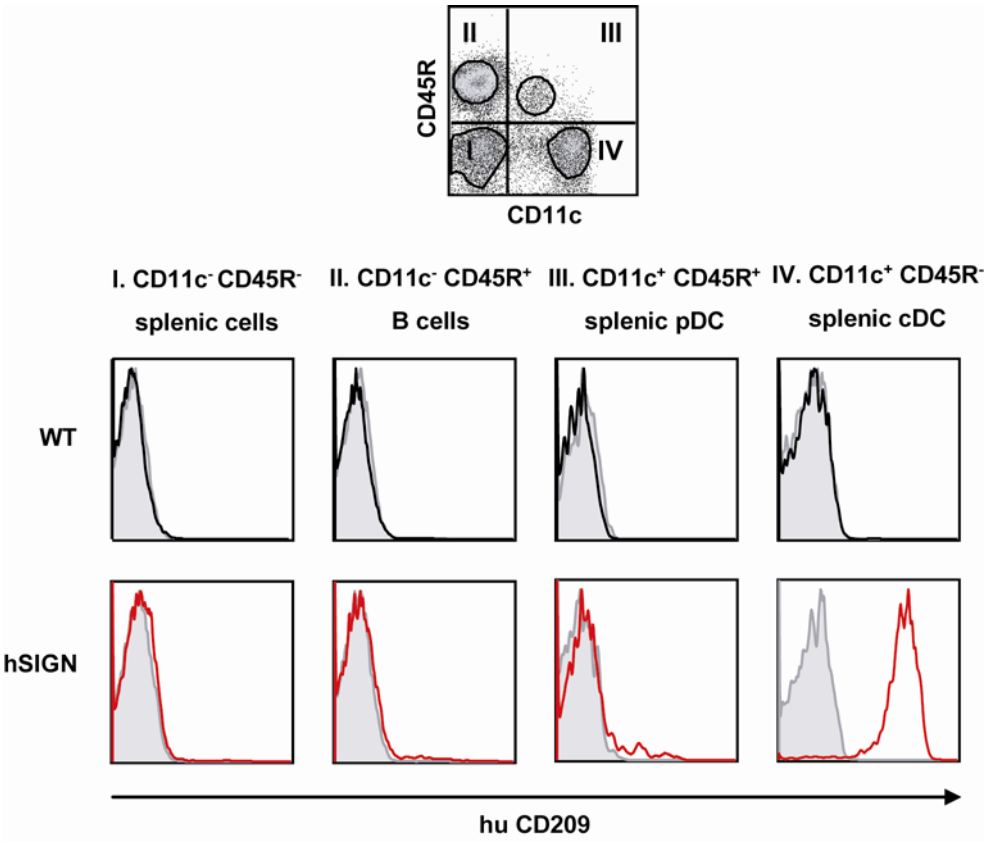
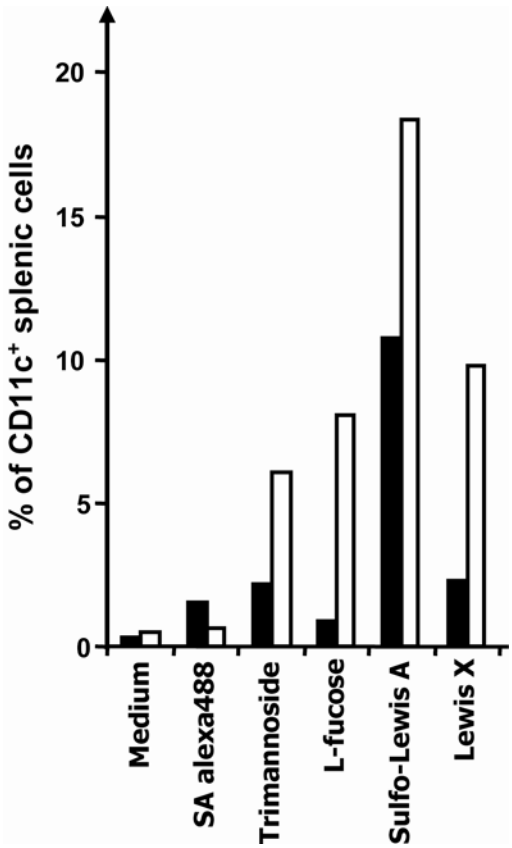


Figure 8: Expression of transgenic human DC-SIGN on splenic cells. Enriched splenic mononuclear cells from WT and DC-SIGN transgenic mice were stained for CD11c, CD45R and CD209. Histograms demonstrate CD209 expression as indicated. Filled histograms: isotype control, solid lines specific CD209 staining (Schaefer, Reiling et al. 2008).

4.3 Lewis antigens specifically bind to hSIGN-derived CD11c$^+$ splenocytes

To study DC-SIGN targeting strategies, it was necessary to determine ligands that specifically bind to huCD209 on hSIGN DCs. In previous experiments it has been demonstrated that highly mannosylated molecules and Lewis cluster such as Lewis X and sulfo Lewis A specifically ligate the CRD of DC-SIGN (Guo, Feinberg et al. 2004). Therefore, *ex vivo* isolated hSIGN and WT CD11c$^+$ splenocytes were pulsed with distinct biotinylated polyacrylamide (PAA) coupled glycans. Subsequently, cells were stained with streptavidin (SA) alexa488 and percentages of glycan positive CD11c$^+$ cells were determined by flow cytometry. As shown in Fig. 9, Trimannoside, L-fucose and especially Lewis cluster preferentially bind to DC-SIGN transgenic cells. Sulfo Lewis A co-stains with 18 % of CD11c$^+$ hSIGN splenocytes and with only 11 % of CD11c$^+$ WT splenocytes whereas Lewis X is captured by 10 % of hSIGN cells and by 3 % of WT cells. Included as control, SA alexa488 is not bound by CD11c$^+$ splenocytes. Based on this result, sulfo Lewis A and Lewis X were selected to target hSIGN DCs *in vivo*.



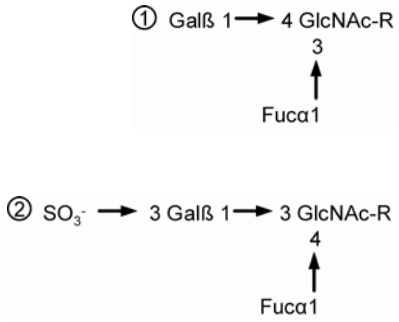


Figure 9: Top: Molecular structures of Lewis X (1) and sulfo Lewis A (2). **Left: Glycan binding to *ex vivo* isolated CD11c splenocytes from WT (filled bars) and hSIGN (open bars) mice.** Cells were incubated with biotinylated PAA coupled glycoconjugates (10 µg/ml) for 30 minutes at 37°C and afterwards stained with streptavidin alexa488 for FACS analysis. Bars show absolute percentage of CD11c splenocytes that have bound glycoconjugates (Singh et al. 2008).

4.4 Generation of OVA-sulfo Lewis A and OVA-Lewis X glycoconjugates

To examine whether sulfo Lewis A and Lewis X are appropriate to target antigen to DC-SIGN *in vivo*, both ligands were chemically conjugated to the model antigen OVA and the glycoconjugates OVA-sulfo Lewis A and OVA-Lewis X were generated. It has been demonstrated that antibodies against OVA or Lewis antigens specifically bind to corresponding moieties confirming that chemical conjugation succeeded and OVA has not been altered during this process (Singh et al. 2008).

OVA protein contains the MHC class II restricted peptide $OVA_{323-339}$ as well as the MHC class I restricted peptide $OVA_{257-264}$ (SIINFEKL). Therefore, we hypothesized that analysis of OVA specific T cell and antibody responses in glycoconjugate-treated hSIGN mice could provide information about the potential of DC-SIGN-targeted vaccines to modulate adaptive immune responses *in vivo*.

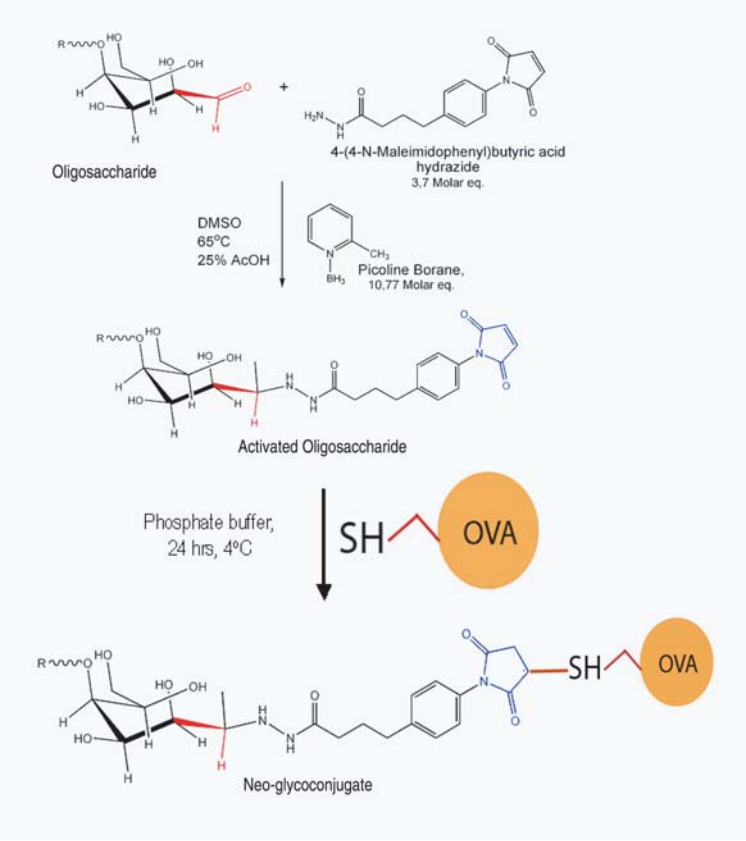


Figure 10: Schematic representation of glycan conjugation to ovalbumin. Oligosaccharides were chemically conjugated to the model antigen Ovalbumin resulting into glycoconjugates such as OVA-Lewis X and OVA-sulfo Lewis A. Specificity of conjugation of glycans to OVA was also analysed in ELISA using specific antibodies against OVA, Lewis X and sulfo Lewis A (Singh et al. 2008).

4.5 Th1 type responses towards glycoconjugates in hSIGN mice

4.5.1 Analysis of OVA specific CD8 T cell responses

Steinman and colleagues demonstrated that targeting antigen to DEC205 on mature DCs increases frequencies of antigen specific Th1 polarized $CD4^+$ T cells, $CD8^+$ CTLs and corresponding isotypes (Bonifaz, Bonnyay et al. 2004). Based on the property of DC-SIGN to load antigen on MHC class I and MHC class II complexes, it has been proposed that similar responses can be induced by DC-SIGN-targeted glycoconjugates. To address this question *in vivo*, hSIGN and WT mice were immunized with OVA-sulfo Lewis A, OVA-Lewis X or unmodified OVA. The B-type CpG-ODN 1826 and aluminium hydroxide (alum) were co-administrated since both adjuvants synergize in promoting Th1 responses (Lipford, Sparwasser et al. 1997; Davis, Weeratna et al. 1998).

On day seven after vaccination, blood was collected from immunized mice, stained for CD8, SIINFEKL-Tetramer and CD62L, an adhesion molecule which becomes downregulated upon activation, and analyzed by FACS.

As shown in Fig. 11, glycoconjugate-treated hSIGN and WT mice produce between 1.34 % and 1.75 % $CD8^+SIINFEKL^+CD62L^-$ T cells at this time point. However, this result does not provide evidence for enhanced OVA specific CD8 T cell responses in vaccinated hSIGN mice. Interestingly, mice which received OVA instead of glycoconjugates mount slightly reduced frequencies of 0.88 % and 1.03 %.

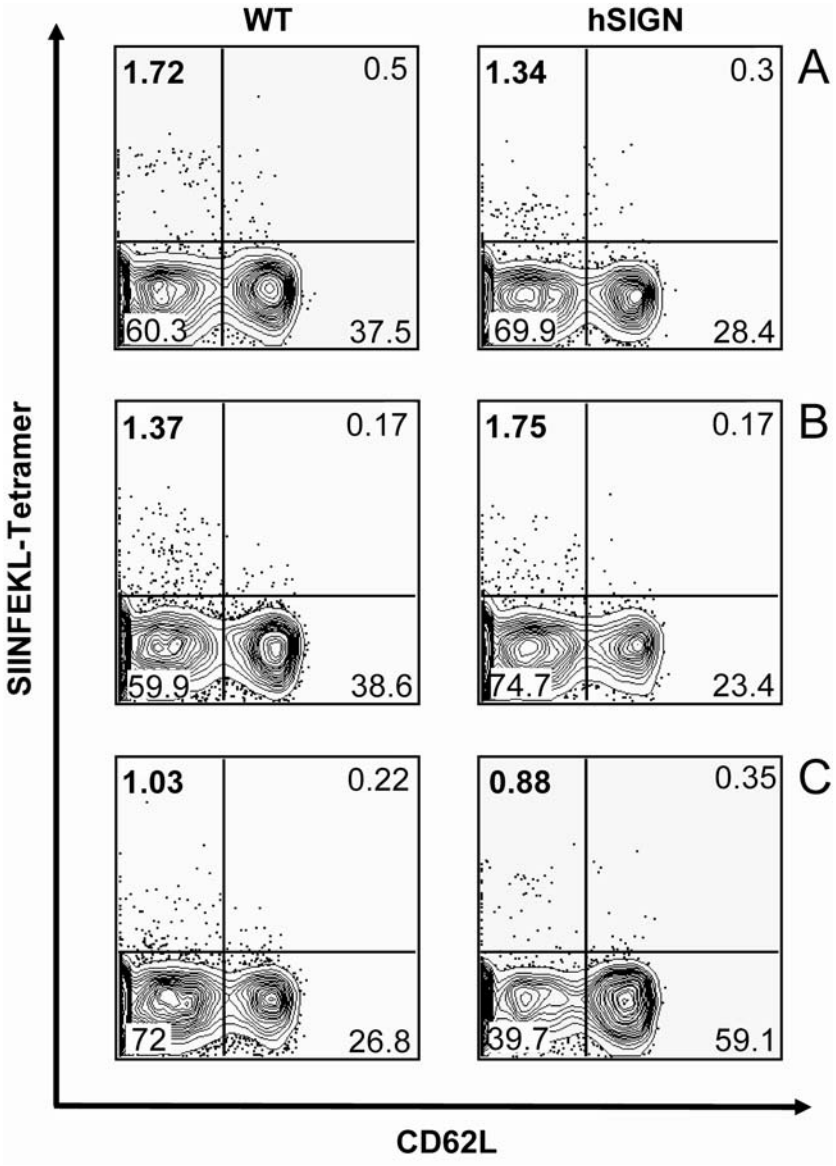


Figure 11: FACS-analysis of *ex vivo* isolated CD8$^+$ T cells 7 days after vaccination. WT and hSIGN mice were injected subcutanously in the tailbase with 10 µg OVA-Lewis X (A), OVA-sulfo Lewis A (B) or unmodified OVA (C) plus 10 nmol CpG-ODN 1826 and 50 µg alum. On day 7 after vaccination, blood from immunized mice was taken, purified from erythrocytes and stained for CD8, CD62L and SIINFEKL-tetramer. FACS plots show SIINFEKL-Tetramer versus CD62L staining of living CD8$^+$ T cells. Results are representative for 3 three independent experiments including 2 mice per group.

Within eight to ten days after vaccination, approximately 95 % of antigen specific effector T cells become eliminated or die from activation-induced cell death and only 5 % enter the memory T cell pool (Chang, Cho et al. 2004). In this context, factors of the initial process including strength of TCR stimulation, expression pattern of co-stimulatory molecules and levels of cytokines such as IL-2 and IL-12 are decisive for T cell differentiation (Langenkamp, Casorati et al. 2002). If quality of T cell priming was distinctly regulated in vaccinated hSIGN and WT mice, it would be possible that a second vaccination could reveil such differences.

To test this hypothesis, WT and DC-SIGN transgenic mice were immunized on day 14 with the same vaccine as on day 1. Spleens of immunized mice were prepared on day 28, stained for CD8, CD62L and SIINFEKL-Tetramer and frequencies of $CD8^+SIINFEKL^+CD62L^-$ T cells were determined by FACS.

As expected, all mice show increased numbers of $CD8^+SIINFEKL^+CD62L^-$ T cells (Fig. 12). Notably, individual OVA-Lewis X-treated DC-SIGN transgenic mice mount up to 4.5 % of $CD8^+SIINFEKL^+CD62L^-$ T cells whereas corresponding frequencies of WT mice do not exceed 2.18 %. In line, even single OVA-sulfo Lewis A-treated hSIGN mice produce higher frequencies of about 2.11 % SIINFEKL specific effector T cells compared to 1.66 % in WT mice. Moreover, we observed again a trend towards decreased frequencies of $CD8^+SIINFEKL^+CD62L^-$ T cells in OVA-treated animals which is apparently not influenced by the presence or absence of DC-SIGN.

