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Human DC-SIGN mediated recognition of mycobacteria in conventional and bacterial artificial chromosome transgenic mouse models

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1 Abbreviations

Ab	Antibody
ADC	Albumin–dextrose complex
APC	Antigen presenting cell
APC	Allophycocyanin (conjugated to ab)
APN	Aminopeptidase N
APS	Ammonium persulphate
AraLAM	Non-mannosylated LAM
ATP	Adenosine tri-phosphate
BAC	Bacterial artificial chromosome
BCA	Bicinchoninic acid
BMDC	Bone marrow derived dendritic cells
BMDM	Bone marrow derived macrophages
B. pertussis	*Bordetella pertussis*
BSA	Bovine serum albumin
C. albicans	Candida albicans
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cDNA	Complementary DNA
CFU	Colony forming unit
CHO	Chinese hamster ovary
CpG	Cytosine-guanosine oligonucleotide
CRD	Carbohydrate recognition domain
CWC	Cell wall components
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing nonintegrin
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetra-acetate

ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
Erk	Extracellular signal-regulated protein kinase
FACS	Fluorescence activated cell sorting
Fc	Fragment crystallisable
FCS	Foetal calf serum
FITC	Fluorescein-5-isothiocyanate
Flt3	FMS-like tyrosine kinase
FoxP3	Forkhead box P3
Gln	Glutamine
GM-CSF	Granulocyte-macrophage colony stimulating factor
h	hours
HBSS	Hank's balanced salt solution
H. pylori	*Helicobacter pylori*
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICAM	Intercellular adhesion molecule
iFABP	Intestinal fatty acid binding promoter
IFN	Interferon
Ig	Immunoglobulin
IκBα	Inhibitor of kappaB alpha
IL	Interleukin
IRF3	Interferon regulatory factor 3
ITAM	Immunoreceptor tyrosine-based activation motif
kB	Kilo-base
kDa	Kilo-Dalton
LAM	Lipoarabinomannan
l	Liter
LB	Luria-Bertani
ManLAM	Mannosylated LAM
mRNA	messenger RNA
LCCM	L-cell conditioned media

Le^x	Lewis x
LRR	Leucine- rich repeats
M	Molar
MAPK	Mitogen-activated protein kinase
MHC	Major Histocompatibility
ml	Milliliter
min	Minutes
MMP	Matrix Metalloproteinases
MOI	Multiplicity of infection
M. bovis BCG	*Mycobacterium bovis* Bacillus Calmette-Guérin
M. tuberculosis	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor-kappaB
NP-40	Nonidet P-40
PAGE	Polyacrylamid gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plamacytoid dendritic cell
PE	Phycoerythrin
Pen	Penicillin
PIM	Phospatidylinositol mannoside
PIM_2	Phospatidylinositol dimannoside
PIM_6	Phospatidylinositol hexamannoside
P. falciparum	*Plasmodium falciparum*
PMSF	Phenylmethylsulphonylfluoride
PNK	Polynucleotide kinase
PRR	Pattern-recognition receptor
RNA	Ribonucleic acid
Rpm	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SD	Standard Deviation

SDS	Sodiumdodecylsulfate
SHP-1	Src homology 2-containing tyrosine phosphatase-1
Strep	Streptomycin
S. pneumoniae	*Streptococcus pneumoniae*
Syk	Spleen tyrosine kinase
TEMED	N, N, N', N'-Tetramethylethyleneamine
TBS	Tris buffered saline
TIMP	Tissue inhibitors of metalloproteases
TIR	Toll/IL-1 receptor
TMB	Tetramethylbezine
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIF	TIR domain-containing adaptor inducing IFN-β
V	Volt
Wt	Wild-type

2 Introduction

2.1 *Mycobacterium tuberculosis*

The causative agent of tuberculosis is the slow growing, acid-fast and rod-shaped bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*). Every second *M. tuberculosis* infects another human being somewhere in the world. With one third of the world's population being infected it's one of the most effective pathogens (WHO 2005).

2.2 The first encounters of *M. tuberculosis*-the macrophages

The first interaction partners of mycobacteria, infecting their host *via* the respiratory route, are alveolar macrophages (Henderson, Dannenberg et al. 1963). To gain entrance into macrophages, pathogenic mycobacteria can use a variety of cell surface receptors. In addition, Cholesterol on the cell surface of macrophages has been shown to act as possible binding site facilitating pathogen-receptor interaction (Gatfield and Pieters 2000). Taken up by a macrophage, the pathogen starts to manipulate the host cell. At an early stage the maturation of the Mycobacterium containing phagosome to a phagolysosome is arrested (Armstrong and Hart 1971). These phagosomes are characterized by the presence of different early endosomal markers, like the transferrin receptor (Clemens and Horwitz 1995), and lack late endosomal markers like the lysosomal protease Cathepsin D (Clemens and Horwitz 1995) and the proton ATPase (Sturgill-Koszycki, Schlesinger et al. 1994). A reduced acidification of mycobacterial phagosomes has been linked to the lack in proton ATPase and the ability of mycobacteria to release high amounts of ammonia (Gordon, Hart et al. 1980). The precise mechanism leading to the arrest in phagolysosomal maturation has still not been elucidated.

2.3 T cell response to mycobacterial infection

Because *M. tuberculosis* lives inside the macrophages of its host, cell mediated immune response plays an important role. Therefore, T cell mediated mechanisms are more important than antibodies. As *M. tuberculosis* persists inside phagolysosomes, the presentation to $CD4^+$ T cells *via* MHC class II is to be expected. Studies using antibody depletion (Muller, Cobbold et al. 1987) or gene deficient mice (Caruso, Serbina et al. 1999) have demonstrated the relevance of $CD4^+$ T cells to control the infection. A main function of $CD4^+$ T cells is thought to be production of IFN-γ, especially in the initial phase of infection (Caruso, Serbina et al. 1999).

Besides $CD4^+$ T cells also $CD8^+$ T cells play an important role in the infection with *M. tuberculosis*. Mice lacking β_2-microglobulin, which do not develop functional $CD8^+$ T cells,

are significantly more susceptible to *M. tuberculosis* infection than control mice (Flynn, Goldstein et al. 1992). The exact role of $CD8^+$ T cells has not been elucidated so far.

Unconventional T cells that express the $\gamma\delta$ T cell receptor seem to be essential for protective immunity in response to very high inocula of *M. tuberculosis* (Ladel, Blum et al. 1995).

2.4 The role of dendritic cells in infection

For a pathogen-specific immune response dendritic cells play a crucial role. Immature dendritic cells, localized in peripheral tissues, are the sensors for invading pathogens. Upon capturing of a pathogen dendritic cells start to differentiate from immature to mature dendritic cells. During this process they relocate along the lymphatic vessels to secondary lymphoid organs, form antigen-Major Histocompatibility Complex II (MHCII) complexes and upregulate costimulatory molecules, which are required to stimulate naïve T-cells.

Several features help dendritic cells to uptake antigens very efficiently. First they are able to phagocytose pathogens, second they can take up particles *via* pinocytosis and third they express different receptors on their cell surface used for ligand mediated endocytosis.

2.5 Receptor mediated antigen recognition and uptake of mycobacteria

Several receptors are important for the receptor mediated recognition and uptake of mycobacteria by dendritic cells.

- Fc receptors for uptake of antibody-antigen complexes
- Complement receptors 3 and 4 are responsible for binding of complement opsonized bacilli
- Toll-like receptors (TLRs) 2, 4 and 9 recognize the pathogen and activate intracellular signalling cascades
- C-type lectins like the mannose receptor (CD206) and DC-SIGN (CD209) are known to be involved in binding and uptake of mycobacteria

The TLRs and the C-type lectins, which are most important for the direct sensing of the pathogen, belong to the family of Pattern Recognition Receptors (PRRs). They are designed to recognize a few highly conserved structures detectable on the cell surface or the envelope of different pathogens.