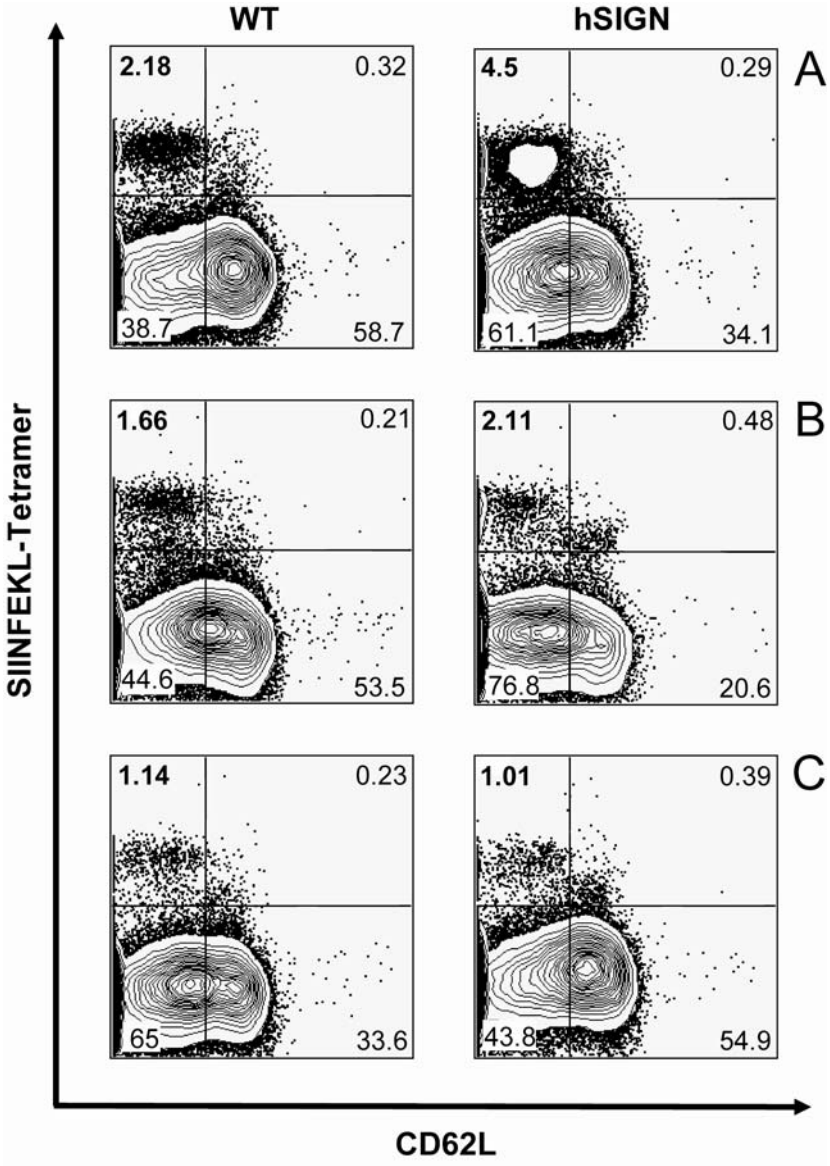


Figure 12: FACS-analysis of *ex vivo* isolated CD8$^+$ T cells 28 days after vaccination. WT and hSIGN mice were injected subcutanously in the tailbase on day 1 and day 14 with 10µg OVA-Lewis X (A), OVA-sulfo Lewis A (B) or unmodified OVA (C) plus 10 nmol CpG-ODN 1826 and 50 µg alum. On day 28, spleens from immunized mice were prepared, stained for CD8, CD62L and SIINFEKL-tetramer and analyzed by flow cytometry. FACS plots show SIINFEKL-Tetramer versus CD62L staining of living CD8$^+$ T cells. The result is representative for three independent experiments including 2 mice per group.

In addition, we asked whether SIINFEKL-specific $CD8^+$ CTLs from vaccinated WT and hSIGN mice display distinct cytotoxic activities. An unique ability of CTLs is to kill cells presenting the corresponding peptide on MHC class I molecules. To analyze the *in vivo* activity of CTLs primed by hSIGN and WT DCs, an *in vivo* cytotoxic T cell assay was performed on day 27 after mice had been immunized on day 1 and day 14 as described above.
Two fractions of *ex vivo* isolated splenocytes from unimmunized WT mice were prepared on day 27. One fraction was stained with a low concentration of 5,6-carboxyfluorescein diacetate succinimidyl ester ($CFSE^{low}$) and loaded with SIINFEKL peptide while a second fraction was labelled with a high concentration of CFSE ($CFSE^{high}$) but not loaded with antigen. Equal numbers of $CFSE^{low}$ and $CFSE^{high}$ cells were adoptively transferred into vaccinated WT and hSIGN as well as into an unimmunized control.
Six hours after adoptive transfer, blood was collected from mice and analyzed for relative percentages of CFSE-labelled cells by flow cytometry. Already at this time point up to 86 % of SIINFEKL loaded $CFSE^{low}$ cells are killed in immunized mice whereas the same fraction is not diminished in the unimmunized control (Fig. 13). Notably, CTLs from glycoconjugate-treated mice show a slightly enhanced cytotoxic activity compared to CTLs from OVA-treated animals.
After 20 h, blood was again taken and relative percentages of $CFSE^{low}$ and $CFSE^{high}$ cells were determined by flow cytometry. As shown in Figure 14, SIINFEKL loaded cells are completely eliminated in vaccinated mice at this time point. A slight decrease of $CFSE^{high}$ cells in the unimmunized control is most probably caused by toxic effects of the dye and reflects the reason for loading antigen on the $CFSE^{low}$ fraction to exclude unspecific cell death.
Although this protocol could not reveal differences between CTL activity of OVA-Lewis X- or OVA-sulfo Lewis A-vaccinated hSIGN and WT animals, it is still possible that analysis of earlier time points might show distinct kinetics.

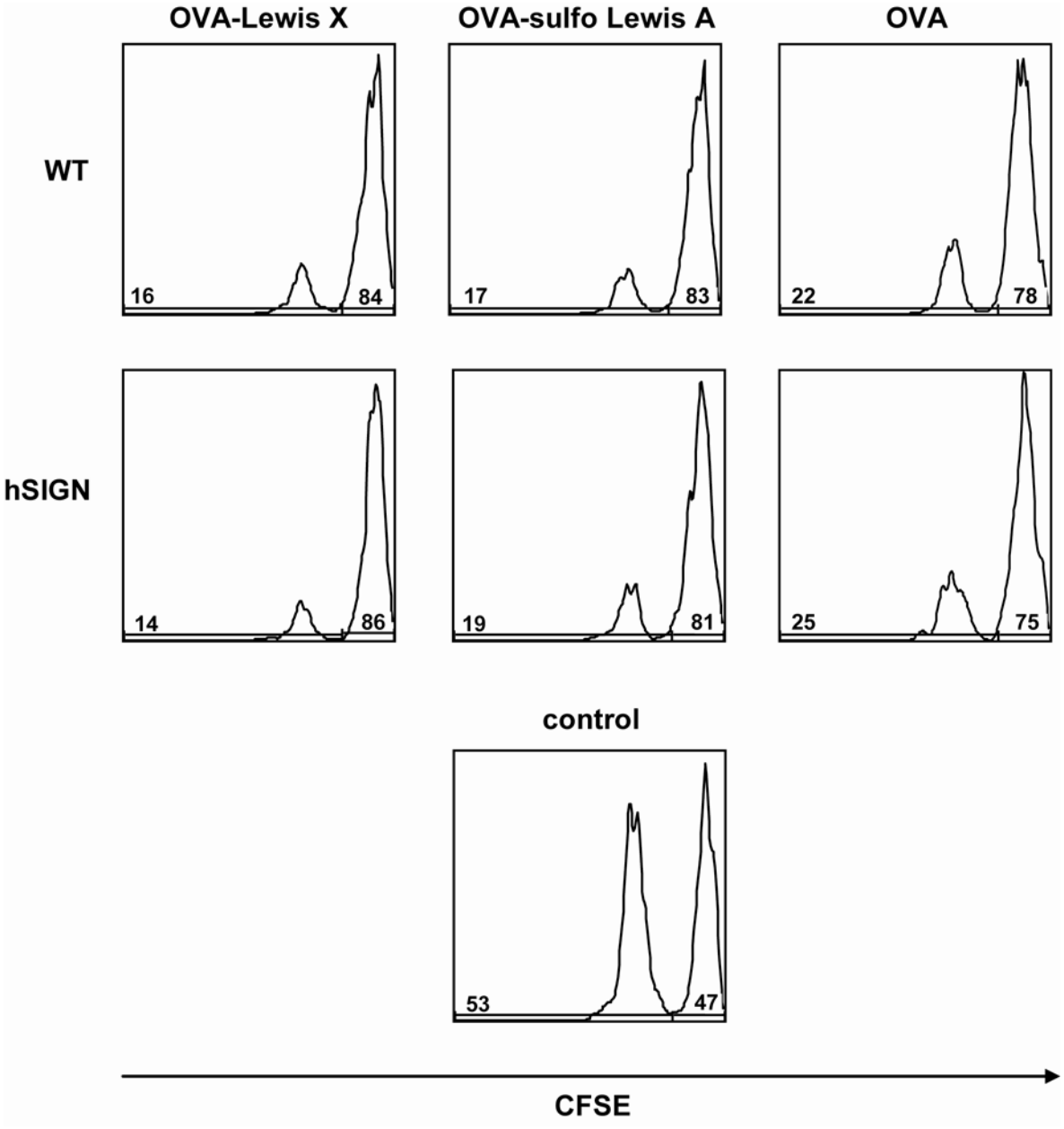


Figure 13: *In vivo* kill of CFSE^{low} SIINFEKL loaded cells in vaccinated WT and hSIGN mice. WT and hSIGN mice were injected subcutanously in the tailbase on day 1 and day 14 with 10 µg OVA-Lewis X (Lewis X), OVA-sulfo Lewis A (sulfo Lewis A) or unmodified OVA plus 10nmol CpG-ODN1826 and 50 µg alum. On day 27, immunized and control animals were injected intravenously with a mixture of 20 x 10^6 CFSE-labelled syngenic splenocytes pulsed with (CFSE^{low}) or without (CFSE^{high}) SIINFEKL peptide. 6 h after adoptive transfer the deletion of CFSE^{low} cells in peripheral blood was measured by FACS as a parameter of specific CTL activity. FACS plots show living cells gated on CFSE positive cells. Results are from one experiment and representative for 2 mice per group.

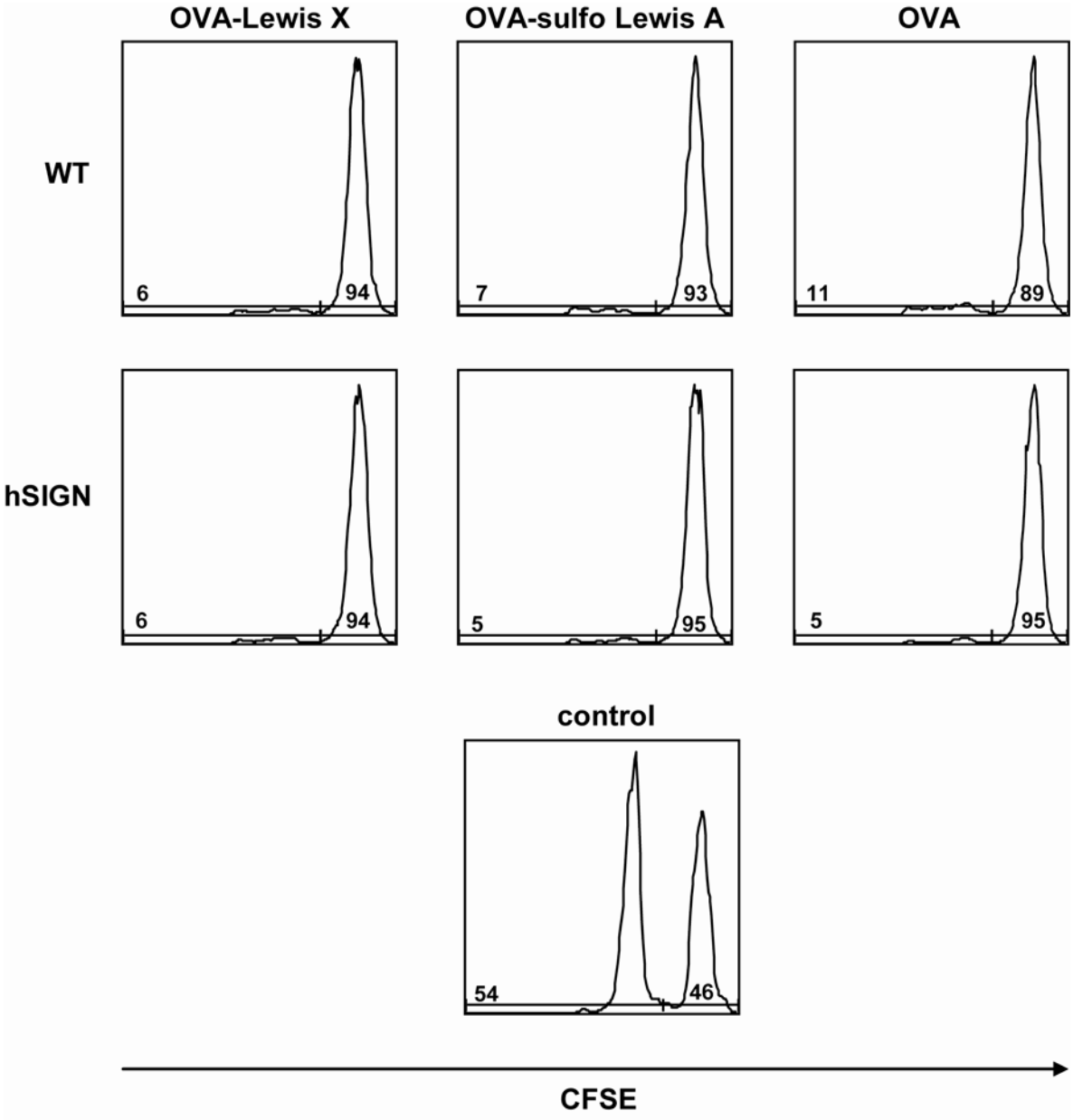


Figure 14: *In vivo* kill of CFSE^{low} SIINFEKL loaded cells in vaccinated WT and hSIGN mice. WT and hSIGN mice were immunized and adoptively transferred with CFSE^{low} SIINFEKL loaded cells and CFSE^{high} cells as described in Fig. 13. After 20 h blood was taken and relative percentages of CFSE^{high} and CFSE^{low} cells were determined by flow cytometry. FACS plots show living cells gated on CFSE positive cells. Results are from one experiment and representative for 2 mice per group.

4.5.2 Analysis of OVA specific CD4 T cell responses

By *in vitro* experiments, Engering et al. demonstrated that anti-DC-SIGN IgG1 isotypes enter MHC-II presenting pathways and enhance proliferation of IgG1 specific $CD4^+$ T cells (Engering, Geijtenbeek et al. 2002). Based on this result, we hypothesized that DC-SIGN-targeted glycoconjugates selectively enhance proliferation of OVA specific $CD4^+$ T cells in hSIGN mice.

To address this question, mice were immunized as indicated with glycoconjugates or OVA plus CpG-ODN 1826 and alum on day 1 and day 14. On day 28 after primary immunization, $CD4^+$ T cells were purified from draining lymphnodes and *ex vivo* restimulated with OVA-pulsed GM-CSF-cultured BMDCs. After 3 days, proliferation of $CD4^+$ T cells was quantified by measuring the concentration of adenosine triphosphate (ATP) from co-cultures.

As shown in Figure 15, $CD4^+$ T cells from OVA-Lewis X- (A), OVA-sulfo-Lewis A- (B) and OVA-treated (C) animals proliferate in a dose dependent manner indicating that glycoconjugate- as well as OVA-derived MHC class II peptides are presented in all immunized mice. Interestingly, $CD4^+$ T cells from mice vaccinated with glycoconjugates show a slightly enhanced proliferation compared to $CD4^+$ T cells from mice treated with OVA. However, this approach did not provide sufficient sensitivity to detect enhanced $CD4^+$ T cell responses in glycoconjugate-vaccinated DC-SIGN transgenic mice.

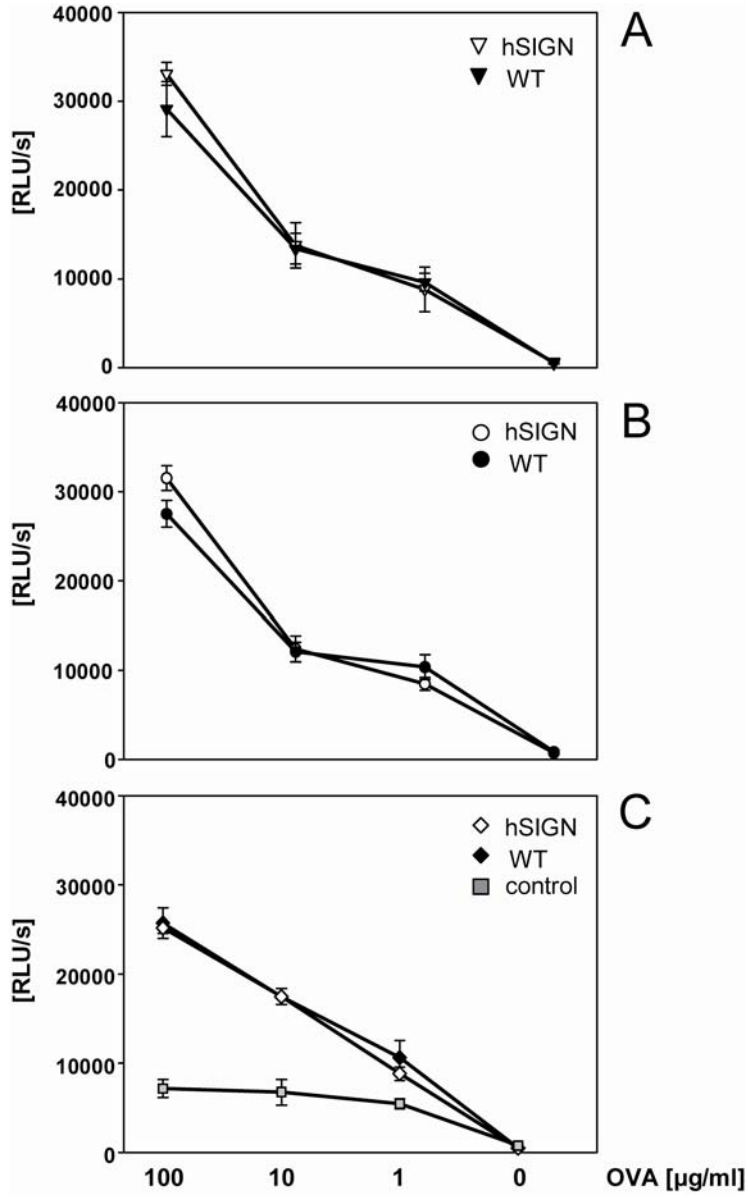


Figure 15: Proliferation of *in vivo* sensitized CD4$^+$ T cells in the presence of OVA-pulsed BMDCs: WT and hSIGN mice were injected subcutanously in the tailbase with 10 µg OVA-Lewis X (A), OVA-sulfo Lewis A (B) or OVA (C) plus 10 nmol CpG-ODN 1826 and 50 µg alum on day 1 and day 14. On day 28 CD4$^+$ T cells were purified from para-aortic lymphnodes of immunized and control animals. CD4$^+$ T cells from 2 mice per group were pooled and cultured for three days in the presence of irradiated GM-CSF BMDCs loaded with OVA protein. T cell proliferation was measured by CellTiter-Glo® Luminescent Cell Viability Assay according to the manufacturer´s protocol and is expressed as relative luminescence/second (RLU). These results are means and standard deviations of triplicates from one experiment.

4.5.3 Analysis of OVA specific isotypes

Dependent on the priming of $CD4^+$ T cells, B-cells produce distinct isotypes. While IL-4 producing $CD4^+$ Th2 cells induce IgG1 and IgE isotypes, IFN-γ producing $CD4^+$ Th1 cells promote generation of IgG2b, IgG2c and IgG3 isotypes (Boscardin, Hafalla et al. 2006). In addition, this process requires co-stimulation by surface molecules such as CD40-CD40L.

To evaluate antibody responses towards DC-SIGN-targeted glycoconjugates, WT and hSIGN mice were immunized with OVA-Lewis X, OVA-sulfo Lewis A or OVA plus CpG-ODN 1826 and alum on day 1 and day 14. On day 28, sera of mice were collected and concentrations of OVA specific IgG2b, IgG2c and IgG1 isotypes were determined by ELISA.

According to applied adjuvants, we observed a strong production of Th1 associated OVA specific IgG2b and IgG2c isotypes (Fig. 16). But we could also detect Th2 associated OVA specific IgG1 isotypes. However, levels of corresponding isotypes do not differ between immunized hSIGN and WT animals. In contrast to results from CD4 and CD8 T cell responses, we could not detect an increased isotype production in glycoconjugate-treated animals compared to animals which received OVA.

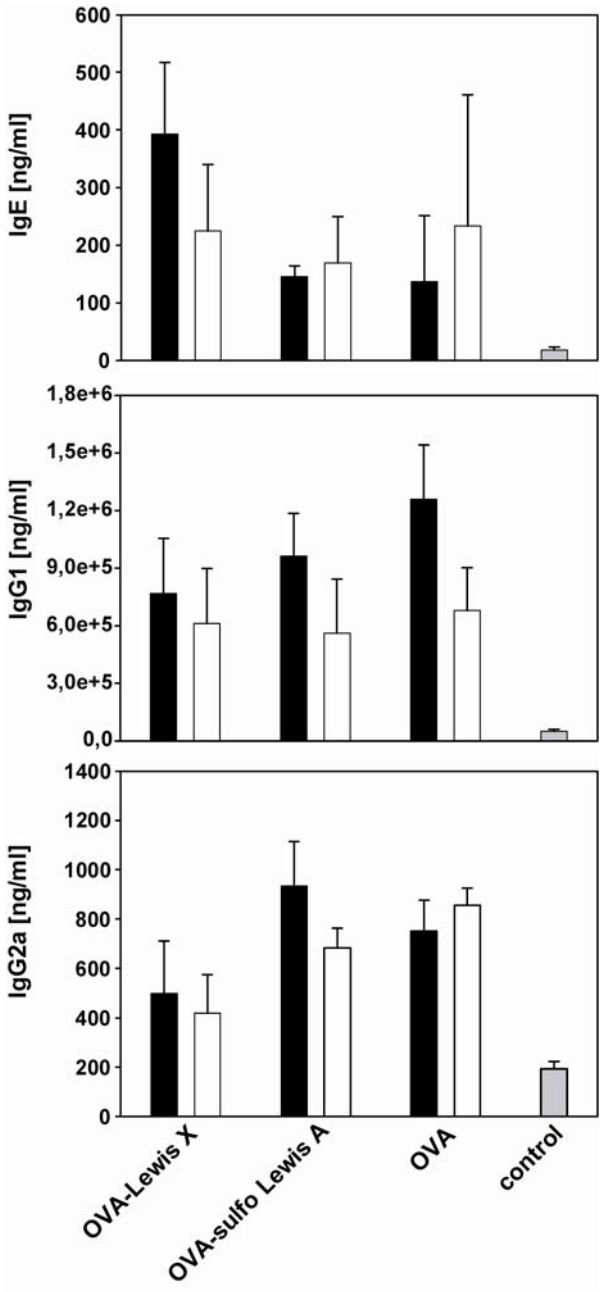


Figure 16: Analysis of OVA specific isotypes: WT (black bars) and hSIGN mice (white bars) were injected subcutanously in the tailbase with 10 µg OVA-Lewis X, OVA-sulfo Lewis A or OVA plus 10 nmol CpG-ODN 1826 and 50 µg alum on day 1 and 14. Blood from vaccinated mice was taken on day 28 and sera were obtained by centrifugation. Relative concentrations of OVA specific IgG2c, IgG2b and IgG1 isotypes were determined by ELISA. Samples from individual mice were measured as duplicates. Results are from two independent experiments including 2 mice per group.

4.5.4 Adoptive transfer of OT-I and OT-II cells

The fact that previous results could not provide clear evidence of enhanced Th1 responses in glycoconjugate-treated hSIGN mice suggests the possibility that protocols lacked sensitivity. In this context, it needs to be considered that in mice only about 1/10,000 naïve T cells responds to a specific antigen (Bonifaz, Bonnyay et al. 2002). Furthermore, this study demonstrated that adoptive transfer of TCR transgenic cells amplifies immune responses and visualizes minimal changes.

In regard to the model antigen OVA, TCR transgenic $CD4^+$ T cells from C57BL/6 mice (OT-II cells) recognize $OVA_{323-339}$ (ISQAVHAAHAEINEAGR) presented on $I\text{-}A^b$ (MHC class II) complexes and TCR transgenic $CD8^+$ T cells (OT-I cells) bind to $H\text{-}2K^b$ (MHC class I) complexes loaded with the octapeptide $OVA_{257-264}$ (SIINFEKL) (Chen, McCluskey et al. 1993; Robertson, Jensen et al. 2000). To detect transferred OT-I and OT-II cells, T cells were purified from TCR transgenic animals expressing the congenic marker CD45.1.

Based on this concept, 2×10^5 $CD45.1^+$ OT-I cells were adoptively transferred into hSIGN and WT mice and the following day mice were immunized with OVA-Lewis X or OVA plus CpG-ODN 1826 and alum.

On day three after immunization, blood was taken and frequencies of $CD45.1^+$ OT-I cells were determined by flow cytometry. Although TCR transgenic cells show an enhanced proliferation in immunized animals, we could not detect differences between DC-SIGN transgenic and WT animals regarding percentages of $CD8^+CD45.1^+$ cells out of all $CD8^+$ T cells (Fig. 17A). On day 5 after vaccination, mice were sacrificed, spleens were collected and stained for CD45.1 and CD8. Here, we observed that mice vaccinated with OVA-Lewis X show approximately 2 % higher frequencies of $CD45.1^+$ OTI cells in comparison to OVA-treated animals (Fig. 17B). However, even at this time point, $CD45.1^+$ OT-I cells show similar proliferation in glycoconjugate-treated hSIGN and WT animals.

In the same manner 2×10^5 $CD45.1^+$ OT-II cells were adoptively transferred into hSIGN and WT mice and the following day mice were immunized with OVA-Lewis X plus CpG-ODN 1826 and alum. On day 3 after immunization, we detected similar frequencies of 0.3 % $CD45.1^+$ OT-II cells in peripheral blood of hSIGN and WT mice (Fig. 18). Finally, mice were sacrificed on day 5, spleens were prepared and frequencies of $CD45.1^+$ OT-II cells were determined by flow cytometry.

Although a clear expansion of TCR transgenic cells up to 3.4 % could be observed in

vaccinated animals, there was no significant difference detectable between DC-SIGN transgenic and WT animals.

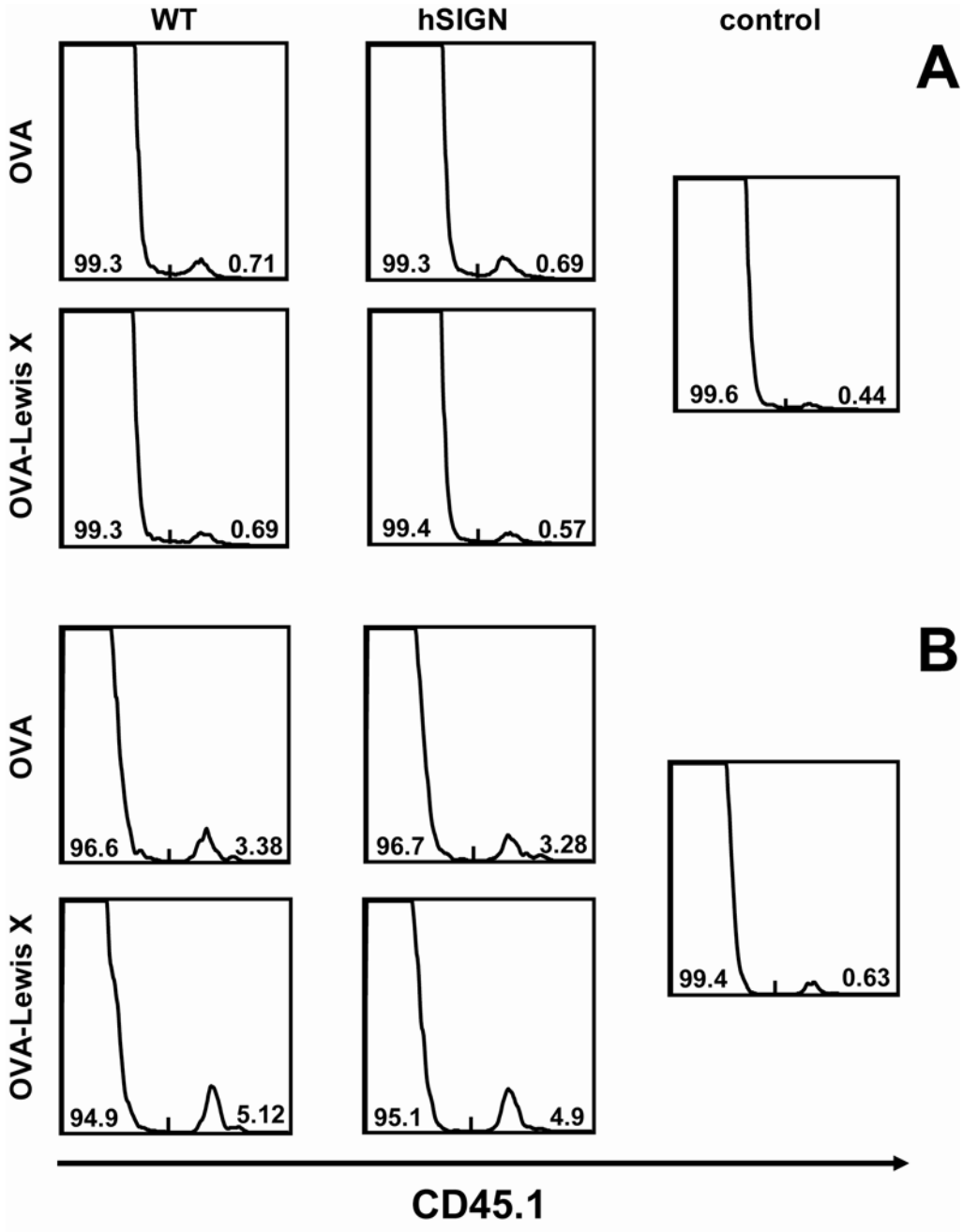


Figure 17: Adoptive transfer of CD45.1$^+$ OT-I cells: CD45.1$^+$ OT-I cells were purified from lymphnodes of CD45.1/OT-I mice by magnetic beads. 2 x 10^5 CD45.1$^+$ OT-I cells were injected i.v. into hSIGN and WT mice. The next day, mice were injected in the tailbase with 10 µg OVA-Lewis X or OVA plus 10 nmol CpG-ODN 1826 and 50 µg alum. On day 3 (A) blood was taken, erythrocytes were lysed and cells were stained for CD8 and CD45.1. On day 5 (B) spleens were collected, purified from erythrocytes and again stained for CD45.1 and CD8. FACS plots show living cells gated on CD8$^+$ cells. Percentages of CD45.1$^+$ OT-I cells out of all CD8$^+$ cells are depicted. Results are representative for three independent experiments including two mice per group.

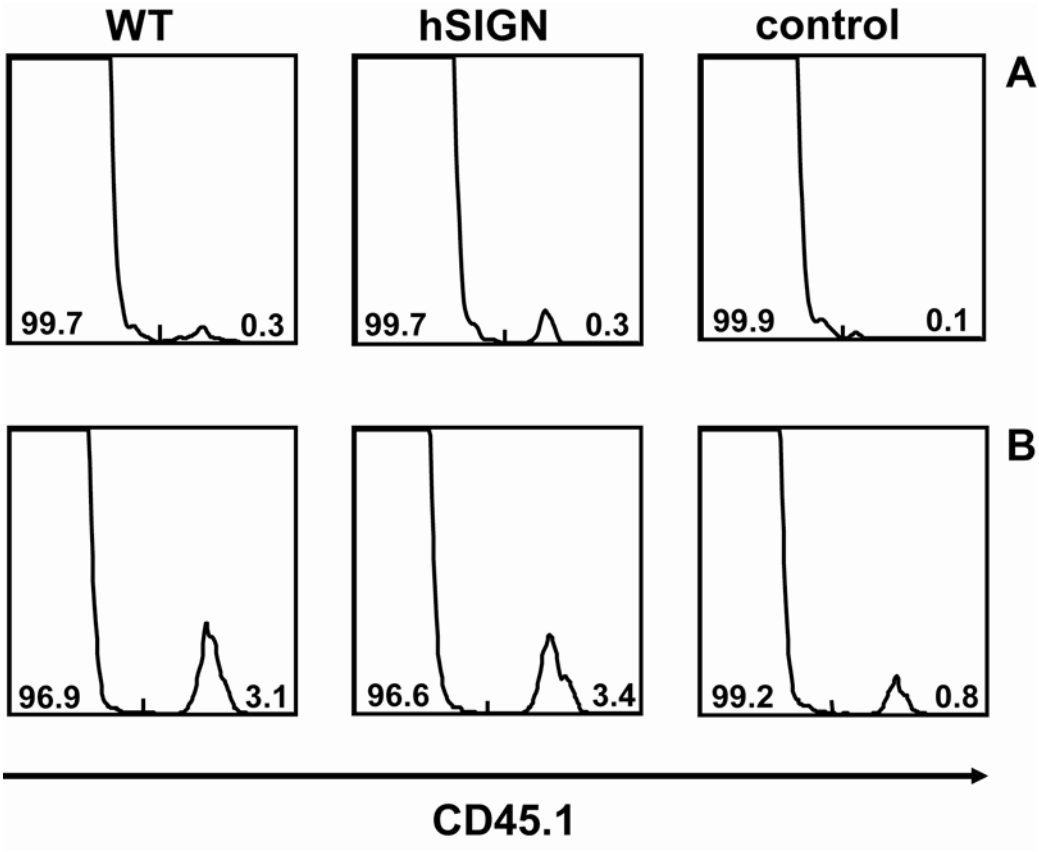


Figure 18: Adpotive transfer of CD45.1$^+$ OT-II cells. CD45.1$^+$ OT-II cells were purified from lymphnodes of CD45.1$^+$ OTII mice and 2 x 10^5 CD45.1$^+$ OT-II cells were injected i.v. into hSIGN and WT mice. The next day, mice were injected subcutanously in the tailbase with 10 µg OVA-Lewis X plus 10nmol CpG-ODN 1826 and 50 µg alum. On day 3 blood was taken, erythrocytes were lysed and cells were stained for CD4 and CD45.1 (A). On day 5 spleens were collected, purified from erythrocytes and stained for CD45.1 and CD4 (B). FACS plots show living cells gated on CD4$^+$ cells. Percentages of CD45.1$^+$ OT-II cells out of all CD4$^+$ T cells are depicted. Results are from one experiment and representative for two mice per group.

4.6 Th2 type responses towards glycoconjugates in hSIGN mice

In addition to Th1 responses, we addressed the question whether DC-SIGN-targeted glycoconjugates modulate Th2 responses in hSIGN mice. Since C57BL/6 mice are quite resistant to Th2 type diseases such as parasite infections or acute hypersensitivity reactions we used hSIGN and WT mice on a BALB/c background to investigate this issue.

The model of allergic airway inflammation has been established to study pathomechanisms and possible treatments of human asthma (Kung, Jones et al. 1994). In the common protocol mice become sensitized with antigen and alum and afterwards animals are challenged with inhalative aerosolized antigen. Similar to human asthma, mice develop airway hyper-responsiveness, show elevated levels of Th2 associated cytokines such as IL-4, IL-5 and IL-13 and produce high levels of antigen specific IgE and IgG1 isotypes. Lung tissue sections are characterized by an influx of eosinophils and an enhanced mucus production.
Accordingly, hSIGN and WT mice were weekly sensitized with glycoconjugates/alum or OVA/alum and in the fourth week mice were challenged on days 24 - 26 with inhalative OVA aerosol. Since the proportion of antigen specific isotypes reflects the priming type of $CD4^+$ T cells, sera from immunized mice were analyzed after the challenge phase for Th2 associated OVA specific IgE and IgG1 isotypes as well as for Th1 associated OVA specific IgG2a isotypes.
Both glycoconjugate- and OVA-treated animals produce slightly higher levels of OVA specific IgG2a than OVA specific IgE (Fig. 19). However, the fact that antigen specific IgG1/IgG2a ratios of all vaccinated mice are clearly elevated demonstrates a Th2 biased response. Furthermore, glycoconjugate-treated hSIGN mice secrete reduced levels of IgG2a compared to hSIGN mice which received OVA. However, vaccinated WT mice tend to produce higher amounts of OVA specific IgG1 isotypes and in response to OVA-Lewis X even higher levels of IgE compared to DC-SIGN transgenic animals.

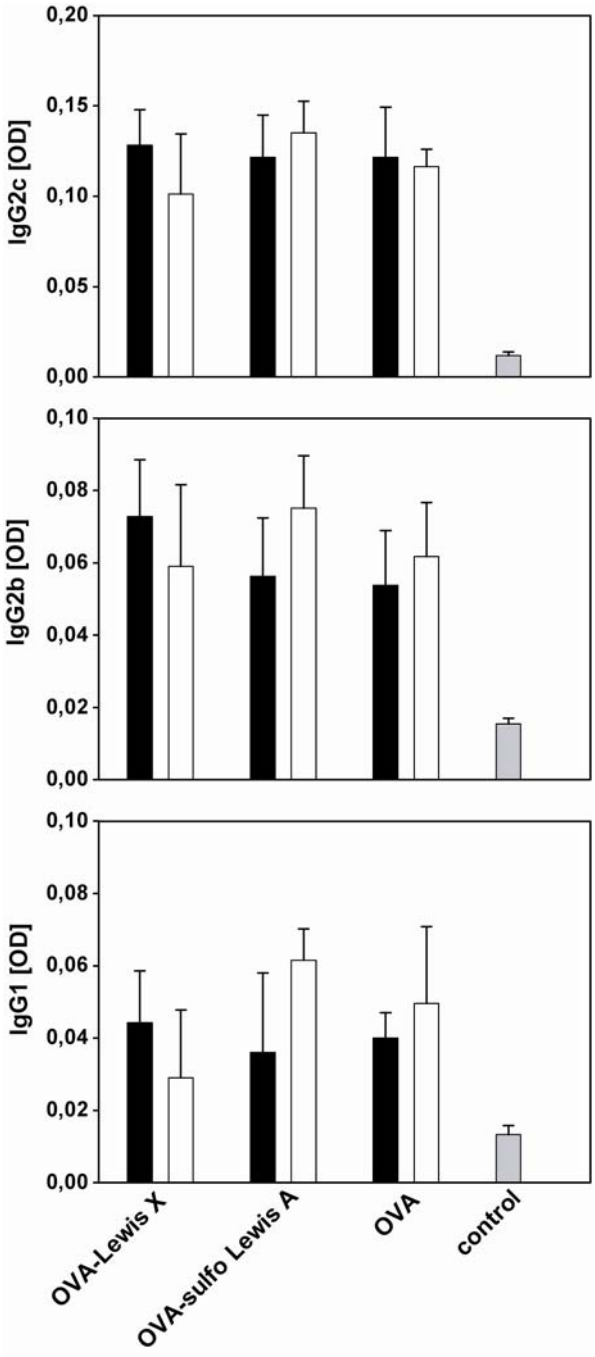


Figure 19: Analysis of OVA specific isotypes: WT (black bars) and hSIGN mice (white bars) were immunized by intraperitoneal injection of 10 µg OVA-Lewis X, OVA-sulfo Lewis A or OVA plus 220 µg alum on day 1, 14 and 21. On day 24, 25 and 26 mice were challenged with inhalative OVA aerosol (10 µg/ml) for 20 min using the Parimaster® nebulizer. On day 28, sera from vaccinated mice and untreated controls were analyzed for OVA specific IgE, IgG1 and IgG2a isotypes by ELISA. Samples from individual mice were measured as duplicates. Results are representative for two independent experiments including 2 mice per group.