2.5.1 Role of TLRs in immune response to mycobacteria

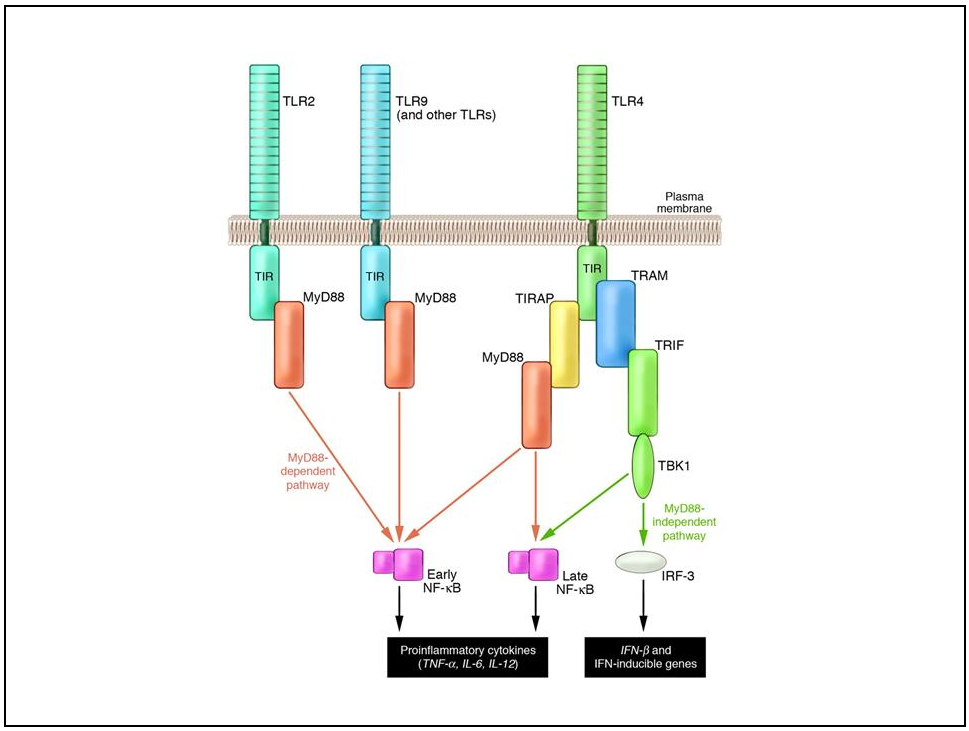

Figure 1: TLRs involved in recognition of *M. tuberculosis*. Most TLRs involved in response to infection with *M. tuberculosis* signal *via* MyD88. TLR4 can additionally transmit signals by using the MyD88-independent pathway (Doherty and Arditi 2004).

As far as known, the family of TLRs consists of 11 members (Akira and Takeda 2004). All members are type I membrane receptors. The extracellular domain contains 19-25 tandem copies of leucine-rich repeats (LRR) and a Toll/Il-1 receptor (TIR) domain is characteristic for the intracellular region of the proteins. Signal transduction initiated by TLRs can be mediated in a MyD88-dependent or –independent pathway. The MyD88-dependent pathway results in activation of NF-κB. Whereas the MyD88-independent pathway which involves another adaptor molecule called Toll-interleukin-1 receptor domain containing adaptor inducing IFNβ (TRIF) initiates activation of interferon regulatory factor 3 (IRF3) and with a delay also NF-κB.

For mycobacterial antigen recognition the involvement of TLR2, TLR4 and TLR9 has been shown. A study using CHO-cells and a murine macrophage cell line demonstrated that viable *M. tuberculosis* induces cellular activation in TLR2 or TLR4 over-expressing cells (Means, Wang et al. 1999). Further aspects of the interaction between TLRs and mycobacteria have been shown by this study. Lipoarabinomannan (LAM) from fast growing mycobacteria signals *via* TLR2 but not TLR4. But LAM from virulent strains like *M. tuberculosis* does not signal *via* TLRs at all. A soluble heat-stable factor mediates a TLR2 dependent activation whereas a cell-associated heat-sensitive mycobacterial factor induces TLR4 dependent activation (Means, Wang et al. 1999). LAMs are anchored to the cell membrane of mycobacteria by phospatidylinositol mannosides (PIM) (Khoo, Dell et al. 1995). The dimannoside (PIM_2) and hexamannoside (PIM_6) forms, which are strongly present in the cell wall of

Mycobacterium bovis Bacillus Calmette-Guérin (*M. bovis* BCG) and *M. tuberculosis*, have been shown to activate primary macrophages through TLR2 (Gilleron, Quesniaux et al. 2003). In addition to cell wall-associated factors also an interaction between the secreted 19-kDa lipoprotein of *M. tuberculosis* and TLR2 has been demonstrated (Brightbill, Libraty et al. 1999). Also mycobacterial CpG-rich DNA has been shown to stimulate immune cells (Tokunaga, Yamamoto et al. 1984). The TLR responsible for recognition of CpG-rich DNA has later been shown to be TLR9 (Hemmi, Takeuchi et al. 2000).

The studies decrypting the recognition of singular mycobacterial compartments by TLRs are based on *in vitro* experiments. As a consequence, mice deficient for different TLRs have been analysed for their response to mycobacterial infection to check the role of TLR signalling *in vivo*.

Studies analysing TLR2 deficient mice reveal conflicting results. Following aerosolic infection with 500 CFU TLR2 deficient mice were not able to control the *M. tuberculosis* infection. Wild-type mice of the same experiment ruled this infectious dose and even appeared in good condition at the end of the experiment (Drennan, Nicolle et al. 2004). In contrast to this experiment other studies show only small differences in the outcome after infection with *M. tuberculosis* (100 CFU) between control and TLR2 deficient mice (Reiling, Holscher et al. 2002; Sugawara, Yamada et al. 2003). Or they can only demonstrate a more severe phenotype of TLR2 deficient mice in comparison to congenic wild-type mice after an infection using a four time higher inoculum (2000 CFUs) (Reiling, Holscher et al. 2002). As TLR2 is known to cooperate with TLR1 and TLR6 in pathogen pattern recognition (Ozinsky, Underhill et al. 2000) also mice deficient in TLR6 were infected with *M. tuberculosis*. After infection with 100 CFU they manage the infection as well as control animals (Sugawara, Yamada et al. 2003). For the *in vivo* role of TLR1 in defending the host against *M. tuberculosis* infection no data are available so far.

The *in vivo* role of TLR4 in resistance to infection with *M. tuberculosis* appears even more complex. Two studies showed in TLR4 deficient animals a significant reduced survival accompanied by a diminished clearance of mycobacteria from the affected lungs after low dose (100 CFU) aerosol infection (Abel, Thieblemont et al. 2002) or high dose ($1*10^5$ CFU) intranasal infection (Branger, Leemans et al. 2004). In sharp contrast to these findings other groups could not show any link between TLR4 deficiency and an increase in susceptibility to infection with *M. tuberculosis* (Reiling, Holscher et al. 2002; Kamath, Alt et al. 2003; Shim, Turner et al. 2003).

A recent study analysed the role of TLR9 in the immune response to mycobacteria. For their study they used single TLR2 and TLR9 as well as TLR2/9 double knockout mice infected by the aerosol route with a low dose (100 CFU) of *M. tuberculosis*. They could show that most TLR2 and TLR9 single knockout mice survive until the end of the experiment at day 200. But the TLR2/9 double deficient mice all died until day 120. This finding came along with an increased pulmonary bacterial burden in TLR2/9 knockout animals at day 42 (Bafica, Scanga et al. 2005). As a consequence TLR9 does not seem to play such an important role in host resistance to *M. tuberculosis* alone, but in cooperation with TLR2.

2.5.2 DC-SIGN and its function in mycobacterial infection

2.5.2.1 Expression and structure of DC-SIGN

The C-type lectin human DC-SIGN (dendritic cell-specific intercellular adhesion molecule3-grabbing nonintegrin) is the main receptor on DCs for *M. tuberculosis* (Tailleux, Schwartz et al. 2003). Expression of DC-SIGN has been reported on DCs in the dermis of the skin, in T cell areas of tonsil, lymph nodes and spleen and in mucosal tissues such as rectum, uterus and lung (Geijtenbeek, Kwon et al. 2000; Geijtenbeek, Torensma et al. 2000; Soilleux, Morris et al. 2002). DC-SIGN is not expressed on CD1a positive DC-like Langerhans cells and plasmacytoid DCs (Geijtenbeek, Torensma et al. 2000; Jameson, Baribaud et al. 2002). Immature DCs, differentiated *in vitro* from monocytes, downregulate expression of DC-SIGN after maturation (Geijtenbeek, Torensma et al. 2000).

As most C-type lectins DC-SIGN is a type II transmembrane protein containing a carbohydrate recognition domain (CRD) (Feinberg, Mitchell et al. 2001). This domain is important for the binding of ManLam, the mannose-capped cell-wall component of *M. tuberculosis* (Geijtenbeek, Van Vliet et al. 2003). A neck domain, which is composed of seven complete and one incomplete tandem repeats, separates the CRD from the transmembrane region of the protein and is thought to be responsible for the forming of DC-SIGN tetramers and thereby subsequently influences carbohydrate specificity (Mitchell, Fadden et al. 2001). The cytoplasmatic tail of DC-SIGN includes a putative internalization motif, such as the di-leucine (LL) motif and the tri-acidic (EEE) clusters (Engering, Geijtenbeek et al. 2002), and an incomplete immunoreceptor tyrosine-based activation motif (ITAM). For internalization and intracellular trafficking only a role of the di-leucine motif has been proven (Lozach, Burleigh et al. 2005).

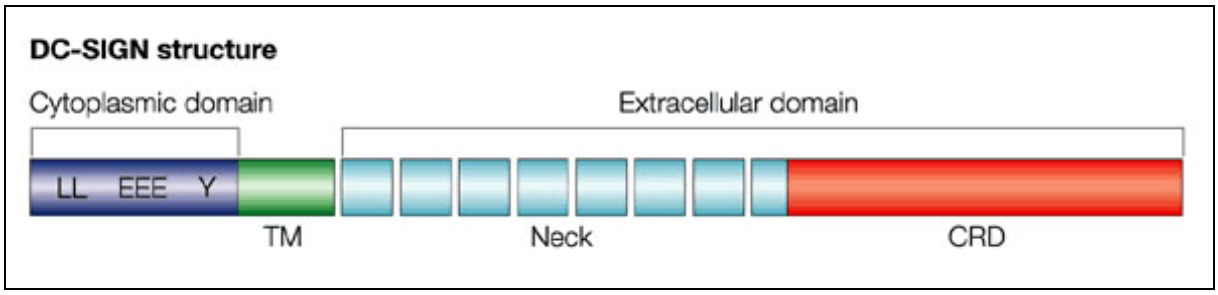

Figure 2: DC-SIGN structure. DC-SIGN consists of a carbohydrate recognition domain (CRD), a neck domain containing seven complete repeats and one incomplete tandem repeat, a transmembrane domain and a cytoplasmic tail. The cytoplasmatic tail includes internalization motifs, such as the dileucine motif, the tri-acidic cluster and an incomplete immunoreceptor tyrosine-based activation motif (ITAM) (van Kooyk and Geijtenbeek 2003).

2.5.2.2 Self and non-self recognition by DC-SIGN

The CRD of DC-SIGN is not only involved in the recognition of carbohydrated structures on pathogens but recognizes also the self-glycoproteins intercellular adhesion molecule 2 and 3 (ICAM2 and ICAM3) (Geijtenbeek, Torensma et al. 2000; Geijtenbeek, Krooshoop et al.

2000). The binding of DC-SIGN, present on the cell surface of DCs, to ICAM2 is important for rolling along endothelial cells and transendothelial migration. Using DC-SIGN blocking antibodies it has been proven that the initial contact between DC and resting T cells and the subsequent proliferation of this T cells is mediated by the binding of DC-SIGN to ICAM3 (Geijtenbeek, Torensma et al. 2000). Therefore it has been speculated, that the initial binding of DC and resting T cell mediated by DC-SIGN and ICAM3 interaction allows efficient TCR engagement by MHC-peptide complexes present on the DCs. A possible way of interaction between DC-SIGN and ICAM2 and ICAM 3 has been demonstrated for ICAM3. ICAM3 has been shown to be highly glycosylated *via* N-linked oligosaccharides containing high mannose-type oligosaccharides (Funatsu, Sato et al. 2001). The removal of this N-linked glycosylations by peptide-N-glycosidase F completely abrogated the binding of DC-SIGN to ICAM3 (Geijtenbeek, van Duijnhoven et al. 2002).

In the first publication describing the discovery of DC-SIGN it was mentioned, that the sequence of this protein was identical to a protein named earlier as HIV-1 envelope glycoprotein gp120 binding C-type lectin (Curtis, Scharnowski et al. 1992; Geijtenbeek, Torensma et al. 2000). This was the first hint for a pathogen interacting with DC-SIGN. The interaction between HIV-1 envelope glycoprotein and DC-SIGN has been shown to be highly dependent on the specific glycosylation pattern of the pathogenic glycoprotein, especially the presence of high mannose N-glycans (Lin, Simmons et al. 2003). Other viral glycoproteins for example from Ebola, Dengue or Hepatitis C virus have been described to bind to DC-SIGN in a similar manner (Lin, Simmons et al. 2003; Lozach, Lortat-Jacob et al. 2003; Lozach, Burleigh et al. 2005). Also a broad range of non-viral pathogens is known to be recognized by DC-SIGN. The gastric pathogen *Helicobacter pylori* and *Schistosoma mansoni* egg antigen bind DC-SIGN through fucose-containing carbohydrate Le^x (Appelmelk, van Die et al. 2003). ManLAM capped with two to three mannose residues is important for the specific interaction of DC-SIGN and *M. tuberculosis* (Maeda, Nigou et al. 2003).

Beside specific carbohydrate structures also the spacing of sugar groups on the surface of the pathogens may be of importance for a high affinity interaction with DC-SIGN (Mitchell, Fadden et al. 2001).

2.5.2.3 Crosstalk between TLRs and DC-SIGN in response to *M. tuberculosis*

Based on the finding that maturation of human DCs by LPS (TLR4-dependent) or viable *M. bovis* BCG (TLR2/4-dependent) can be blocked by simultaneous application of ManLAM, and the evidence that this inhibitory effect can be abrogated by DC-SIGN specific antibodies it has been hypothesised that ligation of DC-SIGN by ManLAM initiates an inhibitory signal interfering with the TLR-mediated DC-maturation events (Geijtenbeek, van Vliet et al. 2003). Moreover, a second remarkable finding has been depicted in this study (Geijtenbeek, van Vliet et al. 2003). LPS stimulated human DCs co-incubated with ManLAM are induced to produce IL-10, dependent on DC-SIGN. As IL-10 transgenic mice are unable to clear an infection with *M. bovis* BCG (Murray, Wang et al. 1997) and as human DCs treated by IL-10 induce a T-cell mediated antigen-specific tolerance (Jonuleit, Schmitt et al. 2000) this might imply DC-SIGN to suppress an active and thereby favour an impaired immune response. The

observed effects are only inducible by ManLAM, derived from virulent mycobacteria strains like *M. tuberculosis*, and not by AraLAM which is abundant in avirulent strains like *M. smegmatis* and lack the mannose cap. The exact mechanism of how DC-SIGN may interfere with TLR-signalling has not been elucidated so far. The incomplete ITAM in the cytoplasmatic tail of human DC-SIGN could be involved in these signalling events.

Summing up the observations it has been postulated, that initially TLRs, expressed on the cell surface of DCs, mediate the recognition of *M. tuberculosis*. Signalling pathways triggered by TLRs induce the activation of NF-κB which leads to an upregulation of co-stimulatory molecules like CD80 and CD86, enables production of proinflammatory cytokines and finally activates an immune response to fight the pathogen. After uptake and digestion of *M. tuberculosis* the release of high amounts of ManLAM, the key ligand of *M. tuberculosis* to DC-SIGN, bind to DC-SIGN and induce an inhibitory signal interfering with TLR-signalling *via* DC-SIGN. This results in downregulation of costimulatory molecules and an increase of IL-10 production, a cytokine known to suppress efficient immune response. Figure 3 recapitulates this hypothesis.

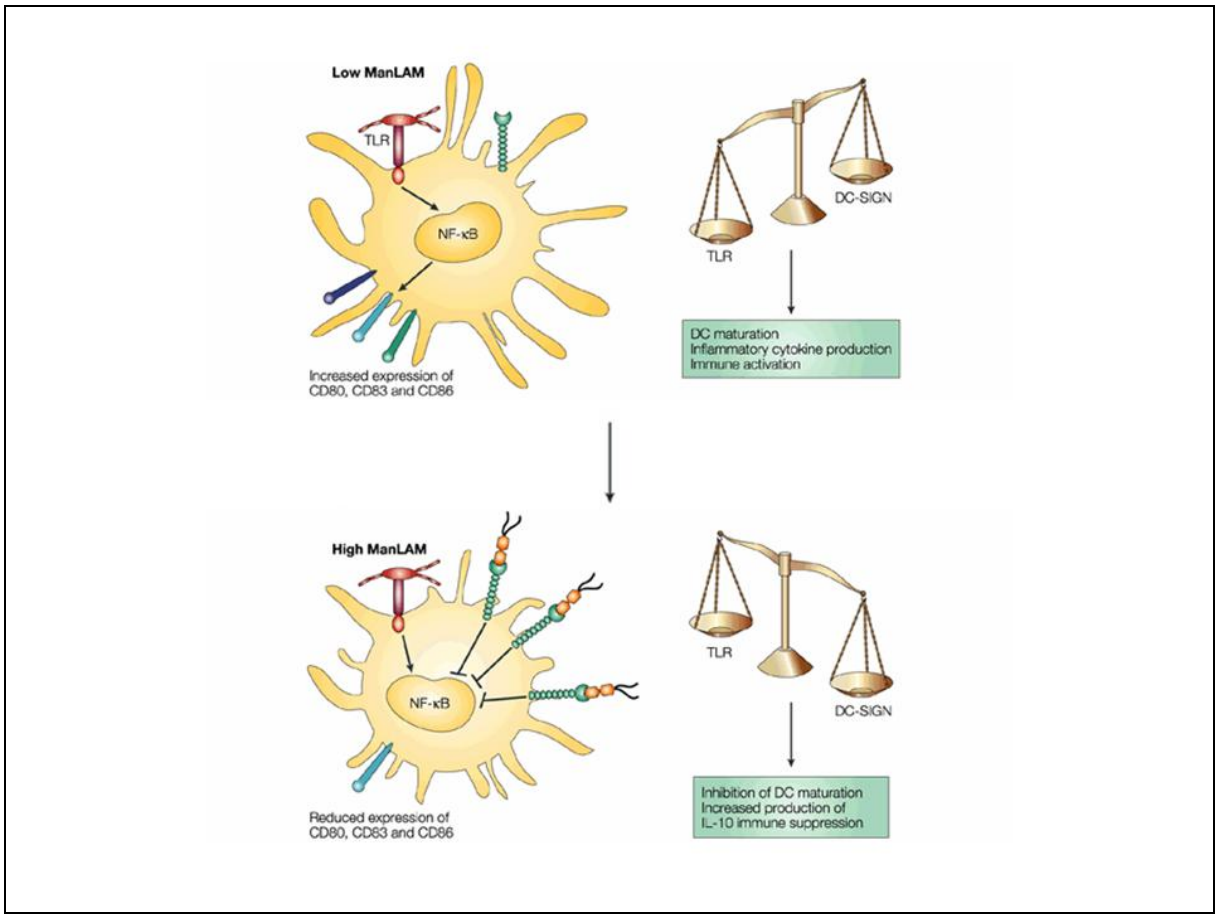

Figure 3: Mycobacteria target DC-SIGN through ManLAM to suppress dendritic cell function. Mycobacteria are recognized by different TLRs . This leads to activation of NF-kB, production of proinflammatory cytokines and upregulation of costimulatory molecules. *In vitro* Data using human DCs demonstrated a DC-SIGN dependent increased IL-10 production and downregulation of costimulatory molecules in response to mycobacterial stimuli. It has been postulated that the release of high amounts of ManLam, which has been shown to bee the key ligand of

M. tuberculosis to DC-SIGN, bind to DC-SIGN and induces an inhibitory effect on TLR signalling *via* DC-SIGN (van Kooyk and Geijtenbeek 2003).