4.7 Tolerogenic T cell responses towards glycoconjugates

The steady state is defined by the absence of inflammatory signals. Here, immature DCs (iDCs) act as sentinels and constantly take up, process and present antigen. However, since iDCs express only low levels of co-stimulatory molecules and MHC-II complexes, iDCs do not induce immunogenic but tolerogenic T cell responses (Shortman and Naik 2007). In this context, it has been demonstrated that targeting anti-DEC205-OVA to iDCs results in only a few divisions of adoptively transferred OT-I cells which subsequently become deleted. In addition, mice show no response to a challenge with OVA in complete Freund´s adjuvant indicating that DEC205-targeted antigens elicit tolerogenic responses during steady state (Bonifaz, Bonnyay et al. 2002).

The fact that human iDCs express high levels of DC-SIGN suggests that DC-SIGN-targeted vaccines might enhance tolerogenic T cell responses as well. To investigate this issue *in vivo*, hSIGN mice were crossed with OT-I TCR transgenic and DEREG BAC transgenic mice (hSIGN/DEREG/OT-I mice). As control we included DEREG/OT-I mice. DEREG mice express green fluorescence protein (GFP) and human diphtheria toxin receptor (huDTR) under control of the murine Foxp3 promoter (Lahl, Loddenkemper et al. 2007). Thus, $Foxp3^+$ Treg cells can be specifically tracked by detection of GFP and selectively depleted by application of diphtheria toxin. The presence of OT-I cells allows analysis of SIINFEKL specific effector T cells ($SIINFEKL^+GFP^-$ cells) as well as antigen specific $SIINFEKL^+GFP^+$ regulatory T cells. Moreover, analysis of $SIINFEKL^-GFP^+$ cells provides information about development of antigen unspecific Treg cells.

Since transgenic mice vary in endogenous frequencies of OT-I cells and GFP^+ Treg cells, it was necessary to analyze corresponding frequencies before vaccination. Therefore, blood was collected from mice and frequencies of $SIINFEKL^+GFP^-$, $SIINFEKL^+$ GFP^+ and $SIINFEKL^-$ GFP^+ cells were determined by FACS (Fig. 20, day 0). Frequencies of $SIINFEKL^+GFP^-$ cells show a remarkable variety from 26 % in triple transgenic mice up to 38 % in double transgenic mice. Only little differences are detectable for $SIINFEKL^+$ GFP^+ (0.05 - 0.08 %) and $SIINFEKL^-GFP^+$ cells (0.3 - 0.4 %).

To compare steady state versus inflammatory state mice were treated with two different regimens. In one group, hSIGN/DEREG/OT-I and DEREG/OT-I mice received OVA-Lewis X without any inflammatory stimulus (Fig. 20 left row) whereas genetically matched animals were treated with OVA-Lewis X plus CpG-ODN 1826 (Fig. 20 right row).

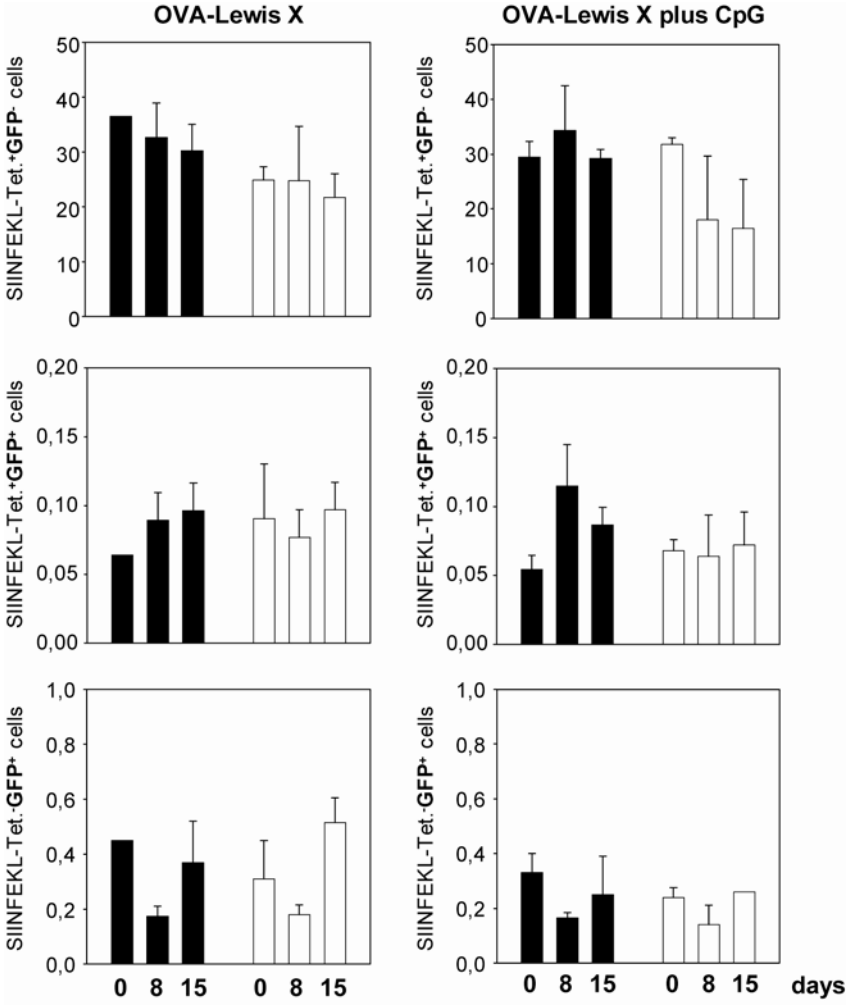


Figure 20: Development of antigen specific effector T cells (SIINFEKL$^+$GFP$^-$), antigen specific regulatory T cells (SIINFEKL$^+$ GFP$^+$) and unspecific regulatory T cells (SIINFEKL$^-$ GFP$^+$) in DEREG/OTI mice (black bars) and hSIGN/DEREG/OTI mice (white bars). On day 0, blood of untreated mice was lysed and stained for SIINFEKL tetramer. Frequencies of described populations were analyzed by flow cytometry. On day 1, mice were immunized with 5 µg OVA-Lewis X i.p. (left row) or 5 µg OVA-Lewis X plus 10 nmol CpG-ODN 1826 i.p. (right row). On day 8, blood was again analyzed as described for day 0. On day 12, all mice received 400 µg OVA protein and 10nmol CpG i.p.. Finally, spleens from mice were collected on day 15, purified from erythrocytes and stained with SIINFEKL tetramer. Depicted populations were analyzed by flow cytometry. Groups included 2 DEREG/OT-I and 3 hSIGN/DEREG/OT-I animals, respectively.

On day 8 after immunization, peripheral blood was again analyzed for described populations. Here, OVA-Lewis X-treated hSIGN/DEREG/OT-I mice show neither a decrease of SIINFEKL$^+$GFP$^-$ cells nor an increase of corresponding antigen specific Treg cells.

Interestingly, two genetic identical mice that received OVA-Lewis X plus CpG-ODN 1826 show a dramatic decrease of SIINFEKL$^+$GFP$^-$ cells. Furthermore, frequencies of SIINFEKL$^-$ GFP$^+$ cells drop in all immunized mice after the first vaccination whereas SIINFEKL$^+$ GFP$^+$

cells are slightly increased in DEREG/OT-I animals. To verify whether T cells primed by steady state DCs are still responsive, all mice were challenged with OVA protein and CpG-ODN 1826 on day 12. On day 15, animals were sacrificed, spleens were collected and stained for CD8 and SIINFEKL tetramer. The following FACS analysis revealed that frequencies of $SIINFEKL^{+}GFP^{-}$ cells are not altered compared to day 8. Moreover, frequencies of $SIINFEKL^{-}GFP^{+}$ cells become slightly enhanced in all mice and reach again percentages of day 0 whereas $SIINFEKL^{+}$ GFP^{+} cells do hardly change compared to frequencies of day 8.
Taken together, we could neither observe a reduction of antigen specific effector T cells nor an expansion of regulatory T cells by targeting glycoconjugates to hSIGN DCs during steady state. However, we did detect a reduction of SIINFEKL specific effector T cells if glycoconjugates were targeted in the presence of TLR9 ligands to hSIGN DCs *in vivo*.

4.8 DC-SIGN in mycobacterial infection

Since infection biology provides important information for the development of vaccines we analyzed the interaction between M. tuberculosis and DC-SIGN which has been described as the major DC receptor for mycobacteria (Tailleux, Schwartz et al. 2003).

4.8.1 M. bovis BCG activates Raf-1 kinase in hSIGN DCs

Geijtenbeek and colleagues demonstrated that the cell wall component ManLam of M. tuberculosis prevents LPS- or M. bovis BCG-induced DC maturation and enhances production of IL-10 (Geijtenbeek, Van Vliet et al. 2003). Furthermore, it has been shown that only if TLR signalling induces nuclear translocation of NFκB subunit p65, ManLAM promotes intranuclear phosphorylation and acetylation of p65 via recruitment of Src and Pak kinases to DC-SIGN which subsequently trigger phosphorylation of Raf-1 kinase. Finally, the crosstalk between TLR and DC-SIGN signalling results in a prolonged transcription of IL-10 (Gringhuis, den Dunnen et al. 2007).

To verify if these signalling pathways are conserved in hSIGN DCs, we analyzed activation of Raf-1 kinase in M. bovis BCG-infected BMDCs. GM-CSF-cultured BMDCs from WT and hSIGN mice were stimulated with M. bovis BCG and afterwards stained with anti-phospho-Raf-1.

By FACS analysis we observed that at least 0.95 % of stimulated hSIGN BMDCs are positive for phosphorylated Raf-1 kinase (phospho-Raf-1) whereas this population is not detectable in M. bovis BCG-infected WT BMDCs (Fig. 21).

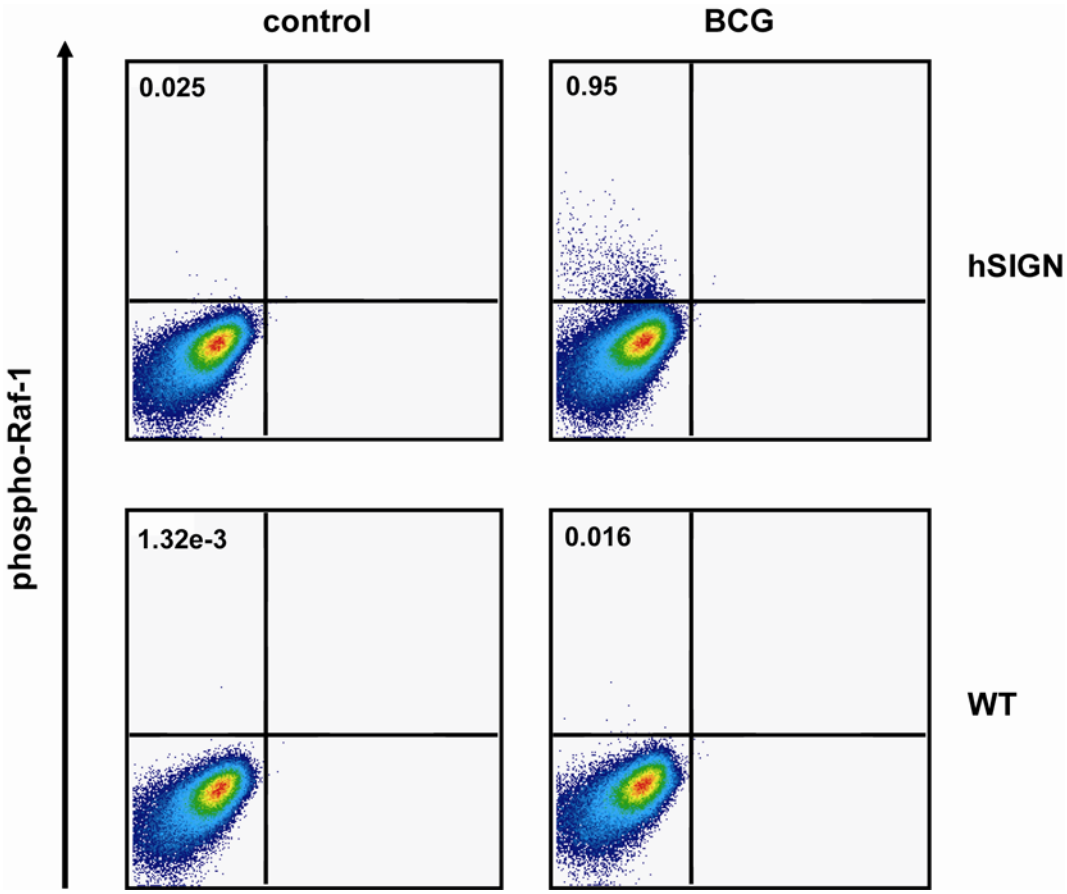


Figure 21: Detection of activated phosphorylated Raf-1 kinase (phospho-Raf-1) in M. bovis BCG stimulated hSIGN BMDCs. On day 7, GM-CSF cultured BMDCs from WT and hSIGN mice were harvested, washed and stained 20 min with EMA. Afterwards BMDCs were stimulated at 37° C with 10 MOI of M. bovis BCG. Cells were fixed with paraformaldeyd (3%) and permeabilized with methanol (90%). For detection of activated Raf-1, cells were stained with anti-phospho-Raf-1 (IgG2 rabbit) and anti-rabbit IgG2-FITC. FACS plots show living cells gated for phospho-Raf-1.

4.8.2 *In vitro* analysis of T cell priming in the presence of M. bovis BCG-infected hSIGN DCs

It has been demonstrated that M. tuberculosis- or M. bovis BCG-infected hSIGN DCs do not secrete enhanced levels of IL-10 but produce significantly reduced levels of IL-12 (Schaefer, Reiling et al. 2008). Thus, since IL-10 and IL-12 counter regulate each other, hSIGN DCs do still mimic human DC physiology. Furthermore, in contrast to the hypothesis that DC-SIGN serves as immune escape mechanism for mycobacteria by downregulating protective anti-microbial responses, we could demonstrate that hSIGN mice show a decreased histopathology and a prolonged survival during M. tuberculosis infection.

Based on this result, we addressed the question whether infected hSIGN DCs specifically alter proliferation and cytokine responses of antigen specific T cells. Therefore, GM-CSF-cultured hSIGN and WT BMDCs were infected with different MOI of OVA expressing M. bovis BCG (BCG-OVA) and co-cultured with OT-I, OT-II or WT CD4 T cells, respectively.

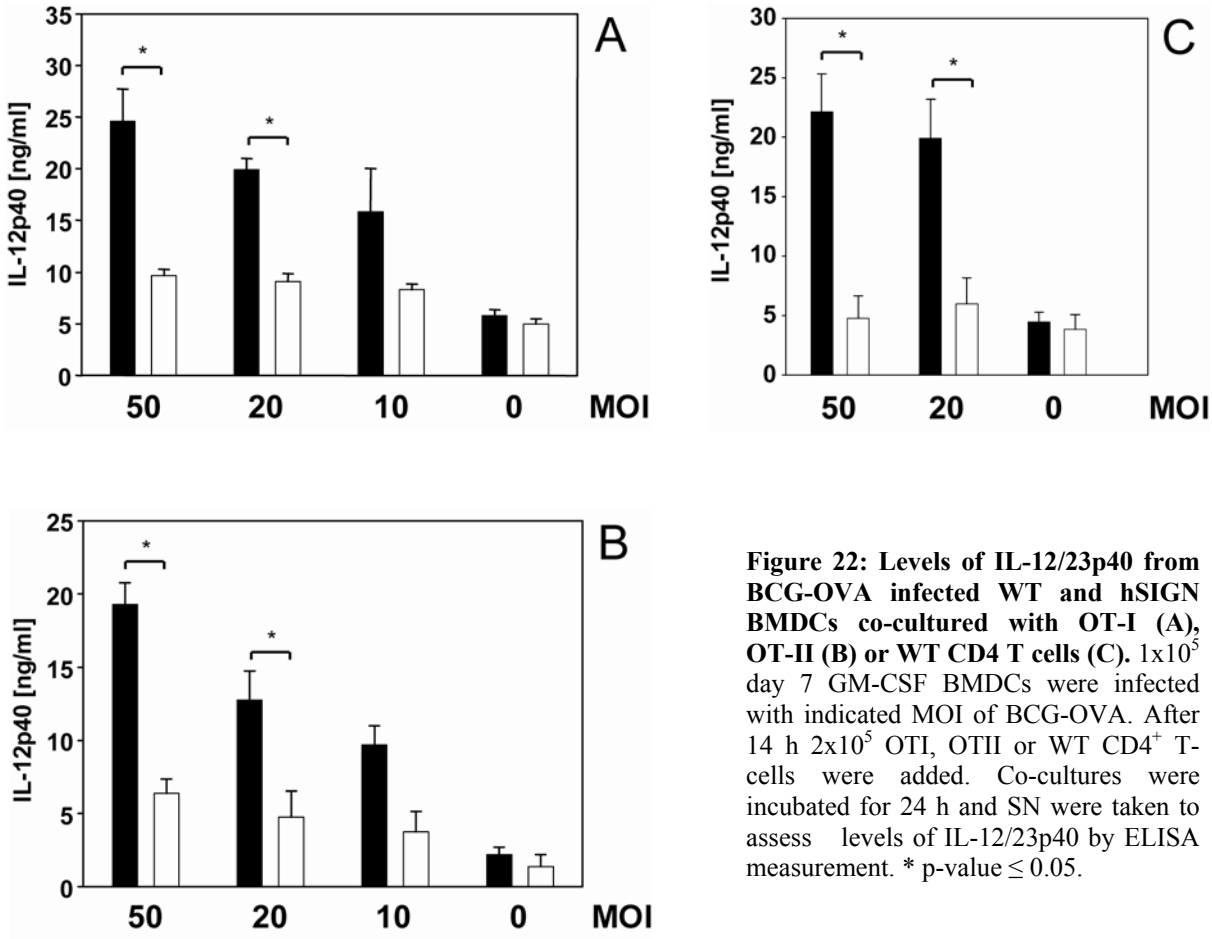


Figure 22: Levels of IL-12/23p40 from BCG-OVA infected WT and hSIGN BMDCs co-cultured with OT-I (A), OT-II (B) or WT CD4 T cells (C). $1x10^5$ day 7 GM-CSF BMDCs were infected with indicated MOI of BCG-OVA. After 14 h $2x10^5$ OTI, OTII or WT $CD4^+$ T-cells were added. Co-cultures were incubated for 24 h and SN were taken to assess levels of IL-12/23p40 by ELISA measurement. * p-value ≤ 0.05.