2.5.3 Aims of the project

Until now, all data supporting the hypothesis of a DC-SIGN mediated immune evasion in mycobacterial infection were obtained from *in vitro* experiments. To address the question of the *in vivo* role of human DC-SIGN, two different human DC-SIGN transgenic mouse models were generated. There are five murine homologues, lacking the human DC-SIGN characteristic internalization and tyrosine motif (incomplete ITAM) (Baribaud, Pohlmann et al. 2001; Park, Takahara et al. 2001). None of the homologues have been shown to be expressed on myeloid DCs. CD209a and CD209b have been shown to be expressed on plasmacytoid preDC (O'Keeffe, Hochrein et al. 2002) and marginal zone macrophages respectively (Geijtenbeek, Groot et al. 2002). Due to the differences in structure and expression profile human DC-SIGN transgenic models should enable us to study the role of DC-SIGN *in vivo*.

At the beginning we analysed the expression of transgenic human DC-SIGN by flow cytometry and RT-PCR on different cell-types and tissue preparations, ranging from different *in vitro* generated DC subsets to spleen, lymph node, peritoneal cells and blood monocytes.

In the second part we investigated the DC-SIGN mediated effects on cytokine production, TLR signalling and NF-κB activation by DCs after infection with mycobacteria *in vitro*.

Finally we tested the different DC-SIGN transgenic mouse models for their susceptibility to infection with *M. tuberculosis in vivo*.

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
Chef Mapper	Bio-Rad, München
Confocal microscope	Zeiss, Jena
Cover slides (24x33 mm)	Roth, Karlsruhe
Cryotom CM 3000	Leica, Bensheim
Curvix 600	Agfa, Köln
Electrophoresis apparatus	Bio-Rad, München
ELISA reader Sunrise	Tecan, Switzerland
FACSCalibur	Becton Dickinson, Heidelberg
Foil for Western blotting	Neolab, Heidelberg
Freezer -20°C	Siemens, München
Fridge	Liebherr, Swizterland
Glass slides Super Frost Plus	Roth, Karlsruhe
Heat block 2Q	VLM, Leopoldshöhe
Hyperfilm ECL	Amersham, Braunschweig
Incubator BBD 6220	Heraeus, Hanau
Microscope	Zeiss, Jena
Multichannel pipettes	ThermoLabsystems, USA
Multipipette plus	Eppendorf, Hamburg
Nanodrop®ND-1000 Spectrophotometer	Nanodrop, Steinfurt
Neubauer counting chamber	Roth, Karlsruhe
Nitrocellulose-Transfer-Membrane	Schleicher&Schuell, Dassel

Nitrogen freezing tank Espace 300	Air Liquide, Düsseldorf
Orion Microplate Luminometer	Berthold Detection Systems, Pforzheim
pH-meter Multical	WTW, Weilheim
Pipettes	Gilson, USA
Plastic ware	NUNC, Wiesbaden; Falcon, USA
Power supply, Power Pac 200	Bio-Rad, München
Radiographic cassette	Dr. Goos-Suprema GmbH, Heidelberg
Sealing apparatus Folio	Severin, Sundern
Shaker (rocker)	Heidolph, Schwabach
Sterile bench	Heraeus, Hanau
Table centrifuge, Biofuge pico	Heraeus, Hanau
Table centrifuge,Biofuge fresco	Heraeus, Hanau
Vortexer Velp	Scientifica, Italy
Waterbath	Julabo, USA
Western Blot apparatus	Bio-Rad, München
Whatman paper	Bio-Rad, München

3.1.2 Chemicals

1 kb ladder GeneRulerTM	Fermentas, St. Leonroth
6x loading buffer	Fermentas, St. Leonroth
Acrylamide	Sigma, Taufkirchen
Ammonium Persulphate	Sigma, Taufkirchen
β-mercaptoethanol 50mM	Invitrogen, Karlsruhe
Bromphenol Blue	Sigma, Taufkirchen
BSA (albumin from bovine serum)	Sigma, Taufkirchen
Chloroform	Baker, Griesheim
Cytofix/Cytoperm	BD Bioscience, Heidelberg
DMEM	Biochrom, Berlin
DMSO	Sigma, Taufkirchen
dNTPs, Roti-Mix® PCR3	Roth, Karlsruhe
EDTA	Sigma, Taufkirchen
Ethanol	Merck, Darmstadt

Ethidiumbromide	Sigma, Taufkirchen
Ethidium monoazide bromide (EMA)	Invitrogen, Karlsruhe
Fetal Calf Serum	Perbiol/HyClone, USA
Fluoromount G	Southern Biotechnology Associates, Inc., USA
Glycerol	Sigma, Taufkirchen
Glycine	Sigma, Taufkirchen
H_2O_2	Sigma, Taufkirchen
Hepes	Sigma, Taufkirchen
Isopropanol	PharmacyMRI
L-Glutamine 200mM	Biochrom AG, Berlin
Methanol	Roth, Karlsruhe
Middlebrook 7H10 Agar	BD Bioscience, Heidelberg
Middlebrook 7H9 Broth	BD Bioscience, Heidelberg
MidRange PFG Marker	NEB, Frankfurt am Main
N,N'-Methylene-bis-acrylamide	Sigma, Taufkirchen
NaCl	Roth, Karlsruhe
NycoPrep	Progen, Heidelberg
OptiPrep	Progen, Heidelberg
Paraformaldehyde	Sigma, Taufkirchen
PBS Dulbeccos	Biochrom AG, Berlin
Penicillin/Streptomycin	Gibco, USA
Precision Plus Protein™ Standard	Bio-Rad, München^
Propidium iodide	Sigma, Taufkirchen
Recombinant Murine IL-4	Peprotech, London UK
Red Blood Cell Lysing Buffer	Sigma, Taufkirchen
Redivue 5'-[γ-^{32}P] ATP, 9.25 MBq	Amersham, Braunschweig
Reporter Lysis Buffer 5x	Promega, Mannheim
SDS (Sodiumdodecylsulfate)	Sigma, Taufkirchen
Sucrose	Sigma, Taufkirchen
Taq polymerase	Roche Diagnostics, USA
TEMED	Sigma, Taufkirchen
Tricine	Sigma, Taufkirchen

Tris-(hydroxymethyl)-aminomethane	Roth, Karlsruhe
TRIzol® Reagent	Invitrogen; Karlsruhe
Trypsin/EDTA	Biochrom, Berlin
Tween 20	Sigma, Taufkirchen
VLE-RPMI1640	Biochrom, Berlin

3.1.3 Expandable items

Cell-culture-plates and petri-plates	Nunc, Wiesbaden
Dialysis membranes	Roth, Karlsruhe
Electroporation cuvettes, 2mm	Bio-Rad, München
Freezing medium	Jung, Nussloch
Insulin-syringe 1ml Sub Q	Becton Dickinson, Heidelberg
MaxiSorp 96-well plates	Nunc, Wiesbaden
Microtubes Noose NS 1,2ml	Alpha, Hampshire, UK
Nitrocellulose membrane SpectraPor®2	Millipore, Schwalbach
Parafilm	Roth, Karlsruhe
PCR tubes 8-strip	Kisker, Steinfurt
Sterile injection-needles, Microlance™3	Becton Dickinson, Heidelberg
Superfrost® Plus slides	Roth, Karlsruhe
Syringe Discardit™ 10ml, 20ml	Becton Dickinson, Heidelberg
Plastique material	Nunc, Wiesbaden
Tissue-Tek® Cryomold 15x15x15mm	Miles Inc, USA
Whatman Paper	Bio-Rad, München

3.1.4 Primer

Table 1: RT-PCR oligonucleotide primers

Name	5'-3' sequence	Company
DC-SIGN-fw	TCTTGGCTGGGCTCCTTGT	MWG Biotech
DC-SIGN-rev	TGCCTGGATTGTTCCTGACTT	MWG Biotech
DC-SIGN-probe	CAAGTGTCCAAGGTCCCCAGCTCCA	MWG Biotech
Beta-actin-fw-mouse	CGTGAAAAGATGACCCAGATCA	MWG Biotech