38 h after infection, supernatants (SN) were harvested and analyzed by ELISA for cytokines. In line with previous results, we observed that hSIGN DCs infected with 50 or 20 MOI BCG-OVA produce significantly reduced levels of IL-12p40 (Fig. 22). Notably, levels of IL-12p40 from co-cultures with OT-I (A), OT-II (B) and WT CD4 T cells (C) do not differ suggesting that the observed reduction of IL-12p40 is independent from the presence of distinct T cell subsets.

As indicator of T cell proliferation, IL-2 was determined. Consistently, T cells co-cultured with BCG-OVA-infected WT DCs secrete increased levels of IL-2 (Fig. 23). In addition, we observed that OVA TCR transgenic cells produce higher levels of IL-2 than WT $CD4^+$ T cells if co-cultured with 50 or 20 MOI of BCG-OVA-infected WT BMDCs. Moreover, OT-II cells release higher levels of IL-2 than OT-I cells.

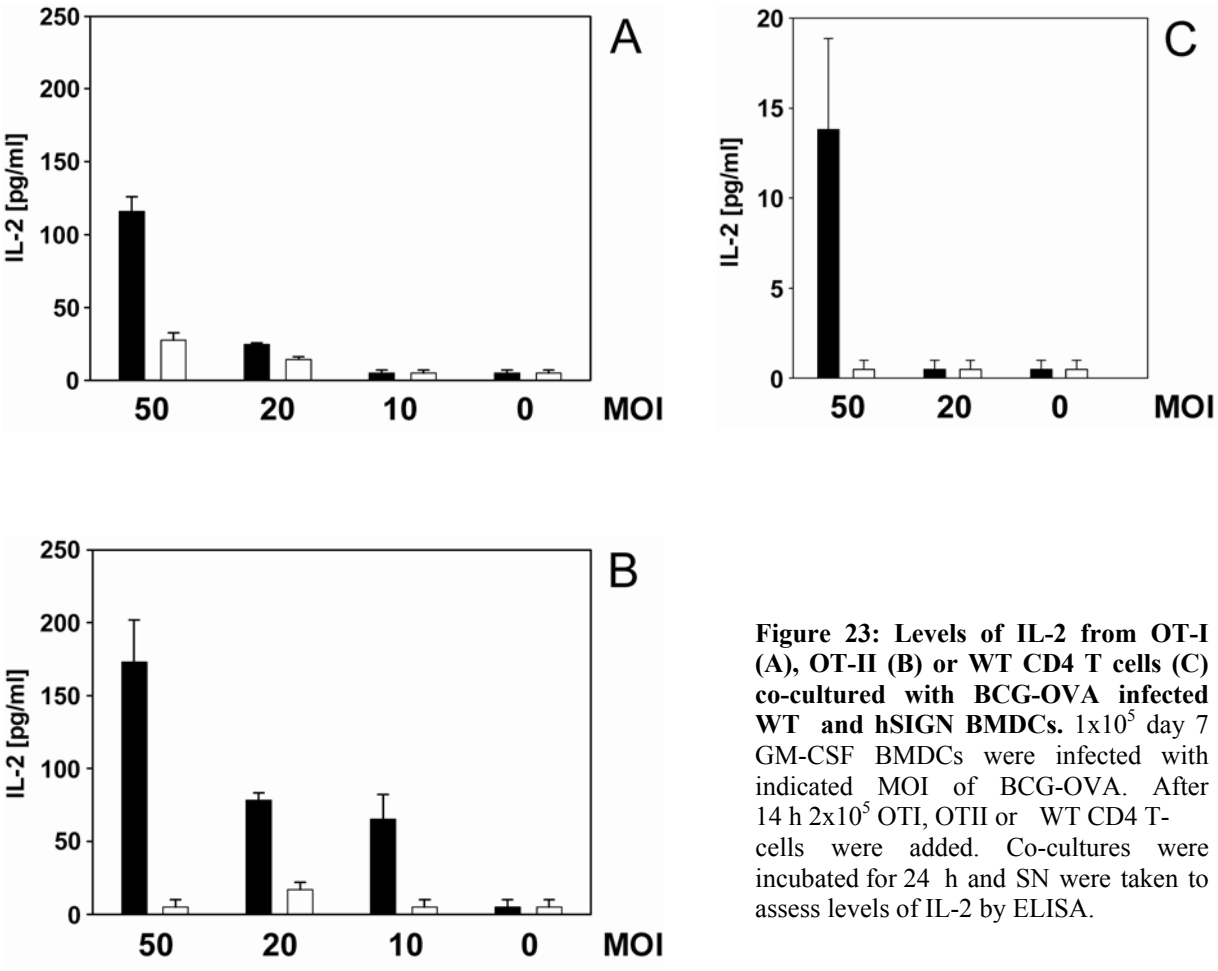


Figure 23: Levels of IL-2 from OT-I (A), OT-II (B) or WT CD4 T cells (C) co-cultured with BCG-OVA infected WT and hSIGN BMDCs. $1x10^5$ day 7 GM-CSF BMDCs were infected with indicated MOI of BCG-OVA. After 14 h $2x10^5$ OTI, OTII or WT CD4 T-cells were added. Co-cultures were incubated for 24 h and SN were taken to assess levels of IL-2 by ELISA.

IL-12p40 and IL-12p35 constitute the cytokine IL-12p70 (IL-12) which plays a crucial role for the priming of IFN-γ producing $CD4^+$ and $CD8^+$ T cells (Sparwasser, Vabulas et al. 2000). To verify whether reduced levels of IL-12p40 from BCG-OVA-infected hSIGN BMDCs alter the priming of T cells, concentrations of IFN-γ were measured by ELISA. Indeed, we observed that OT-I, OT-II as well as WT CD4 T cells produce reduced levels of IFN-γ in the presence of BCG-OVA-infected hSIGN BMDCs (Fig. 24). Again, as a proof of antigen specificity OT-II cells release still higher amounts of IFN-γ than WT CD4 T cells. Furthermore, both OT-II cells and WT CD4 T cells secrete higher levels of IFN-γ than OT-I cells.

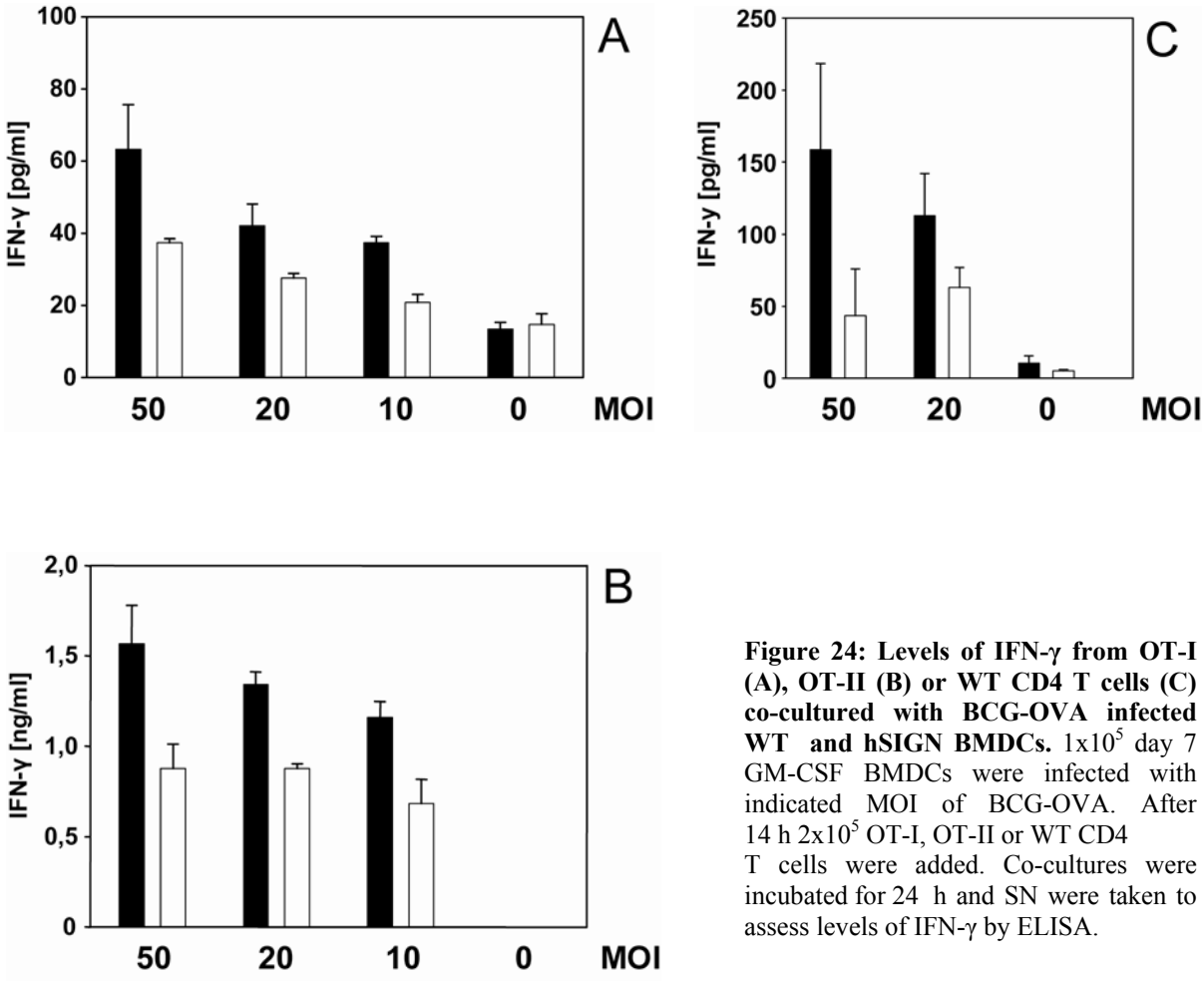


Figure 24: Levels of IFN-γ from OT-I (A), OT-II (B) or WT CD4 T cells (C) co-cultured with BCG-OVA infected WT and hSIGN BMDCs. $1x10^5$ day 7 GM-CSF BMDCs were infected with indicated MOI of BCG-OVA. After 14 h $2x10^5$ OT-I, OT-II or WT CD4 T cells were added. Co-cultures were incubated for 24 h and SN were taken to assess levels of IFN-γ by ELISA.

If IL-12p40 dimerizes with IL-23p19, IL-23 is formed, a cytokine which contributes to induction and maintenance of $CD4^+$ Th17 cells and which drives priming of IL-17 producing $CD8^+$ T cells (He, Wu et al. 2006; Veldhoen, Hocking et al. 2006).

The measurement of IL-17 from SN showed that TCR transgenic OT-I as well as OT-II cells produce decreased levels of IL-17 if co-cultured with BCG-OVA-infected hSIGN BMDCs (Fig. 25). Moreover, SN from OT-II cells contain 10 fold higher concentrations of IL-17 than SN from OT-I cells.

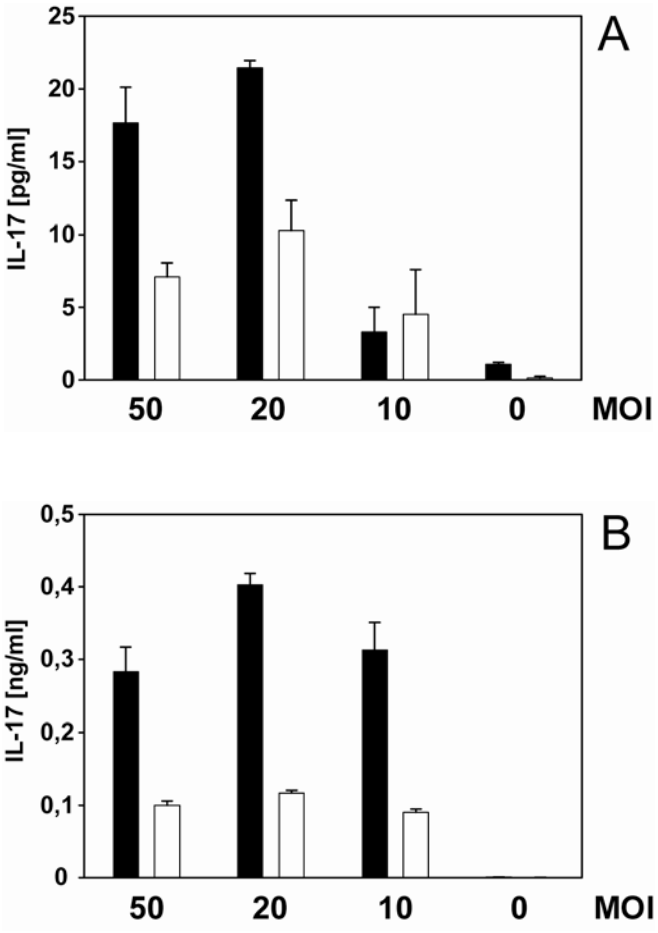


Figure 25: Levels of IL-17 from OT-I (A), OT-II (B) cells co-cultured with BCG-OVA infected WT and hSIGN BMDCs. $1x10^5$ day 7 GMCSF BMDCs were infected with indicated MOI of BCG-OVA. After 14 hr $2x10^5$ OT-I or OT-II cells were added. Co-cultures were incubated for 24 h and SN were taken to assess levels of IL-17 by ELISA.

Taken together, our results demonstrate that M. bovis BCG-infected hSIGN BMDCs secrete decreased levels of IL-12p40 and thereby limit T cell proliferation and restrict the priming of Th1 and Th17 cells.

4.9 Generation of human TLR9 BAC transgenic mice

According to studies in mice, TLR9 ligands represent very potent adjuvants which induce protective Th1 polarized immune responses *in vivo* (Lipford, Sparwasser et al. 2000). However, first human trials could hardly reproduce results from animal experiments (Krieg 2006). Most probably, distinct species specific expression pattern of TLR9 are responsible for this discrepancy. Consequently, since there is a great demand for an improved *in vivo* model to evaluate the potential of huTLR9-targeted adjuvants, this work aimed at generating huTLR9 BAC transgenic mice, termed HUT9 mice.

The gene of huTLR9 contains 5082 bp. Its start codon (635-637) is flanked by two untranslated regions (UTRs: 1-634, 638-1851). The coding sequence consists of 3096 bp (CDS:1852-4947) and is again flanked by a 3′ UTR (4948-5082). For generation of HUT9 mice a BAC clone was chosen that comprised 145 kb 5′ and 50 kb 3′ of the huTLR9 gene. The huTLR9 BAC was linearized by PmeI digest and was purified by agarose gel electrophoresis for microinjection into pronuclei of fertilized C57BL/6 eggs.

By PCR, 42 putative HUT9 founder mice were screened for the presence of the CDS of huTLR9 as well as for 5′ and 3′ sequences. A fragment of the CDS could be amplified in one sample whereas neither 5′ nor 3′ PCRs succeeded in detecting specific fragments as indicated by the positive control (Fig. 26). Thus, we obtained one HUT9 founder carrying at least the CDS of huTLR9.

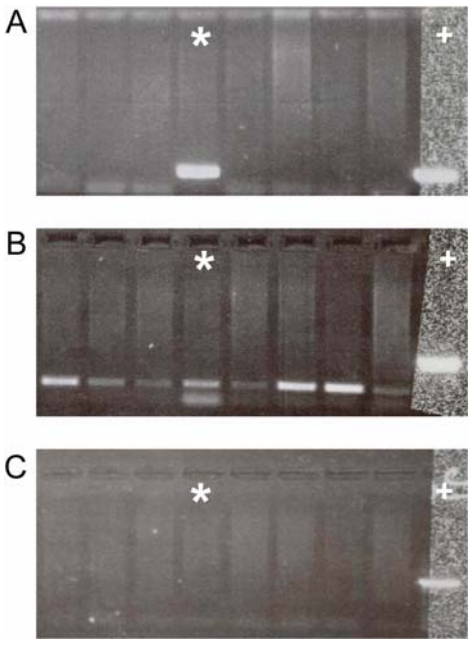


Figure 26: Screening PCRs for putative huTLR9 BAC transgenic founder mice: DNA samples were purified by phenol/chloroform extraction from tails of 42 putative founder mice. Three corresponding sections show results from 8 out of 42 putative huTLR9 transgenic mice. The huTLR9 CDS is successfully amplified in one founder (A *). PCRs for 5′ (B) and 3′ (C) parts of the huTLR9BAC are negative for all samples. As positive control the huTLR9 BAC is included (+)

To study detailed functions of huTLR9 in HUT9 mice, it was necessary to use ligands that specifically ligate the transgene. It has been demonstrated that GT*CG*TT motifs specifically activate huTLR9 whereas GA*CG*TT motifs specifically bind to the murine homologue (Bauer, Kirschning et al. 2001). However, since muTLR9 shares 75,5 % amino-acid identity with huTLR9, human specific ligands still activate muTLR9. In Fig. 27, murine splenocytes and murine FLT3-L-cultured BMDCs were stimulated with equal concentrations of mouse specific B-type CpG-ODN 1826 or human specific B-type CpG-ODN 2006. As expected, CpG-ODN 2006 upregulates CD25 expression on splenic B-cells and CD86 expression on FLT3-L cultured pDCs even though both effects are stronger in response to CpG-ODN 1826.

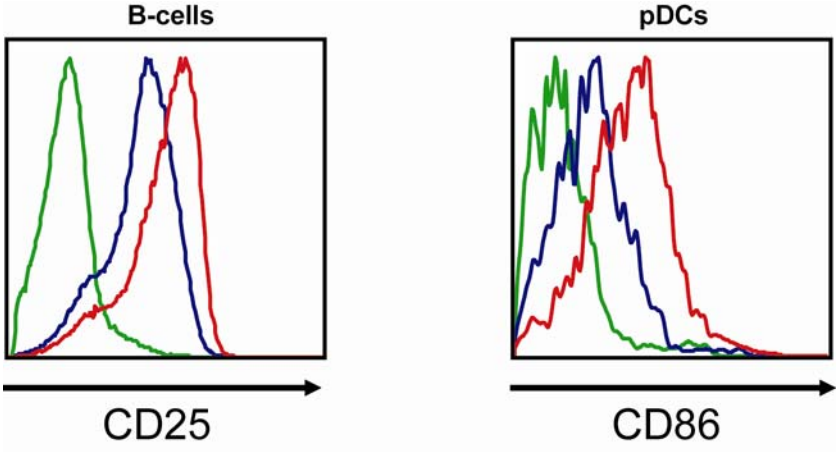


Figure 27: Upregulation of CD25 on murine B-cells and CD86 on murine pDCs in response to murine and human specific CpG-ODNs: *Ex vivo* isolated splenocytes and day 10 FL-BMDCs were stimulated with 500 pmol CpG-ODN 1826 or 500 pmol CpG-ODN 2006. After 14 h splenocytes were stained for B220 and CD25 and FL BMDCs were stained for CD11c, B220 and CD86. Histograms show living cells. Left histogram: expression of CD25 on $B220^+$ splenic B-cells Right histogram: expression of CD86 on $CD11c^+B220^+$ pDCs. CpG-ODN 1826 stimulated cells (red), CpG-ODN 2006 stimulated cells (blue) and unstimulated cells (green).

Due to these overlapping activities of murine and human specific CpG-ODNs, the HUT9 founder was bred to a muTLR9 deficient background to obtain HUT9/muTLR9$^{-/-}$ mice in the F2 generation which provide an optimal genetic background for targeting huTLR9. By PCR, DNA samples from 13 F1 mice were screened for the CDS of huTLR9. As shown in Figure 28, we could detect eight transgenic F1 offspring.

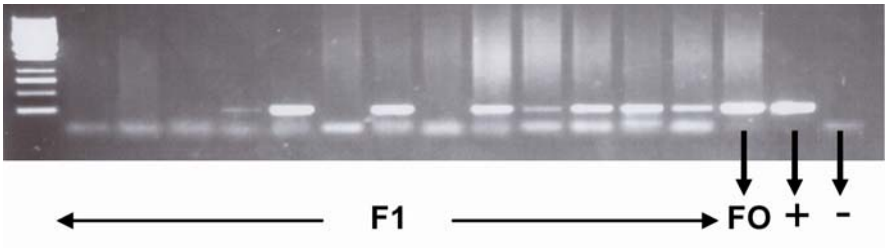


Figure 28: Screening PCR for huTLR9 CDS in F1 mice: DNA samples were purified by phenol/chloroform extraction from tails of F1 mice. Founder DNA (FO) and huTLR9 BAC DNA (+) are included as positive controls. One PCR mix without template is included as negative control (-).

F1 HUT9 mice were again bred with muTLR9 deficient animals. In total, 26 F2 mice were obtained and screened by PCR for the CDS of huTLR9. As shown in Fig. 29 A, eleven F2 mice carried the CDS of huTLR9. In a second PCR, F2 animals were screened for muTLR9 and a third PCR was performed to detect the corresponding muTLR9 knockout allele (Fig. 29 B, C). In total, we obtained five out of 11 HUT9 F2 animals that were deficient for muTLR9.

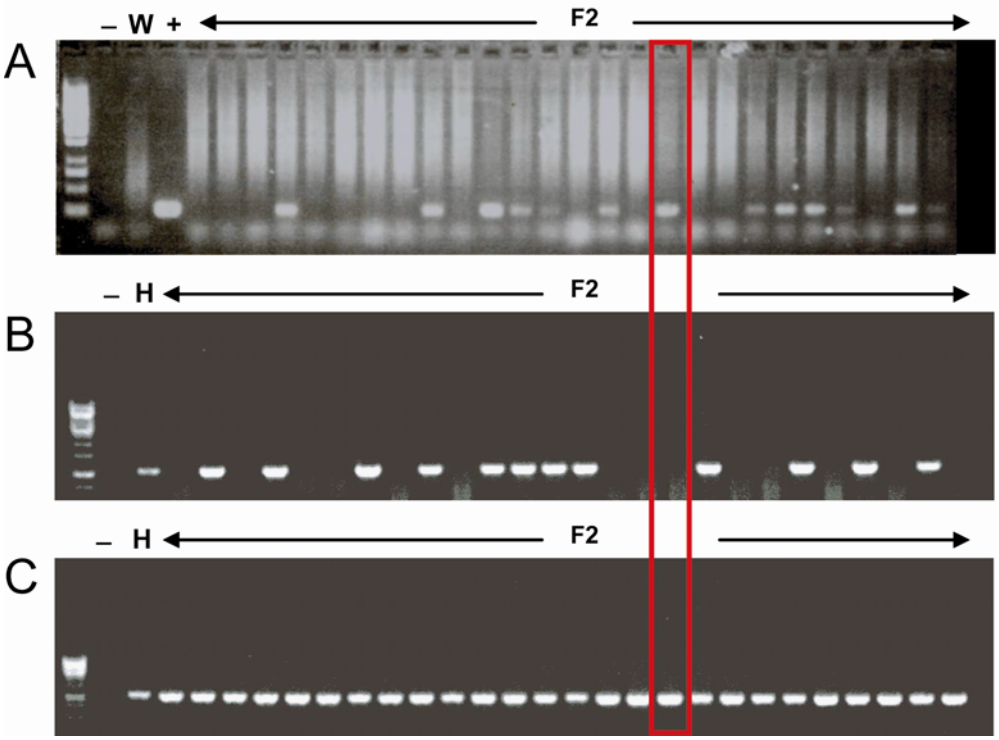


Figure 29: PCRs for huTLR9 BAC (A), muTLR9 (B) and muTLR9 knockout allele (C) in F2 mice. DNA samples of 26 F2 mice were prepared from tails by phenol/chloroform extraction. Controlled by PCR mix without template (-), WT DNA, huTLR9 BAC DNA (+) and DNA of muTLR9 heterozygous mice (H) five F2 mice are huTLR9 BAC transgenic and deficient for muTLR9. One example is indicated in red.

4.9.1 Functional analysis of HUT9 mice

To verify if huTLR9 is functionally expressed in HUT9 mice WT, muTLR9$^{-/-}$ and muTLR9$^{-/-}$/ HUT9 mice were injected with CpG-ODN 2006. After two hours, blood was collected from mice and levels of IL-6 were determined by ELISA. Fig. 30 A shows that WT mice produce higher levels of IL-6 than muTLR9$^{-/-}$ and muTLR9$^{-/-}$/HUT9 animals without a remarkable difference between the two latter genotypes. In addition, spleens were collected from injected mice and *ex vivo* stimulated with CpG-ODN 2006. After 12 h, SN were harvested and analyzed by Elisa for production of IFN-α and IL-6. In line with results from sera, splenocytes from muTLR9$^{-/-}$ mice secrete reduced levels of IFN-α and IL-6 independent from the presence of huTLR9 (Fig. 30 B, C).

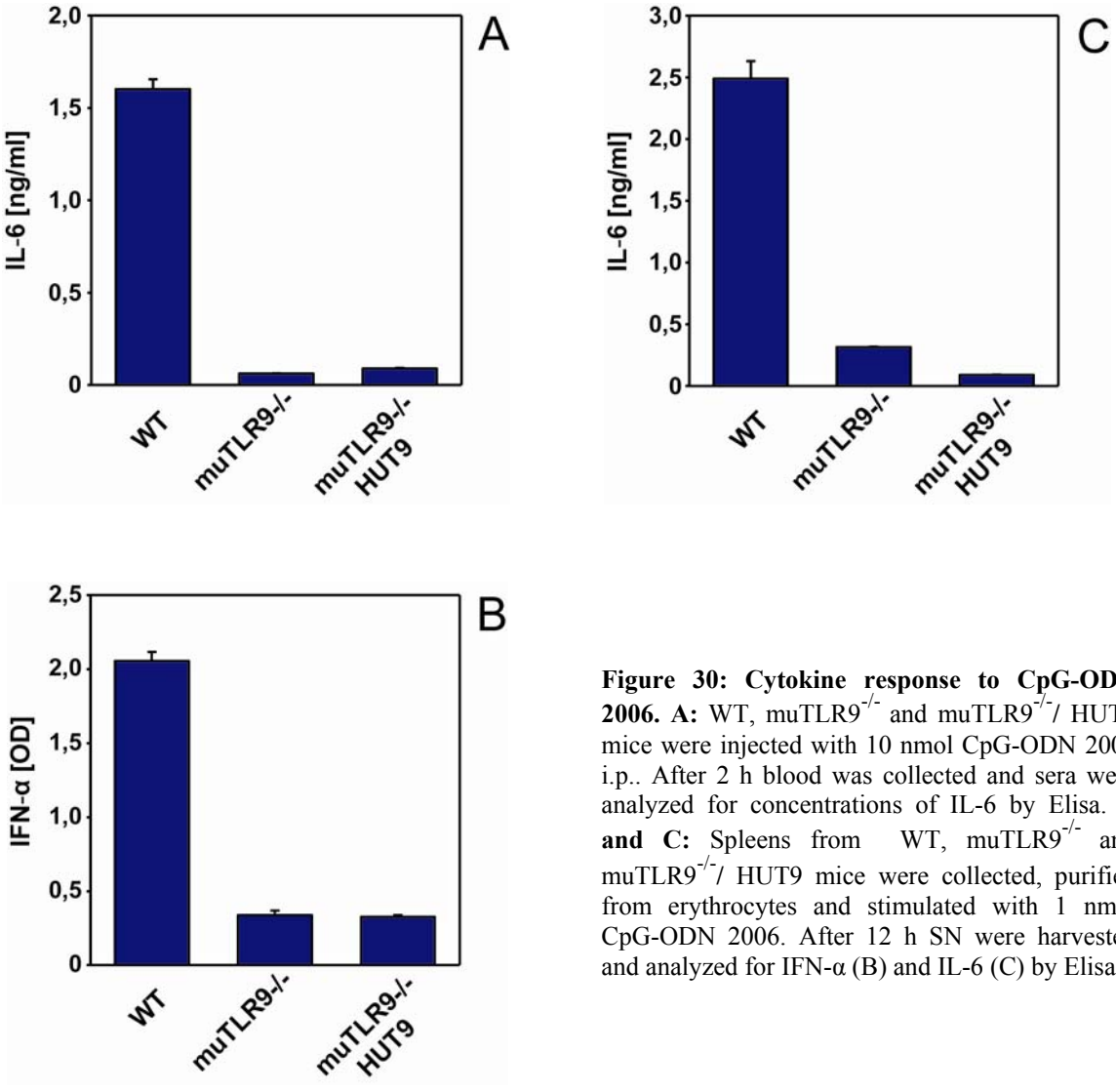


Figure 30: Cytokine response to CpG-ODN 2006. A: WT, muTLR9$^{-/-}$ and muTLR9$^{-/-}$/ HUT9 mice were injected with 10 nmol CpG-ODN 2006 i.p.. After 2 h blood was collected and sera were analyzed for concentrations of IL-6 by Elisa. **B and C:** Spleens from WT, muTLR9$^{-/-}$ and muTLR9$^{-/-}$/ HUT9 mice were collected, purified from erythrocytes and stimulated with 1 nmol CpG-ODN 2006. After 12 h SN were harvested and analyzed for IFN-α (B) and IL-6 (C) by Elisa.

Moreover, *ex vivo* isolated splenocytes and FLT3-L-cultured BMDCs from WT, muTLR9$^{-/-}$ and muTLR9$^{-/-}$/HUT9 mice were stimulated with CpG-ODN 2006. After 12 h, cells were harvested, splenocytes were stained for B220 and CD25 and FLT3-L BMDCs were stained for CD11c, B220 and CD86. By FACS-analysis, we observed that only B220^{+} B-cells and CD11c^{+}B220^{+} pDCs from WT mice upregulate CD25 and CD86 expression, respectively, whereas this effect was not detectable on muTLR9$^{-/-}$ or muTLR9$^{-/-}$/HUT9 cells. (Fig. 31, 32).

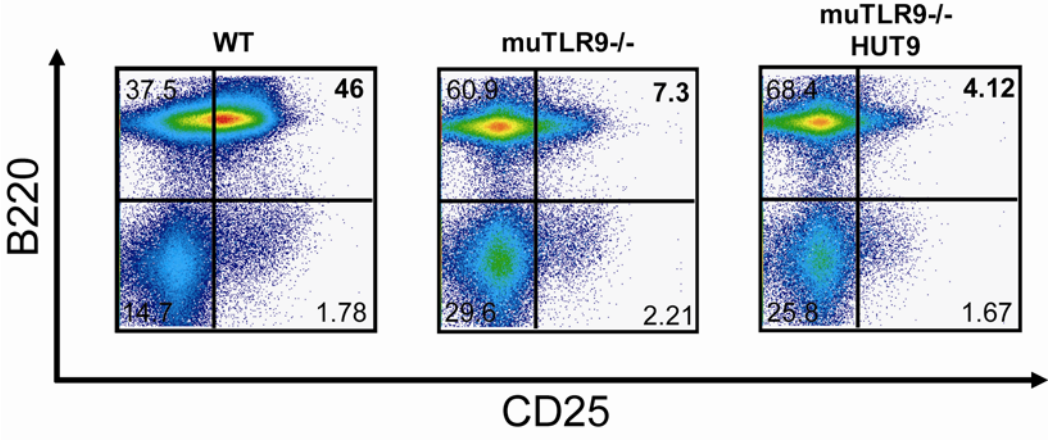


Figure 31: Activation of splenic B220^{+} B-cells in response to CpG-ODN 2006. WT, muTLR9$^{-/-}$ as well as muTLR9$^{-/-}$/HUT9 splenocytes were stimulated *ex vivo* with 1 nmol CpG-ODN 2006. After 12h cells were harvested and stained for B220 and CD25. FACS plots show expression of B220 and CD25 on living splenocytes.

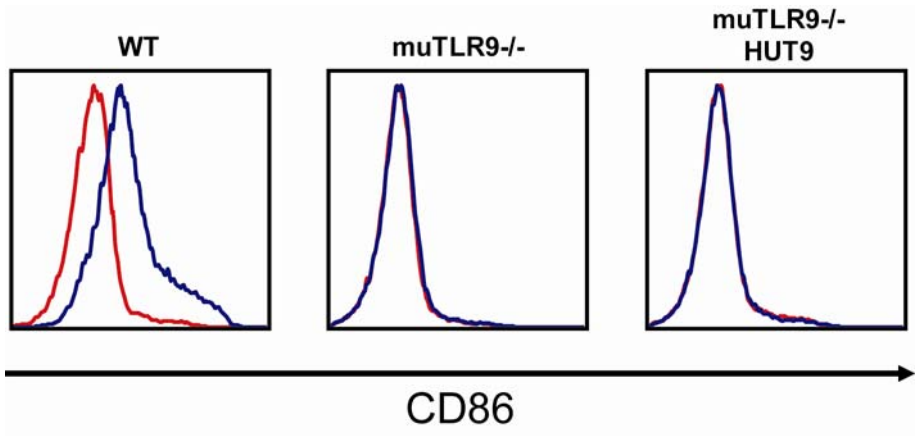


Figure 32: Activation of FLT3-L cultured BMDCs in response to CpG-ODN 2006. WT, muTLR9$^{-/-}$ and muTLR9$^{-/-}$/HUT9 FLT3-L cultured BMDCs were stimulated with 1 nmol CpG-ODN 2006. After 12 h cells were harvested and stained for CD11c, B220 and CD86. Histograms demonstrate CD86 expression on electronically gated living CD11c^{+}B220^{+} pDCs. red histogram: unstimulated control; blue histogram: stimulated cells.

5 Discussion

5.1 hSIGN mice – the first *in vivo* model for DC-SIGN

As key regulators of T- and B-cell immunity, DCs moved into the focus of vaccinology. Compared to other APCs, such as B-cells and macrophages, DCs express 10-100 times more MHC molecules and only one DC is sufficient to activate 100-3000 T cells (Banchereau and Steinman 1998). In line, DCs were also assigned as "nature´s adjuvant" (Austyn 1993). The identification of DC receptors has offered new opportunities to perform highly specific DC targeting strategies. In this context, especially CLRs are suitable targets recognizing glycoproteins through conserved carbohydrate recognition domains (CRD) and routing ligands to antigen presenting pathways (Robinson, Sancho et al. 2006). Accordingly, studies succeeded in protecting mice against tumours and infectious diseases by targeting vaccines to CLRs such as DEC205 and MR (Bonifaz, Bonnyay et al. 2004; He, Crocker et al. 2007).
However, first human trials in this field could hardly reproduce results from animal experiments (Tacken, de Vries et al. 2007). One explanation for this discrepancy is given by species specific expression pattern of PRRs. For example, mice express DEC205 exclusively on DCs whereas human DEC205 is also detectable on monocytes, B-cells and NK-cells (Swiggard, Mirza et al. 1995; Kato, McDonald et al. 2006). By contrast, expression of the human CLR DC-SIGN is restricted to DCs in skin, mucosal tissues and draining lymphnodes (Geijtenbeek, Torensma et al. 2000). Therefore, DC-SIGN represents a very attractive target for DC-based immunotherapy in humans. Although *in vitro* experiments could already confirm this hypothesis by showing that DC-SIGN-targeted vaccines enhance antigen specific T cell responses, evidence from an appropriate *in vivo* model is still lacking (Engering, Geijtenbeek et al. 2002).
Mice express eight homologues of DC-SIGN, termed SIGNR1 – SIGNR8 (Powlesland, Ward et al. 2006). Except SIGNR6 which is probably a pseudogene and SIGNR2 which lacks its membrane anchor, murine homologues encode for transmembrane receptors. The CRDs of SIGNR1, -R3 and -R7 show a preferential binding of fucose over mannose similar to the CRD of DC-SIGN whereas SIGNR5 (mDC-SIGN/CIRE) and SIGNR8 preferentially bind mannose. However, only SIGNR5 is expressed on pDCs and $CD8^-$ DCs while other homologues are detectable at lower levels in various cells according to RT-PCR and Northern blot analysis on RNA level (Caminschi, Lucas et al. 2001; Park, Takahara et al. 2001).