Name	5'-3' sequence	Company
Beta-actin-rev-mouse	CACAGCCTGGATGGCTACGT	MWG Biotech
Beta-actin-probe-mouse	TTTGAGACCTTCAACACCCCAGCCA	MWG Biotech

3.1.5 Antibodies used for FACS

Table 2: antibodies used for flow cytometry

Antigen	recognized Species	Isotype	Conjugate	Dilutions for FACS	Company
CD11c (Integrin α-chain)	mouse	armenian hamster, $IgG_{1\lambda}$	FITC	1:100	BD Bioscience
CD11c (Integrin α-chain)	mouse	armenian hamster, $IgG_{1\lambda}$	R-PE	1:100	BD Bioscience
CD11c (Integrin α-chain)	mouse	armenian hamster, $IgG_{1\lambda}$	APC	1:100	eBioscience
CD209	mouse	mouse, IgG2B	R-PE	1:500	R & D Systems
CD40	mouse	rat, $IgG_{2\alpha,\kappa}$	R-PE	1:100	BD Bioscience
CD45R/B220	mouse	rat, $IgG_{2\alpha}$	Biotin	1:500	eBioscience
CD45R/B220	mouse	rat, $IgG_{2\alpha}$	FITC	1:200	eBioscience
CD16/CD32	mouse	rat, $IgG2_{\alpha,\lambda}$	pure	1:20	eBioscience
(MHCII) I-A/I-E	mouse	rat, $IgG2_{\alpha,\kappa}$	FITC	1:500	BD Bioscience
CD80 (B7-1)	mouse	armenian hamster, IgG2,k	R-PE	1:100	BD Bioscience
CD86	mouse	rat, $IgG2_{b,\kappa}$	Biotin	1:200	BD Bioscience
Biotin		Streptavidin	Alexa647	1:500	Molecular Probes

3.1.6 Antibodies used for Western Blot

Table 3: antibodies used for Western Blot

Antigen	recognized Species	Isotype	Conjugate	Dilution	Company
ß-Actin	mouse, human	mouse, IgG1		1:10000	Sigma Aldrich
Phospho-p44/42 (Erk1 and Erk2)	mouse	rabbit		1:1000	Cell Signaling Technology
p44/42	mouse	rabbit		1:1000	Cell Signaling Technology
Phospho-p38	mouse	rabbit		1:1000	Cell Signaling Technology
p38	mouse	rabbit		1:1000	Cell Signaling Technology
Phospho SAPK/JNK	mouse	rabbit		1:1000	Cell Signaling Technology
SAPK/JNK	mouse	rabbit		1:1000	Cell Signaling Technology

3.2 Methods

3.2.1 Cell biology

3.2.1.1 Media used for the culture of eukaryotic cells

RPMI 1640	500	ml RPMI 1640
	50	ml heat inactivated FCS
	5	ml Penicillin/Streptomycin
	5	ml L-Glutamine 200 mM
	500	µl β-mercaptoethanol 50 mM

DMEM	500	ml DMEM
	50	ml FCS
	5	ml Penicillin/Streptomycin
	5	ml L-Glutamine 200 mM
	500	µl β-mercaptoethanol 50 mM

3.2.1.2 Culture of HEK293 cells

HEK293 cells were cultured in DMEM medium at 37°C, 5% CO_2 and 85% humidity. Cells were cultured to 60-70 % confluency. To split the cells, they were washed once with 1xPBS and then incubated with 1 ml of Trypsin/EDTA at 37°C for 5 min to detach the cells. Trypsin was inhibited by the addition of 9 ml of medium. The cells were gently resuspended and 1/5th to 1/10th of this solution was transferred to a new cell culture dish containing fresh medium. This procedure was carried out every two to three days.

3.2.1.3 Preparation and culture of Bone Marrow derived murine Macrophages

Bone marrow of one to two mice was rinsed with sterile PBS using a syringe (best needle 27G) into a 10 ml petri dish. On day three or day four 1ml of LCCM (10% supernatant in RPM complete medium) was added. At day 5 cells were washed once with PBS. One ml of Accutase was added and cells were incubated for 5'. After addition of 9 ml of PBS cells were counted and plates for stimulation on next day.

3.2.1.4 Preparation and culture of Bone Marrow derived mouse Dendritic Cells

The mice were sacrificed, the hind legs were cut off and the 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 1xPBS using an injection needle. After centrifugation at 1300 rpm for 7 min, the supernatant was discarded and the cells were taken up in RPMI medium, supplemented with 10% FCS, Pen/Strep/Gln, β-Mercaptoethanol and 1% GM-CSF, plated in a total volume of 10 ml each on petri dishes and incubated at 37°C. Two days after preparation 10 ml of GM-CSF containing medium were added to each petri dish. 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 transferred to cell culture plates. 10 ml per plate were transferred directly, the remaining 10 ml were centrifuged, resuspended and 10 ml each added to the cell culture plates. After 8 days of cultivation, cells were ready to use.

3.2.2 Mice

All mice were backcrossed at least 10 generations onto a C57BL/6 backgound. All 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 local government. Experiments were performed with 10-12 week old mice both sexes, unless otherwise stated. Prior to the experiment all the mice were genotyped 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
	900	ml H_2O and autoclaved at 121 °C for 10 min
		add 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
Liquid LB-media:	10	g Bacto-Trypton
	5	g Bacto-Yeast-extract
	10	g NaCl
	900	ml H_2O, pH 7.0 with NaOH
		add 1 l H_2O

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.

SOB-media:	20	g Bacto-Trypton
	5	g Bacto-Yeast-extract
	0.5	g NaCl
	2,5	ml 1 M KCl

		ad 900 ml H_2O
		adjust pH to 7.0 with 10 M NaOH
		ad 990 ml H_2O
		sterilize by autoclaving and add 10 ml sterile 1 M $MgCl_2$ before use
SOC-media	1	l SOB-media
	1	ml 1 M $MgCl_2$
	20	ml 1 M glucose
Ampicillin	50	μg/ml (stored in H_2O at –20°C)
Chloramphenicol	34	μg/ml (stored in EtOH at –20°C)

3.2.3.2 Bacteria and Infection

M. bovis BCG was grown in Middlebrook 7H9 broth supplemented with Middlebrook OADC-Enrichment. Midlog phase cultures 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. For i.p. infection with *M. bovis* BCG, mice were infected with $10*10^6$ CFU mycobacteria.

3.2.3.3 Uptake and binding assay

Thawed *M. bovis* BCG samples were pelleted and suspended in 1 ml of RPMI 1640 and clumps were disrupted by multiple passages through a 25-gauge needle. Cells (10^6) were exposed to BCG at a 10:1 ratio in 1 ml of medium in six-well plates for the noted incubation periods. Partially attached, non ingested bacteria were removed by 10 min treatment with trypsin-EDTA at 37°C and extensive washing with HBSS. Tenfold serial dilutions of lysates were plated onto Middlebrook 7H10 agar plates containing Middlebrook OADC-Enrichment and incubated at 37°C for 19–21 days. Finally colonies on the plates were enumerated.

3.2.3.4 Colony enumeration assay

Bacterial loads in the spleen of infected mice were evaluated at different time points after infection with *M. bovis* BCG. The organ was weighed and defined aliquots were homogenized in PBS. Tenfold serial dilution of organ homogenates were plated onto Middlebrook 7H10 agar plates containing Middlebrook OADC-Enrichment and incubated at 37°C for 19–21 days. Colonies on plates were counted and results are expressed as $\log_{10}$ CFU per organ. In the case of aerogenical infection with *M. tuberculosis* the lungs were prepared and analysed according to the formed described procedure.

3.2.4 Protein biochemistry

3.2.4.1 Protein biochemistry buffers and solutions

Erk-buffer:	50	mM Hepes pH 7.5
	150	mM NaCl
	1	mM EDTA
	1%	Triton
	10	mM $Na_4P_2O_7$
	10	% Glycerol
Lysis buffer:	7	ml Erk-buffer
	140	µl NaF (500mM)
	35	µl Na-ortho-vanadate (200mM)
	140	µl 50x Protease-inhibitor cocktail
Acrylamide solution :	30	% (w/v) Acrylamide
	0.8	% (w/v) Bisacrylamide
		bring to 200 ml with H_2O_{dd}
4x Resolving gel buffer:	1.5	M Tris-HCl pH 8.8
4x Stacking gel buffer:	0.5	M Tris-HCl pH 6.8
10% SDS:	10	% SDS in H_2O_{dd}
10% APS:	10	% Ammonium persulphate in H_2O_{dd}
6x sample buffer:	7	ml stacking gel buffer
	1	g SDS
	3	ml Glycerol
	0.9	g DTT
	0.06	% Bromphenol blue

Tank buffer:	0.025	M Tris
	0.129	M Glycine
	0.1	% SDS
		bring to 10 l with H_2O_{dd}
Water-saturated n-butanol:	50	ml n-butanol
	5	ml H_2O_{dd}
Transfer buffer	0.025	M Tris
	0.129	M Glycine
	200	ml EtOH
		bring to 1 l with H_2O_{dd}

3.2.4.2 Cell lysis

Cell lysis was carried out on ice. After stimulation the cells were washed twice with cold 1xPBS and lysed in 200 µl of lysis buffer for 30 min. After cell lysis the probes were centrifuged for 5 minutes at max. speed in a tabletop centrifuge to remove debris and nuclei. The supernatants were transferred to 1.5 ml tubes and protein concentration was determined using the BCA Protein Assay Reagent Kit (PIERCE).

3.2.4.3 Protein determination

This assay is based on reduction or Cu^{2+} to Cu^{1+} by protein in alkaline medium. Cu^{1+} forms purple-colored complexes with bicinchoninic acid (BCA), which exhibit a strongabsorbance at 562 nm. This absorbance is almost linear with increasing protein concentrations.The assay was performed in 96well plates. 10 µl of each sample or BSA standard were added to the plate. The BSA standards had been prepared resulting in the following concentrations: 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, 0.025 and zero µg/ml. Then 200 µl/well of working reagent (50:1, Reagent A:B) were added, the plate was mixed for 30 sec on a shaker and incubated for 30 min at 37°C. Afterwards the plate was cooled to RT and the absorbance was measured at 562 nm (Ref.: 0) in the ELISA reader. Analysis of the data was carried out using the software Excel.

3.2.4.4 SDS-Polyacrylamid gel electrophoresis (PAGE) of proteins

SDS-PAGE was carried out using gels with a thickness of 1.5 mm.

The resolving gel was poured first and covered immediately with butanol. After polymerization the butanol was poured off, the gels were rinsed with water, the stacking gel was poured and the comb was inserted. The comb was removed after complete

polymerization of the stacking gel, gels were installed and overlayed with tank buffer. To remove unreacted acrylamide, wells were rinsed with tank buffer. The cell lysates were mixed 1:6 with 6x sample buffer and incubated at 95°C for 5 min. 20 µl of each sample were loaded on the gel. For determination of the protein size, 5 µl of a protein standard were loaded on the gel. Electrophoresis took place at 80 V for the first 15 min and at 200 V for 1-2 hours. The procedure was carried out on ice to avoid overheating of the electrophoresis chamber.

Table 4: SDS-PAGE gel recipes

	Resolving Gel					Stacking Gel
	5 %	7.5 %	10 %	12.5 %	15 %	4 %
Acrylamide solution	3.3 ml	5 ml	6.7 ml	8.3 ml	10 ml	0.88 ml
4x Resolving gel buffer	5 ml	5 ml	5 ml	5 ml	5 ml	-
4x Stacking gel buffer	-	-	-	-	-	1. 66 ml
10 % SDS	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	66 µl
H_2O_{dd}	11.4 ml	9.7 ml	8 ml	6.4 ml	4.7 ml	4.06 ml
TEMED	6.7 µl	6.7 µl	6.7 µl	6.7 µl	6.7 µl	3.3 µl
10 % APS	100 µl	100 µl	100 µl	100 µl	100 µl	33.4 µl

3.2.4.5 Western Blot analysis

To transfer the proteins from the gel to a nitrocellulose membrane the tank blot method was carried out. The gel, the nitrocellulose membrane, two Whatman papers and two sponges were equilibrated in transfer buffer before setting up the blot. The complete set up was installed in the transfer chamber and the chamber was filled up with transfer buffer. The transfer took place at 110 V for 1 h on ice. After transfer, the membrane was incubated in water for 5 min on the shaker to remove remaining methanol. To ensure equal protein loadings for all lanes the membrane was stained with Ponceau S solution for 5 min. To remove the staining afterwards the membrane was incubated in water again. To avoid unspecific binding of the antibody, the membrane was blocked in 1xTBS-T 5% BSA for 1 h on the shaker. Afterwards three washings steps in 1xTBS-T for 10 min were carried out.

Incubation with the primary antibody (diluted in 1xTBS-T 5% BSA) took place at 4°C overnight. The next day, the membrane was washed 3x10 min in TBS-T and incubated with the secondary antibody (diluted in 1xTBS-T 5% BSA) for 1 h at room temperature on the shaker. After three additional washing steps, proteins were detected using Lumi-Light Western Blot Substrate. Before incubation of the membrane with a different antibody, a stripping protocol was carried out to remove all antibodies from the membrane. To achieve this, the membrane was incubated in H_2O for 5 min, in 0.2 N NaOH for 20 min and again in H_2O for 5 min. All procedures were carried out on the shaker. Additionally, the membrane

was blocked again for 1 h in 1xTBS-T 5% BSA. After 3 washing steps incubation with a different primary antibody was possible.

3.2.5 Molecular biology

3.2.5.1 Buffers and solutions for molecular biology

10 x DNA running buffer:	50	mg bromphenole blue
	3	ml 150 mM Tris pH 7.6
	60	ml glycerol
	7	ml H_2O_{dd}
10 x TBE:	108	g Tris
	55	g boric acid
	900	ml H_2O_{dd}
	40	ml 0.5 M Na_2EDTA, pH 8.0
		ad 1 l H_2O_{dd}
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_2O_{dd}

3.2.5.2 Basic tools for molecular genetic approaches

Minipreps and Maxipreps were performed using the appropriate kits from QIAGEN following the manufacturer's protocol. For gel extraction and DNA-purification, the kits from QIAGEN were used as well. Ligations were done with the Quick Ligation Kit from New England Biolabs following their instructions. As not indicated otherwise fragments were used in a 3:1-ration of insert to vector.

3.2.5.3 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 a 1 kb ladder were used. Electrophoresis took place at 70 V.

3.2.5.4 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	ng DNA in 1 µl
Primer mix: 5’ and 3’ primers	10	pMol

In general amplification occurred 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	∞	

3.2.5.5 EMSA - Electrophoretic Mobility Shift Assay

3.2.5.5.1 EMSA Solutions

Totex-Buffer:	20	mM HEPES (pH7,9)
	0.35	M NaCl
	20	% Glycerol
	1	% NP-40
	1	mM $MgCl_2$
	0.5	mM EDTA (pH8,0)
	0.1	mM EGTA
	10	mg/ml Leupeptin
	10	mg/mlAprotinin
	100	mM PMSF
	100	mM DTT
10X Buffer L:	0.1	M Tris pH 7.5
	0.1	M $MgCl_2$
	0.01	M DTT

Cells ($5x10^6$) were cultured in 6-wells plates in 2% FCS medium overnight following stimulation for the indicated periods. The cells were washed with PBS and centrifuged for 15 min, 13000 rpm (Biofuge fresco) at 4°C. The supernatant was removed and the pellet was resuspended in 50 µl of Totex buffer, left on ice for 30 min and finally centrifuged for 5 min, 13000 rpm, 4°C. The supernatant was put into new tube and frozen immediately in ethanol-dry ice bath. The protein concentration was estimated by application of BCA protein kit following the manufacturer's protocol.

The NF-κB oligonucleotides were labelled as follows:

Annealing mix:	1	µl NF-κB sense oligo
	1	µl NF-κB antisense oligo
	5	µl 10x buffer L
	14.5	µl H_2O_{dd}

The annealing mixture was incubated for 15 min at 55°C followed by incubation at room temperature for another 15 min.

For the labelling of the annealed NF-kB oligonucleotides:

Labelling mix:	8	µl annealing mix
	2	µl 10x T4 PNK buffer
	5	µl γ-^{32}P-ATP
	1	µl T4 PNK
	4	µl H_2O_{dd}

The mixture was incubated 30 min at 37°C, refilled with water to the volume of 50 µl and purified on the BioRad column at 2000 rpm for 4 min. The probe was kept at -20°C.

EMSA mix:	40	µl BSA 10 µg/ µl
	40	µl poly-dIdC
	80	µl buffer F
	40	µl buffer D
	100	µl H_2O_{dd}
	4 – 12	µl radioactive probe (depending on the radioactivity)

10-15 µg of the nuclear extracts were used for EMSA. If the volume of the extracts was lower than 5 µl, the probe was refilled with water to the volume of 5 µl. 15 µl of EMSA mix was added to the nuclear extracts probe and after vortexing the probes were incubated for 25 min at room temperature. In this time 6% EMSA gel was pre-run at 100V for 20-25 min with 6 µl of marker in the last slot.

6%-EMSA gel:	12.5	ml Acrylamide-bisacrylamide (29:1)

5	m 10xTBE
82.5	ml ddH_2O
75	µl TEMED
750	µl 10% (w/v) APS

After incubation time, the probes were loaded on the gel and run at 220V for ca. 1.5 h, which should correspond to the distance of 10 cm measured from the slot end. When the run was finished, the gel was put on Whatman paper, covered with plastic foil and dried 1 h at 80°C. Finally, the dried blot was exposed on the phospho-screen over night followed by exposure on the X-ray film.