Taken together, none of the murine homologues fulfils function and expression pattern of DC-SIGN and consequently, WT mice cannot serve as a model system to analyze *in vivo* function of DC-SIGN. For this reason a conventional DC-SIGN transgenic mouse model, termed hSIGN mouse, was generated (Schaefer, Reiling et al. 2008). It has been demonstrated that the murine CD11c promoter drives DC-specific expression of transgenes in mice (Jung, Unutmaz et al. 2002). Accordingly, hSIGN mice express DC-SIGN, controlled by the murine CD11c promoter, exclusively on DCs and thereby mimic human specific expression pattern (Figure 7, 8). Thus, hSIGN mice provide for the first time an appropriate model to evaluate the potential of DC-SIGN-targeted vaccines *in vivo*.

Until now, most studies employed antibody-conjugates for in vivo targeting of DC receptors. Since the CRD of DC-SIGN is well characterized, it is also possible to use glycans for targeting antigen to DC-SIGN. Mansour et al. showed that DC-SIGN contributes to uptake and presentation of mannoproteins from *Cryptococcus neoformans* (Mansour, Latz et al. 2006). However, *in vitro* data suggest that the MR binds mannosylated proteins with higher affinity compared to DC-SIGN. By contrast, DC-SIGN surpasses the MR in binding fucosylated proteins and Lewis antigens (Frison, Taylor et al. 2003). Even more important for human immunotherapy is the finding that both DC-SIGN and L-SIGN bind in a similar manner to mannose residues but only DC-SIGN is able to capture fucose containing Lewis antigens such as Lewis X and sulfo Lewis A (Guo, Feinberg et al. 2004).

By *in vitro* experiments we could demonstrate that both Lewis X and sulfo-Lewis A are bound by a higher percentage of hSIGN-derived $CD11c^+$ splenocytes than by corresponding WT cells indicating that murine DC receptors do not exceed the affinity of DC-SIGN for Lewis cluster (Figure 9). Moreover, hSIGN DCs do even process and present ligands similar to human DCs (Singh 2008). Based on this result, each Lewis cluster was chemically conjugated to the model antigen OVA in order to employ the neo-glycoconjugates OVA-Lewis X and OVA-sulfo Lewis A for targeting antigen to DC-SIGN.

5.2 Th1 responses towards DC-SIGN-targeted glycoconjugates

Th1 type responses including $CD8^+$ CTLs, IFN-γ producing $CD4^+$ T cells and corresponding isotypes play an essential role for immune surveillance of tumours and infectious diseases. It has been demonstrated that vaccines targeted to the DC-SIGN-related type II CLRs Dectin-1 and Dectin-2 elicit Th1 responses *in vivo* (Carter, Thompson et al. 2006).

Regarding DC-SIGN, Engering et al. observed that anti-huCD209 antibodies reside in lysosomal compartments of immature DCs whereas mature DCs route DC-SIGN specific antibodies into neutral early endosomes. In line, targeting DC-SIGN specific IgG1 isotypes to immature DCs results in proliferation of IgG1 specific $CD4^+$ T cells (Engering, Geijtenbeek et al. 2002). In addition, DC-SIGN routes HIV virions onto MHC class I complexes (Moris, Nobile et al. 2004). Moreover, *ex vivo* isolated DCs from patients immunized with keyhole limpet hemocyanin (KLH) induce comparable proliferation of autologous peripheral blood lymphocytes (PBL) upon restimulation with KLH or a 100 fold lower concentration of anti-huCD209-KLH conjugate (Tacken, de Vries et al. 2005). Notably, proliferation can be blocked by isotype controls against MHC class I and MHC class II molecules, respectively. Apart from strategies using antibody conjugates, Aarnoudse et al. demonstrated that DC-SIGN facilitates uptake of the highly mannosylated melanoma antigen gp100 and DCs subsequently induce expansion of antigen specific $CD4^+$ T cells (Aarnoudse, Bax et al. 2008).
We observed that hSIGN mice immunized with glycoconjugates, CpG-ODN and alum produce OVA specific $CD8^+$ CTLs, $CD4^+$ T cells, as well as OVA specific antibodies (Figure 11-18). Based on our protocol, we could not detect significant differences between hSIGN and WT animals in regard to production of OVA specific $CD4^+$ T cells and OVA specific IgG2b, IgG2c and IgG1 isotypes (Figure 15, 16). However, individual DC-SIGN transgenic mice produced enhanced frequencies of SIINFEKL specific CTLs (Figure 12). Unfortunately, we could not confirm this result by adoptive transfer experiments with TCR transgenic OT-I cells (Figure 17). Thus, our results indicate that DC-SIGN-targeted glycoconjugates probably enhance antigen uptake and MHC class I presentation *in vivo* but at the same time we have clear indications that other murine receptors compete for uptake of glycoconjugates and thereby diminish DC-SIGN specific effects.
According to the glycan binding assay, such competitive receptors are probably not or at least not only expressed on DCs (Figure 9). The murine macrophage galactose-type C-type lectin 1 (mMGL1) is expressed on immature DCs and on macrophages (Denda-Nagai, Kubota et al. 2002). *In vitro* experiments could show that mMGL1 exhibits a carbohydrate-specificity for Lewis X-soluble conjugated polyacrylamides (Tsuiji, Fujimori et al. 2002). However, it is still unknown if this property reflects the *in vivo* function of mMGL1 and if mMGL1 is able to route ligands to MHC presenting pathways. The MR represents a second receptor which might compete for DC-SIGN-targeted glycoconjugates (Wileman, Lennartz et al. 1986). It traffics soluble OVA exclusively to MHC class I positive early endosomes and routes

mannan, a mannose polymer, onto MHC class II complexes (Sallusto, Cella et al. 1995; Burgdorf, Kautz et al. 2007). The exracellular part of the MR contains one distal cystein-rich (Cys-MR) domain and eight membrane-proximal C-type CRDs (Leteux, Chai et al. 2000). While the former binds sulfated carbohydrates, eight proximal CRDs capture mannose-, N-acetylglucosamine- and fucose-terminating oligosaccharides. According to the structure of sulfo Lewis A and Lewis X it is possible that both glycoconjugates bind to proximal CRDs and OVA-sulfo Lewis A ligates the Cys-MR domain as well. In addition, the MR is not only expressed on DCs but also highly abundant on macrophages. Thus, although DC-SIGN exhibits a higher affinity for Lewis antigens, it is possible that a broader expression pattern of the MR compensates for its reduced specificity. To exclude competitive effects of the MR, hSIGN mice will be crossed onto a MR deficient background. This strategy will allow us to target antigen more specifically to DC-SIGN transgenic DCs *in vivo*.

Pertaining to the amount of applied glycoconjugates, it has been demonstrated that 4 µg of anti-DEC205-OVA conjugate containing approximately 400 ng OVA elicit maximal proliferation of adoptively transfered OT-I cells, whereas 400 µg of OVA are necessary to induce comparable frequencies (Bonifaz, Bonnyay et al. 2002). Since the binding of glycoconjugates can not compete with specificity of anti-huCD209 isotypes, it is possible that the dose of glycoconjugates needs to be increased. In line, it has been demonstrated that DCs require relatively high amounts of antigen to facilitate cross priming of CTLs (Kurts, Miller et al. 1998). Furthermore, Wagner and colleagues demonstrated that conjugation of antigen to TLR9 ligands enhances MHC class I presentation and competes with the efficacy of ´live´ vaccines (Wagner, Heit et al. 2004). Consequently, it could be possible to improve OVA specific CTL responses in hSIGN mice by applying glycoconjugates coupled to CpG-ODNs. Finally, if discussed improvements are not sufficient to enhance Th1 responses in hSIGN mice, we will employ DC-SIGN specific antibody conjugates, a strategy which has succeeded in targeting antigen to several CLRs.

5.3 Th2 responses towards DC-SIGN-targeted glycoconjugates

Upon binding through DC-SIGN to Lewis cluster from H. pylori or from eggs of S. mansoni, DCs promote Th2 priming of $CD4^+$ T cells (Bergman, Engering et al. 2004; van Liempt, van Vliet et al. 2007). According to these *in vitro* results, immune responses towards DC-SIGN-targeted glycoconjugates might become more evident in a Th2 model. It has been

demonstrated that mice sensitized with OVA plus alum and challenged with inhalative OVA aerosol develop Th2 polarized OVA specific T cell and isotype responses causing airway inflammation similar to the pathology of allergic asthma (Brusselle, Kips et al. 1994). Antigen uptake via DC receptors plays most probably an important role for the etiology of asthma since *in vivo* depletion of lung $CD11c^+$ DCs during the challenge phase prevents asthmatic features in mice (van Rijt, Jung et al. 2005).

DC-SIGN transgenic mice which were sensitized with glycoconjugates and challenged with OVA produce slightly decreased levels of OVA specific IgG2a isotypes compared to hSIGN mice sensitized and challenged with OVA indicating that glycoconjugates support Th2 responses (Figure 19). However, OVA-Lewis X-sensitized WT animals show the same trend and produce even slightly elevated levels of OVA specific IgG1 and IgE isotypes. On the one hand, this result indicates that additional receptors compete for uptake of glycoconjugates. Notably, the Th2 cytokine IL-4 upregulates more than 10-fold surface expression and 15-fold activity of the murine MR on peritoneal macrophages and concomitantly enhances MHC class II expression (Stein, Keshav et al. 1992). Therefore, even this model can be improved by using hSIGN mice on a MR deficient background. On the other hand, it needs to be considered that even DC-SIGN ligands without Lewis cluster promote Th2 responses. Shreffler et al. demonstrated that the glycoallergen Ara h 1 from Arachis hypogaea containing xylosylated glycans acts as a Th2 adjuvant by ligating DC-SIGN (Shreffler, Castro et al. 2006). Moreover, ManLam from M. tuberculosis engages DC-SIGN for enhancing IL-10 release in the presence of TLR signalling. Thus, Th2 responses towards DC-SIGN ligands probably require ligation and activation of additional PRRs. Data from Eisenbarth et al. show that low dose inhalative LPS enhances Th2 responses. (Eisenbarth, Piggott et al. 2002). If this effect can reveal unique Th2 responses towards DC-SIGN-targeted glycoconjugates needs to be evaluated in future experiments.

5.4 Tolerogenic responses towards DC-SIGN-targeted glycoconjugates

During steady state, immature DCs (iDCs), also referred to as tolerogenic DCs, take up, process and present self as well as harmless non-self antigens to T cells. However, since iDCs express low levels of MHC class II and co-stimulatory molecules, T cells do not receive adequate signals for activation and proliferation (Shortman and Naik 2007). In contrast, iDCs induce and maintain T cell anergy or even delete auto-reactive T cells by

expressing indoleamine 2,3-dioxygenase (IDO) and inhibitory molecules such as programmed cell death 1 ligand (PDL1), CD95 ligand (CD95L) and TNF-related apoptosis-inducing ligand (TRAIL) (Morelli and Thomson 2007). Moreover, by interacting through CD80/86 with cytotoxic T-lymphocyte antigen 4 (CTLA4) and by secreting IL-10 and TGF-ß, iDCs promote expansion and *de novo* generation of adaptive Treg cells. In line with these properties, it has been proven that tolerogenic DCs are able to suppress autoimmunity and to prolong allograft survival (Fu, Li et al. 1996; Verginis, Li et al. 2005). Until now, most studies aimed to induce tolerogenic responses *in vivo* by transferring *in vitro* cultured iDCs or by applying immunosuppressive drugs such as corticosteroids, cyclosporine, rapamycin, deoxyspergualin (DSG) or mycophenolate mofetil (MMF) preventing DC maturation and/or activation. In a different approach, Bonifaz et al. demonstrated that upon application of anti-DEC205-OVA conjugate, OT-I cells expand independently from the presence or absence of anti-CD40. However, OT-I cells induced by anti-DEC205-OVA and anti-CD40 produce enhanced levels of IL-2 and IFN-γ. Moreover, only OT-I cells primed by mature DCs respond to a challenge with OVA in complete Freund´s adjuvant indicating that DEC205-targeted vaccines induce and maintain T cell anergy during steady state (Bonifaz, Bonnyay et al. 2002). In analogous experiments, Hawiger et al. showed that this strategy even succeeds in inducing peripheral $CD4^+$ T cell tolerance (Hawiger, Inaba et al. 2001). The fact that DC-SIGN is highly abundant on iDCs suggests that DC-SIGN-targeted vaccines promote tolerogenic T cell responses as well (Geijtenbeek, Torensma et al. 2000). This hypothesis is strengthened by the finding that commensal microbes selectively target DC-SIGN on iDCs to prime IL-10 producing T cells which suppress proliferation of effector cells (Smits, Engering et al. 2005). Similar to DEC205 targeting, OVA-Lewis X was applied in the absence or presence of TLR9 ligands to double transgenic DEREG/OT-I and triple transgenic hSIGN/DEREG/OT-I mice and subsequently, all mice were challenged with OVA and CpG-ODNs. According to data from von Boehmer and colleagues, especially low antigen doses do efficiently induce antigen specific tolerance (Kretschmer, Apostolou et al. 2005). Therefore, mice were primarily immunized with only 5 µg OVA-Lewis X. Upon immunization with OVA-Lewis X in the absence of CpG-ODNs, triple and double transgenic mice produced equal frequencies of SIINFEKL specific effector T cells ($SIINFEKL^+GFP^-$ cells) (Figure 20). However, it is possible that slight changes occurred in secondary lymphoid organs such as spleen and lymphnodes and escaped from analysis of peripheral blood. Interestingly, the same population of both strains showed no response to a challenge with OVA and CpG-ODNs

indicating that OVA-Lewis X induces T cell anergy during steady state independent from the presence of DC-SIGN. Furthermore, we could detect an increase of $SIINFEKL^{+}GFP^{+}$ cells in DEREG/OT-I mice and a slight expansion of $SIINFEKL^{-}GFP^{+}$ cells in both strains. Strikingly, upon immunization with OVA-Lewis X and CpG-ODNs, $SIINFEKL^{+}GFP^{-}$ cells dramatically decreased in 2 out of 3 hSIGN/DEREG/OT-I mice whereas SIINFEKL specific effector cells did not change in corresponding DEREG/OT-I mice. In this context, Mellor et al. showed that a subpopulation of $CD11c^{+}CD19^{+}$ splenic DCs upregulates indoleamine 2,3-dioxygenase expression in response to CpG-ODNs and thereby acquires a T cell suppressive function indicating that activation of TLR9 includes not only immune stimulatory but also immune regulatory effects (Mellor, Baban et al. 2005). Since we could not detect an expansion of corresponding $SIINFEKL^{+}GFP^{+}$ or $SIINFEKL^{-}GFP^{+}$ cells, this result suggests that DC-SIGN transgenic DCs may suppress or even delete antigen specific effector T cells if targeted by glycoconjugates in the presence of CpG-ODNs. In further experiments we will analyze functional aspects of described populations and we will employ MR deficient animals to confirm these preliminary data.

5.5 DC-SIGN in mycobacterial infection

The understanding of infection biology is a fundamental requirement for development of novel vaccines. In this context, we focused on the interaction between M. tuberculosis (Mtb) and DC-SIGN representing the major receptor for Mtb on DCs (Tailleux, Schwartz et al. 2003). Geijtenbeek et al. demonstrated that DC-SIGN-bound ManLam inhibits DC maturation and enhances IL-10 production (Geijtenbeek, Van Vliet et al. 2003). Furthermore, it has been shown that this process requires recruitment of Src and Pak kinases to DC-SIGN which subsequently activate Raf-1 kinase by phosphorylating S338, Y340 and Y341 residues (Gringhuis, den Dunnen et al. 2007). Consistent with these data, we observed that hSIGN DCs show an enhanced phosphorylation of Raf-1 kinase in response to M. bovis BCG indicating that upstream signalling pathways are identical to human DCs (Figure 21). To what extent downstream pathways, including intranuclear phosphorylation and acetylation of p65 match between human and hSIGN DCs needs to be investigated in future studies. Nevertheless, this result confirms that hSIGN mice represent an appropriate model to study the *in vivo* function of DC-SIGN during mycobacterial infection.

Since neutralization of IL-10 increases resistance to mycobacterial infection and blockade of

the IL-10 receptor improves vaccination and anti-mycobacterial chemotherapy, it has been hypothesized that DC-SIGN serves as an immune escape mechanism for the pathogen (Denis and Ghadirian 1993; Silva, Pais et al. 2001; van Kooyk and Geijtenbeek 2003). Interestingly, Mtb-infected hSIGN DCs do not secrete increased levels of IL-10 but instead produce decreased levels of IL-12p40 (Schaefer, Reiling et al. 2008). However, since IL-10 and IL-12 antagonize each other by inhibiting or rather supporting Th1 responses, hSIGN DCs do still mimic human DC physiology. Surprisingly, DC-SIGN transgenic mice show a prolonged survival and a decreased histopathology during mycobacterial infection. This result confirms epidemiological data demonstrating a positive correlation between an increased expression of DC-SIGN and an improved infection status of Tbc patients (Barreiro, Neyrolles et al. 2006). Together these results clearly indicate that DC-SIGN plays rather a protective role for the host than for the pathogen. Therefore, we hypothesized that DC-SIGN modulates T cell responses during mycobacterial infection and thereby provides an optimal anti-microbial immune response without endangering self-integrity. By *in vitro* experiments, we analyzed the priming of naïve OT-I, OT-II and WT $CD4^+$ T cells in the presence of BCG-OVA-infected WT or hSIGN BMDCs. The measurement of IL-12p40 from co-cultures confirmed that BCG-OVA-infected hSIGN BMDCs produce significantly decreased levels of IL-12p40 (Figure 22). In line, it has been demonstrated that IL-12p40-transgenic mice which produce 14 fold higher amounts of IL-12p70 have neither an improved granuloma function nor a decreased mycobacterial load (Olleros, Vesin et al. 2007). Although the absence of IL-12p40 makes mice highly susceptible towards mycobacterial infection, our results indicate that it is even necessary to limit IL-12p40 production (Cooper, Kipnis et al. 2002). Regarding the role of IL-2 during Mtb infection, it has been reported that IL-2/IFN-γ-dual secreting cells are highly abundant during the active state whereas additional IL-2-only secreting cells become induced during the inactive state (Millington, Innes et al. 2007). This kinetic suggests that IL-2 plays an important role for control of mycobacterial growth. However, patients suffering from pulmonary tuberculosis did not benefit from co-administration of IL-2 with standard chemotherapy in a randomised trial (Johnson, Ssekasanvu et al. 2003). We observed that T cells in co-culture with BCG-OVA-infected hSIGN BMDCs secrete reduced levels of IL-2 indicating that mice benefit from decreased T cell proliferation during Mtb infection (Figure 23). As a proof of antigen specificity, OT-II cells produce increased levels of IL-2 compared to WT $CD4^+$ T cells. Similar to IL-12p40, also IFN-γ deficient animals are highly susceptible towards mycobacterial infection. By contrast, genetically manipulated mice that produce

elevated levels of IL-12p40 and IFN-γ die from hyper-inflammatory immune responses during mycobacterial infection (Holscher, Holscher et al. 2005). Correspondingly, T cells co-cultured with BCG-OVA-infected hSIGN BMDCs secrete decreased levels of IFN-γ (Figure 24). The fact that OT-II cells release higher amounts of IFN-γ than OT-I cells confirms results from Ladel et al. demonstrating that MHC class II deficient A beta$^{-/-}$ mice are not able to produce IFN-γ and succumb to M. bovis BCG infection whereas MHC class I deficient ß2-microglobulin$^{-/-}$ mice are protected by high amounts of IFN-γ (Ladel, Daugelat et al. 1995). Moreover, *in vitro* depletion of human CD4^{+} T cells but not CD8^{+} T cells prior to stimulation with Mtb abrogates IFN-γ production (Shams, Wizel et al. 2001).