3.2.5.6 mRNA Isolation using TRIzol® Reagent

5 *10^6 cells per well of a 6-well plate were lysed in 1 ml of TRIzol® Reagent. After incubation for 5 minutes at room temperature 200 µl of chloroform were added. Sample tubes were carefully closed. The tubes were vigorously shaken by hands for 15 seconds and incubated for 3 minutes at room temperature. Next the samples were centrifuged at 12000 g for 15 minutes at 4°C. After centrifugation the aqueous phase was removed and mixed with 500 µl isopropyl alcohol by hand. This mixture was incubated at room temperature for 10 minutes. After another centrifugation step at 12000 g for 10 minutes at 4°C the precipitated RNA got visible. The supernatant was removed. The pellet was resuspended in 1 ml of ethanol by vortexing and subsequently centrifuged at 7500 g for 5 minutes at 4°C. At the end of the procedure the supernatant was removed and the pellet was allowed to dry briefly. The RNA was dissolved in 20 µl of H_2O at 56°C for 10 minutes.

3.2.5.7 TaqMan primers and fluorogenic probes

TaqMan primers (MWG Biotec, Ebersberg, Germany) and probes (PerkinElmer, Weiterstadt, Germany) were designed using the primer design software Primer Express (PE Applied Biosystems, Foster City, CA). All probes were synthesized by PerkinElmer and labeled with the reporter dye 6-carboxyfluorescein at the 5' end and the quencher dye 6-carboxytetramethylrhodamin at the 3' end. Primer and probe sequences are shown in Table 1. Primers and probes were chosen to span exon junctions or to lie in different exons to prevent amplification of genomic DNA.

3.2.5.8 TaqMan PCR procedure

Five µl of RNA was transcribed into cDNA in a total volume of 50 µl using 50 U of MultiScribe reverse transcriptase (PerkinElmer), according to the manufacturer's instructions. PCR was performed in a volume of 30 µl on the ABI PRISM 7700 sequence detection system (PerkinElmer). For each run, a master mix was prepared on ice containing 15 µl of Universal Master Mix (PE Applied Biosystems), primers (0.5 µmol/L for β-Actin and 0.5 µmol/L for human DC-SIGN), flourogenic probes (80 nmol/L for β-Actin and 80 nmol/L for human DC-SIGN), and H_2O. To each well of a 96-well plate, 25 µl of master mix and 5 µl of cDNA samples were added. All PCRs were performed in duplicate. Thermal cycling was initiated

with an incubation step at 50°C for 2 minutes, followed by a first denaturation step at 95°C for 15 minutes, and continued with 40 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds.

3.2.6 Immunology

3.2.6.1 Enzyme linked immunosorbent assay (ELISA)

3.2.6.1.1 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 epitop of the antigen. The detection antibody is tagged with an enzyme (e.g. peroxidase) which transforms a colorless substrate into a colored product. By measurement of the intensity of the color the level of cytokine production, for example, can be determined.

3.2.6.1.2 ELISA buffers and solutions

Coating solution:	1	x PBS
Blocking buffer:	1	x PBS
	1	% BSA
	5	% Sucrose
Reagent Diluent:	1	x PBS
	1	% BSA
Washing buffer:	1	xPBS
	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:	50	µl HRP buffer
	10	ml reagent diluent
Substrate reagent:	1	tablette Tetramethylbezine (TMB)
	10	ml 0.05 M Phosphate-Citrate buffer
	2	µl H_2O_2 30%
Phosphate-Citrate buffer:	25.7	ml 0.2 M Na_2HPO_4 l
	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	2	N H_2SO_4

Plates were coated with 50 µl per well of capture antibody (720ng/ml) and incubated overnight at 4°C. The next day the plate was 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 3x 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 folllowed 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 3x afterwards followed by 20 min of incubation with conjugation buffer (50 µl/well). Finally the plate was washed 3x again and fresh substrate reagent was added (100 µl/well). The plate was incubated in the dark because the substrate reagent contains H_2O_2, which is known to be light sensitive. 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).

3.2.6.2 Flow cytometry

3.2.6.2.1 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 1 N HCl)

ad 1000 ml H_2O_{dd}

3.2.6.3 Analysis of cell surface antigens by flow cytometry

Up to $1*10^6$ 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 2000 rpm, 4°C, 1 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 one more time. The cells were resuspended in 50 µl of the ready made 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 2000 rpm, 4°C, 1 min). Finally the supernatant was discarded and cells were resuspended in 150 µl washing buffer containing propidium iodide (2 mg/ ml, dilution 1:1000), which stains dead cells, and transferred to FACS tubes. Stained samples were analyzed by flow cytometry using a FACSCalibur and the data was analysed using the software FlowJo.

In the case of analysing peripheral blood samples, these were treated with Erythrocyte lysis buffer after preparation and before starting general staining procedures.

3.2.6.4 Analysis of intracellular antigens by flow cytometry

For intracellular staining, the cells were stained for 20 min with ethidium monoazide bromide, also called EMA, (1mg/ml, 1:1000 dilution in FACS-buffer) after performing the Fc-block. By exposing the samples to direct light EMA was covalently linked to DNA in dead cells. Next, samples were washed three times. Prior to the incubation with staining solutions the samples were fixed and permeabilized by treatment with BD Cytofix/Cytoperm according to the manufactures protocol.

3.2.6.5 Isolation of mouse dendritic cells from spleen or lymphnodes on a discontinous OptiPrep™ gradient

3.2.6.5.1 OptiPrep™ gradient solutions

Solution A:		OptiPrep™ (60 % iodixanol solution)
Solution B:		HBSS without $Ca^{2+}$and Mg^{2+}
Solution C:	0.88	% NaCl
	1	mM EDTA
	0.5	% BSA
	10	mM Hepes-NaOH

		adjusted to pH 7.4
Solution D:	400	U/ml Collagenase
	100	µg/ml DNAse
		in RPMI 1640
Solution E:	11.5	% iodixanol (1:4.2 mixture of Solution A and C)
Solution F:	15	% iodixanol (1:3 mixture of Solution A and B)

The Spleen was put into a cell strainer (70 µm) in a well of a 6-well plate. Solution D was added and the spleen was injected with the mixture using an insulin syringe. After 30' of digestion at 37 °C the organ was pressed through the strainer using the plunger of an insulin syringe. The digest was continued at 37 °C for another 30' with the last 5' in the presence of 10 mM EDTA (1 ml EDTA 0.1 M / 10 ml solution D). The cell suspension was vigorously pipetted several times to disrupt the remaining tissue and filtered. Following steps were all performed at 4 °C. The cells were pelleted at 1300 rpm at 4 °C using the Multifuge centrifuge. This step was repeated twice, using solution B for resuspension. After the last washing step the pellet was resuspended in 4 ml of solution F. This solution was overlayed with 5 ml of solution E and 3 ml of solution B. The tube containing these stacked solutions was centrifuged at 600 g for 15 minutes at room temperature. The rotor was set to decelerate without break. After centrifugation the dendritic cell population could be harvested from the top of the 12 % iodixanol layer. To recover the cells, the interphase between the top and middle layer was put into a tube containing 10 ml of PBS, mixed quite well and centrifuged at 1700 rpm for 10 minutes. These cells were then used for further experiments.

3.2.6.6 Isolation of mouse dendritic cells from spleen on a discontinous NycoPrep™ gradient

3.2.6.6.1 NycoPrep gradient solutions

Solution A:		Nycoprep™
Solution B:	400	U/ml Collagenase
	100	µg/ml DNAse
		in RPMI 1640
Solution C:		PBS

The Spleen was put into a cell strainer (70 µm) in a well of a 6-well plate. Solution B was added and the spleen was rinsed with mixture using an insulin syringe. After 30' of digestion at 37 °C the organ was pressed through the strainer using the plunger of an insulin syringe. The digest was continued at 37 °C for another 30' with the last 5' in the presence of 10 mM EDTA (1 ml EDTA 0.1 M / 10 ml solution B). The cell suspension was vigorously pipetted several times to disrupt the remaining tissue and filtered. Following steps were all performed at 4 °C. The cells were pelleted at 1300 rpm at 4 °C using the Multifuge centrifuge. This step

was repeated twice, using solution C for resuspension. After the last washing step the pellet was resuspended in 4 ml of solution A. This solution was overlayed with 2 ml of solution C. The tube containing these stacked solutions was centrifuged at 600 g for 15 minutes at room temperature. The rotor was set to decelerate without break. After centrifugation the dendritic cell population could be harvested from the interphase between the two solutions. To recover the cells, the interphase between the top and lower layer was put into a tube containing 10 ml of PBS, mixtured quite well and centrifuged at 1700 rpm for 10 minutes. These cells were then used for further experiments.