Th17 cells represent a further inflammatory subset that becomes induced during mycobacterial infection (Khader and Cooper 2008). Cua and colleagues showed that TGF-ß and IL-6 drive the initial priming of Th17 cells whereas IL-23 promotes pathogenicity of this subset (McGeachy, Bak-Jensen et al. 2007). More recently, it has been demonstrated that IL-23 is even sufficient to prime human Th17 cells in the presence of TGF-ß (Volpe, Servant et al. 2008). Interestingly, the absence IL-23p19 does not increase susceptibility of mice towards mycobacteria (Chackerian, Chen et al. 2006). However, vaccinated mice which lack IL-17 show an impaired Th1 memory response upon mycobacterial infection (Khader, Bell et al. 2007). We observed that BCG-OVA-infected hSIGN as well as WT BMDCs induce IL-17 production in OT-I and OT-II cells (Figure 25). The fact that T cells secrete decreased levels of IL-17 in the presence of infected hSIGN BMDCs indicates that IL-23 contributes to the priming of CD4^{+} Th17 and IL-17 producing CD8^{+} T cells. Furthermore, this result implicates that decreased frequencies of both subsets improve survival of hSIGN mice during mycobacterial infection. Therefore, we propose that DC-SIGN protects the host from overwhelming hyper-inflammatory responses during mycobacterial infection by balancing Th1 as well as Th17 responses. Moreover, this result allows to speculate that DC-SIGN-targeted vaccines might have a potential to treat exacerbated infectious as well as non-infectious immune disorders caused by an inadequate activation of Th1 and/or Th17 cells.

5.6 Generation of huTLR9 BAC transgenic mice

In 1984, Tokunaga et al. observed that mice mount significant anti-tumour responses upon treatment with a fraction of 97 % ss DNA isolated from M. bovis BCG (Tokunaga, Yamamoto et al. 1984). Moreover, it has been demonstrated that CpG-ODNs activate DCs by upregulating CD80 and CD86 expression and by inducing Th1 associated cytokines such as IL-12 (Sparwasser, Koch et al. 1998). On the one hand, this property has been successfully utilized to induce protective antigen specific Th1 responses against tumours and viral infections while on the other hand, high doses of CpG-rich bacterial DNA cause septic shock and inhibitory CpG sequences can again rescue mice from polymicrobial sepsis (Sparwasser, Miethke et al. 1997; Weiner, Liu et al. 1997; Vabulas, Pircher et al. 2000; Plitas, Burt et al. 2008). Furthermore, it has been demonstrated that co-administration of antigen and CpG-ODNs prevents development of Th2 type responses in a murine model of asthma (Kline, Waldschmidt et al. 1998).

Based on these clear results from animal experiments, CpG-ODNs became introduced into first basic human studies. In a Phase II randomized controlled trial, chemotherapy-naïve patients suffering from unresectable non-small cell lung cancer received either standard chemotherapy alone or in combination with a B-type CpG-ODN (Manegold 2005). According to the Response Evaluation Criteria in Solid Tumours (RECIST), patients developed a significant improved response rate if treated with CpG-ODNs. However, there was no significant difference in terms of survival. During a phase Ib trial, 60 HCV-infected patients were treated with C-class CpG ODNs as a mono-therapy and showed a dose dependent decrease in blood viral RNA levels (McHutchison, Bacon et al. 2007). In terms of vaccination, a phase I/II study demonstrated that addition of K/B-type CpG-ODNs to an alum-absorbed HBV vaccine results in a faster and enhanced production of antigen specific antibodies (Cooper, Davis et al. 2004). Furthermore, CpG-ODNs were also tested in a placebo-controlled phase II trial which included 25 patients suffering from allergy to ragweed-pollen antigen AMB a 1 (Creticos, Schroeder et al. 2006). Patients treated with AMB a 1 conjugated to phosphorothioate CpG-ODN yielded an improved life quality score and showed significant fewer symptoms such as rhinitis. In addition, CpG-ODN treated patients switched from AMB a 1 specific IgE to corresponding IgG isotypes and showed reduced numbers of IL-4-positive basophils in peripheral blood.

Taken together, human trials could partially reproduce results from mouse experiments but

have not yet succeeded in establishing safe indications for the employment of TLR9 ligands in human immunotherapy. In addition, it has to be considered that studies included only small numbers of patients who usually suffered from severe and advanced diseases. Moreover, observation periods took at maximum six months and thereby limit information about long term effects of huTLR9 ligands. This again is of particular interest to assess the risk for autoimmune diseases such as lupus erythematosus which is associated with mutations in the TLR9 signalling pathway (Marshak-Rothstein 2006).

A possible reason for divergent results in mice and men is the fact that huTLR9 is only expressed on pDCs and B-cells whereas muTLR9 is also detectable on cDCs. Consequently, TLR9 ligands induce species specific cytokine pattern promoting distinct immune responses. In order to verify the therapeutic potential and risk of huTLR9-targeted adjuvants without endangering the health of individuals, we generated huTLR9 BAC transgenic (HUT9) mice. BAC technology allows introduction of large DNA fragments containing 100-300 kb which provide a high probability for faithful and specific expression of transgenes due to inclusion of all important epigenetic elements (Heintz 2001; Sparwasser and Eberl 2007). In case of the gene encoding murine CD4, enhancer elements are separated by 24 kb from the transcriptional start site (Ellmeier, Sawada et al. 1999). To ensure the presence of all regulatory elements required for huTLR9 expression, a BAC clone was injected that comprised 201.393 bp with 145.023 bp 5′ and 51.288 bp 3′ of the huTLR9 CDS (5082 bp). By PCR we could detect the CDS of huTLR9 in one out of 42 offspring whereas we did not succeed in amplifying 5′ (101.901-102.769 kb) and 3′ (153.267-154.043) fragments of the huTLR9 BAC (Figure 26). Thus, although it could not be excluded that the huTLR9 BAC became damaged during microinjection, at least one HUT9 founder carried the CDS of huTLR9 flanked by at maximum 100 kb 5′ and 3 kb 3′. Takeshita et al. showed that the 5′ flanking region of huTLR9 including 3.227 bp enhances more than 15 fold the expression of huTLR9 and even deletion of the 5′end from 3227 - 700 bp does not alter promoter activity (Takeshita, Suzuki et al. 2004). According to these results, HUT9 mice had still a high probability to express huTLR9. Since huTLR9 and muTLR9 possess overlapping ligand specificities, it was necessary to cross HUT9 mice onto a muTLR9 deficient background (Figure 27). Here, we observed that the huTLR9 BAC was transmitted in a mendelian ratio to 61.5 % of F1 mice and to 42 % of F2 mice demonstrating that it was integrated in the murine genome (Figure 28, 29). Finally, HUT9/muTLR9$^{-/-}$ mice provided an optimal model to target huTLR9 *in vivo*. Upon injection of human specific CpG-ODN 2006 both muTLR9$^{-/-}$ and

HUT9/muTLR9$^{-/-}$ mice produced dramatically decreased levels of IL-6 compared to WT animals indicating that at most very low levels of huTLR9 were expressed (Figure 30A). Therefore, we increased sensitivity by analyzing responsiveness of *ex vivo* isolated B-cells and FLT3-L cultured BMDCs. However, even in this approach we could not observe differences between cells from muTLR9$^{-/-}$ and HUT9/muTLR9$^{-/-}$ with respect to cytokine production and upregulation of co-stimulatory molecules (Figure 30-32). Thus, we concluded that huTLR9 was not expressed or at least not functional in HUT9 mice. It has been demonstrated that the 5´ upstream region from 700 - 68 bp of huTLR9 contains several cis-acting elements including CRE, 5´-PU box, 3´-PU box and a C/EBP site that synergistically regulate huTLR9 promoter activity by interacting with corresponding CREB1, Ets2, Elf1, Elk1 and C/EBPα trans-activating elements (Takeshita, Suzuki et al. 2004). A deletion of this sequence results in more than 80 % reduction of huTLR9 expression. Therfore, it is reasonable that a dissection of this region caused the loss of regulatory elements in HUT9 mice. Furthermore, it is still possible that despite a successful transcription of huTLR9, mice lack appropriate factors regulating translation, protein editing and endosomal trafficking of the transgene. Currently, offspring from a new huTLR9 BAC injection were analyzed and we could detect 5´ as well as 3´ sequences indicating that the BAC is intact and transgenic mice probably express huTLR9.

6 Literature

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8 Zusammenfassung

Aktuell ist circa ein Drittel der Weltbevölkerung mit Mycobacterium tuberculosis (Mtb), dem Erreger der Tuberkulose infiziert, und mehr als 40 Millionen Menschen sind als HIV-positiv registriert. Allerdings ist es trotz der erfolgreichen Entwicklung von Vakzinen gegen letale Infektionserkrankungen wie Diphtherie und Polio bislang nicht gelungen, Impfstoffe zur Prävention von HIV oder Tuberkulose herzustellen. Nach heutigem Wissensstand spielen dendritische Zellen (DZ) eine Schlüsselrolle für die Initiation von Immunantworten. Über spezifische Rezeptoren können DZ Antigene erkennen, aufnehmen und mittels MHC-Molekülen T-Zellen präsentieren. Eine besondere Rolle spielen hierbei Rezeptoren der PRR (pathogen recognition receptors)-Familie, die charakteristische Motive von Mikroorganismen detektieren. Die Ligation von PRR kann sowohl zur Aktivierung von DZ führen als auch eine spezifische Antigenaufnahme vermitteln. Letztere Eigenschaft zeichnet insbesondere die PRR-Subfamilie der C-Typ-Lektin-Rezeptoren (CLR) aus. Hierbei handelt es sich überwiegend um Transmembran-Rezeptoren, wie DEC205 und DC-SIGN, die spezifische Glykolisierungsmuster erkennen und konsekutiv Antigen internalisieren. Im Mausmodell wurde gezeigt, dass die Antigenpräsentation durch Kopplung von Antigen an DEC205-Antikörper bis zu 1000-fach gesteigert werden kann. Bei gleichzeitiger DZ-Aktivierung können reife DZ verstärkte antigenspezifische Immunantworten generieren, während unreife DZ bei Aufnahme von DEC205-Liganden antigenspezifische Toleranz induzieren. In diesem Zusammenhang deuten *in vitro* Ergebnisse darauf hin, dass auch DC-SIGN über ähnliche Eigenschaften verfügt. Allerdings exprimieren Mäuse acht DC-SIGN Homologe (SIGNR1 – SIGNR8), die im Vergleich zu DC-SIGN unterschiedliche Liganden-Spezifitäten, Funktionsmotive und Expressionsmuster aufweisen. Um dennoch grundlegende Funktionen von DC-SIGN *in vivo* zu studieren, wurde ein konventionell transgenes Mausmodell, genannt hSIGN-Maus, generiert. Unter Anwendung des murinen CD11c-Promotors wird DC-SIGN, analog zum humanen Expressionsmuster, fast ausschließlich auf konventionellen DZ von hSIGN-Mäusen exprimiert. Somit bietet die hSIGN-Maus ein geeignetes Modell, um die Anwendung von DC-SIGN-basierten Vakzinen *in vivo* zu untersuchen.

Im Unterschied zu bisherigen Studien wurden in dieser Arbeit nicht Antikörper-Konjugate verwendet, sondern Glykokonjugate bestehend aus dem Modellantigen Ovalbumin (OVA) und den kovalent gebundenen Oligosacchariden Lewis X bzw. sulfo Lewis A. *In vitro* Experimente haben gezeigt, dass beide Lewis-Cluster eine erhöhte Affinität zu DC-SIGN-

transgenen DZ aufweisen. Folglich sollten OVA-spezifische Immunantworten in Glykokonjugat-vakzinierten hSIGN-Mäusen darüber Aufschluss geben, in welcher Art und in welcher Stärke adaptive Immunantworten mittels einer DC-SIGN-spezifischen Vakzine moduliert werden können. In einem ersten Vakzinierungsprotokoll wurden OVA-spezifische Th1-Antworten in hSIGN-Mäusen analysiert, die mit Glykokonjugaten und den Adjuvanzien CpG-ODN 1826 und Aluminiumhydroxid immunisiert worden waren. Während bei der Analyse OVA-spezifischer Antikörper sowie OVA-spezifischer $CD4^+$ T-Zell-Antworten keine Unterschiede zwischen vakzinierten hSIGN- und WT-Mäusen detektierbar waren, konnten jedoch in einzelnen Glykokonjugat-vakzinierten transgenen Mäusen erhöhte Frequenzen OVA-spezifischer $CD8^+$ T-Zellen nachgewiesen werden. Die Tatsache, dass es uns mittels TCR-transgener Systeme nicht gelang, diese Einzelresultate zu bestätigen, führte zu der Schlussfolgerung, dass sehr wahrscheinlich murine Rezeptoren an der Aufnahme von Glykokonjugaten beteiligt sind. Diese Annahme wurde auch durch die Untersuchung von Th2-Antworten und die Analyse regulatorischer T-Zell-Antworten bestätigt. In diesem Zusammenhang spielt möglicherweise der Mannose-Rezeptor (MR) eine entscheidende Rolle, da er als CLR mit sehr hoher Effizienz die Aufnahme von Glykoproteinen vermittelt und neben DZ auch auf Makrophagen exprimiert wird. Daher wird aktuell durch Kreuzung von hSIGN-Mäusen mit MR-defizienten Tieren das DC-SIGN transgene Mausmodell optimiert.

Neben Lewis-Antigenen ist auch der Mtb Zellwandbestandteil mannosyliertes Lipoarabinomannan (ManLam) ein spezifischer Ligand von DC-SIGN. Es wurde gezeigt, dass die Bindung von ManLam an DC-SIGN zu einer Aktivierung des Ras/Raf-1-Signalweges führt, der bei simultaner Aktivierung von Toll-like-Rezeptoren (TLR) eine verstärkte Produktion von Interleukin 10 (IL-10) induziert. In dieser Arbeit konnte gezeigt werden, dass die Infektion von hSIGN-DZ mit M. bovis BCG ebenfalls eine Aktivierung der Raf-1 Kinase bewirkt. Dieses Ergebnis verdeutlicht, dass hSIGN-Mäuse ein adäquates Modell zur Untersuchung der *in vivo* Funktion von DC-SIGN darstellen. Im Gegensatz zu humanen DZ sezernieren Mtb-infizierte hSIGN-DZ jedoch nicht vermehrt IL-10, sondern verminderte Mengen IL-12. Die Tatsache, dass sich IL-10 und IL-12 funktionell antagonisieren, zeigt, dass hSIGN-Mäuse dennoch die biologische Funktion humaner DZ reflektieren. Da IL-10 Th1-Antworten inhibiert, die wiederum entscheidend zur Kontrolle der Tbc Infektion beitragen, wurde die Hypothese erstellt, dass DC-SIGN eine Immunevasion von Mtb begünstigt. Erstaunlicherweise zeigen jedoch Mtb-infizierte hSIGN-Mäuse ein verlängertes Überleben bei vermindertem Gewebsschaden und folglich scheint DC-SIGN

eine Protektion des Wirtsorganismus durch Restriktion hyper-inflammatorischer T-Zell-Antworten zu vermitteln. Zur Verifizierung dieser Theorie wurde das Aktivierungsprofil naiver T-Zellen in Gegenwart von M. bovis BCG-infizierten hSIGN-DZ analysiert. Hierbei konnte gezeigt werden, dass T-Zellen, die mit infizierten DC-SIGN-transgenen DZ kultiviert wurden, vermindert IL-2, IFN-γ und IL-17 produzieren im Vergleich zu T-Zellen in Gegenwart von infizierten WT-DZ. Dieses Ergebnis impliziert, dass Vakzine, die in analoger Weise DC-SIGN aktivieren, sowohl zur Therapie der Tbc als auch zur Therapie chronisch entzündlicher Erkrankungen eingesetzt werden können.

Ebenso wie DC-SIGN zählt auch Toll-like-Rezepor 9 (TLR9) zu den PRR. Im Gegensatz zu DC-SIGN vermittelt TLR9 keine Antigenaufnahme, sondern detektiert als endosomaler Transmembranrezeptor bakterielle unmethylierte DNS. Im humanen Immunsystem wird TLR9 auf plasmazytoiden DZs (pDZ) und B-Zellen exprimiert. Die Ligation von TLR9 bewirkt eine Aktivierung von DZ und die Sekretion pro-inflammatorischer Zytokine mit konsekutiver Induktion von Th1-Antworten. Bislang ist es nur im Mausmodell gelungen, diese Funktion zu nutzen, um protektive Th1-Antworten gegen Krebs- und Infektionserkrankungen zu generieren. Eine wesentliche Ursache für die unzureichende Reproduzierbarkeit dieser Ergebnisse in humanen Studien ist darauf zurückzuführen, dass der murine TLR9 (muTLR9) nicht nur auf pDZ und B-Zellen, sondern auch auf konventionellen DZ (kDZ) exprimiert wird. Aus diesem Grund wurde unter Anwendung der bacterial artificial chromosome (BAC) Technologie ein transgenes Mausmodell für den humanen TLR9 (huTLR9) generiert, genannt HUT9-Maus. Ziel war es, das humane Expressionsmuster von TLR9 in HUT9-Mäusen zu imitieren. Um Interferenzen mit dem murinen Homolog auszuschließen, wurden HUT9-Mäuse mit muTLR9-defizienten Tieren verpaart.

Es wurde festgestellt, dass weder die *in vivo*-Applikation von huTLR9-Liganden noch die *in vitro* Stimulation von HUT9 B-Zellen und pDZ mit huTLR9-Liganden eine signifikante Zytokinantwort oder Zellaktivierung bewirken konnte. Demnach war anzunehmen, dass HUT9-Mäuse das Transgen nicht exprimierten oder zusätzlich funktionell relevante Elemente fehlten. Ursächlich hierfür kann eine Schädigung des BAC mit Verlust regulatorischer Promotorelemente sein. Im Rahmen einer weiteren Injektion des huTLR9-BAC wurden erneut HUT9-Mäuse generiert, die aktuell hinsichtlich der Expression des Transgens analysiert werden.