4 Results

4.1 Generation of transgenic mice

4.1.1 Generation of human DC-SIGN transgenic mouse models

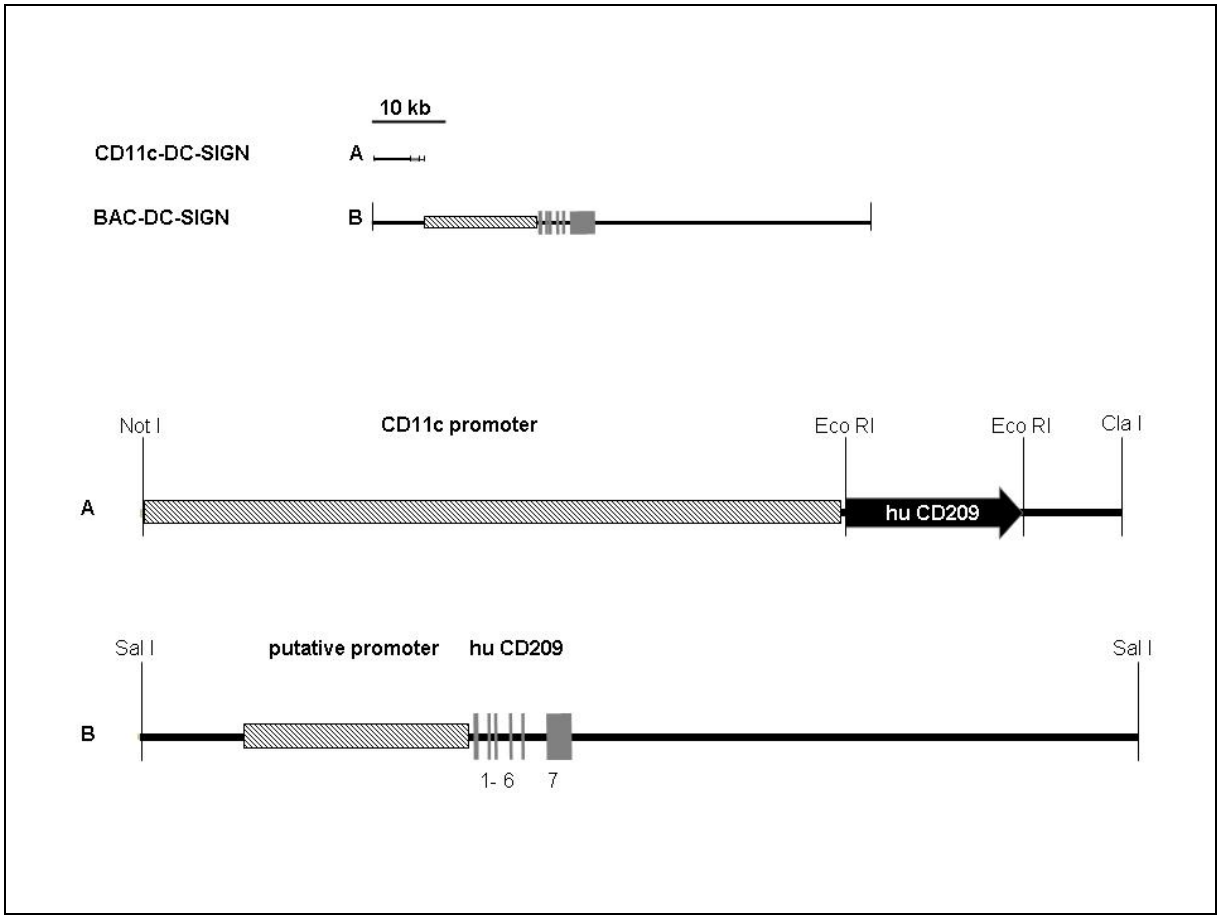

Figure 4: Schematic representation of the human DC-SIGN transgenic constructs. Upper panel: scaled view of the two generated constructs.

In humans DC-SIGN (CD209) is mainly expressed on myeloid dendritic cells. The complete promoter has not been identified so far. To mirror physiologic expression of human DC-SIGN in the mouse, two different transgenic constructs were generated. First, a conventional transgenic model using the human DC-SIGN cDNA sequence under the control of the murine CD11c promoter (Brocker, Riedinger et al. 1997) was designed. A 1.3-kb cDNA of human DC-SIGN was amplified by PCR using total cDNA from human peripheral blood derived monocytes as template. The amplified cDNA was cloned 3' of the 5.5-kb CD11c promoter *via* Eco RI restriction sites. An appropriate polyadenylation signal was provided by the vector containing the CD11c promoter. Finally, the DNA fragment for injection was obtained by a

combined Not I/Cla I digest and subsequent agarose gel electrophoresis driven purification. This construct was named CD11c-DC-SIGN.

An independent approach was demonstrated by a bacterial artificial chromosome transgenic mouse model. A Sal I digest was performed on the commercial available bacterial artificial chromosome AC008812. The resulting fragment of approximately 70-kb, containing the complete human *CD209* gene locus including the putative promoter, was purified by pulse field gel electrophoresis. This transgenic model was called BAC-DC-SIGN.

These constructs were successfully transferred into pronuclei of C57BL/6 mice by microinjection.

4.2 Analysis of transgene expression

4.2.1 Transgene expression in BMDCs

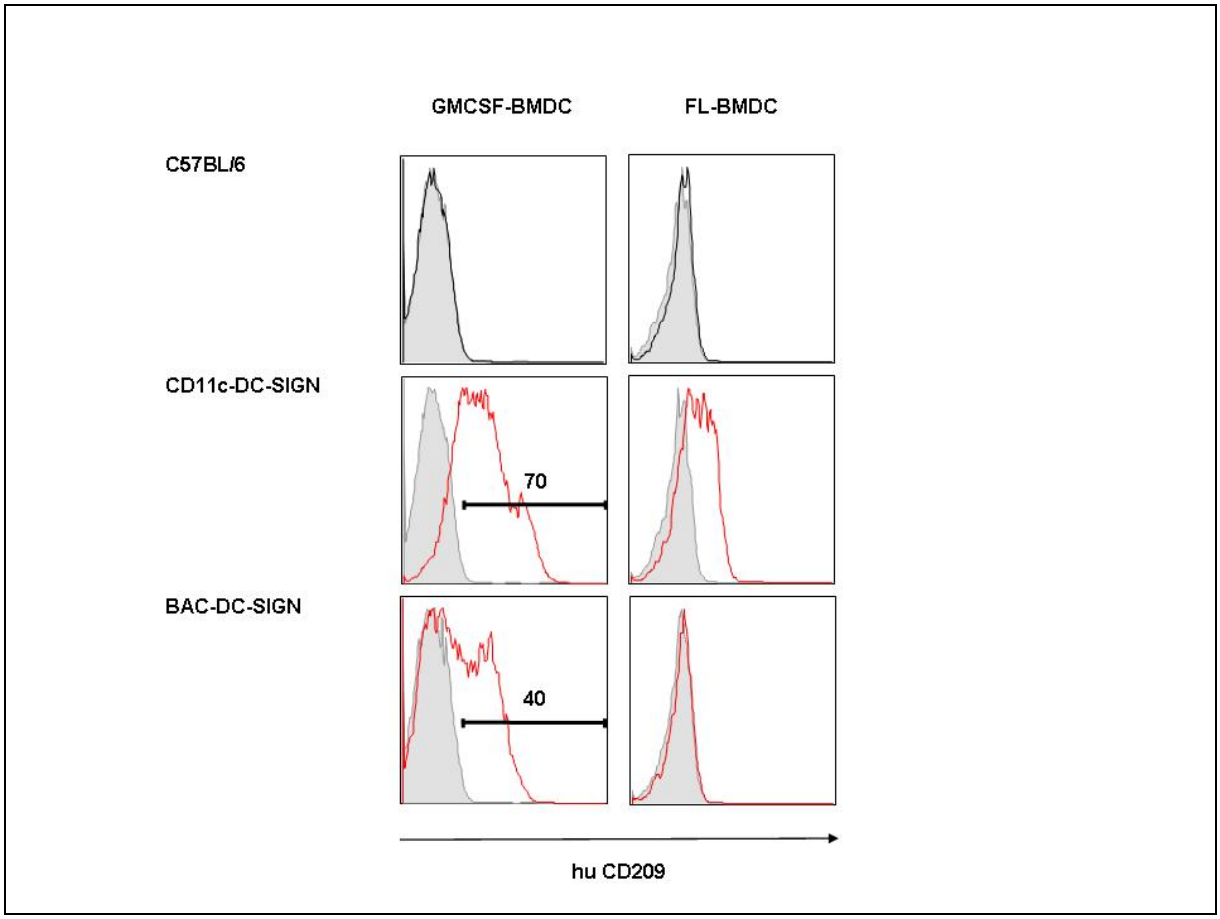

Figure 5: Detection of transgenic human DC-SIGN on *in vitro* generated BMDCs by FACS analysis. The cells were cultured for eight days. GMCSF-BMDCs were stained for CD11c/CD209 whereas FL-BMDCs were analysed for CD11c/CD45R/CD209. Histograms demonstrate CD209 expression of electronically gated living and $CD11c^+$ (GMCSF-BMDC) or $CD11c^+/CD45R^+$ (FL-BMDC) cells. Filled histograms: isotype control, solid lines: specific CD209 staining.

The transgene was expected to be expressed on DCs. For that reason it was initially tried to detect human DC-SIGN on bone marrow-derived dendritic cells (BMDCs) from both transgenic lines. Culture of bone marrow cells in the presence of GMCSF or Flt3-ligand has been shown to induce development of DCs. Culturing with GMCSF supplemented medium gives rise to cells demonstrating a myeloid phenotype (Inaba, Inaba et al. 1992; Brasel, De Smedt et al. 2000). Addition of Flt3-ligand instead of GMCSF favours the formation of plasmacytoid DCs and conventional DC-populations, as found in *ex vivo* isolated splenic DCs (Naik, Proietto et al. 2005). On day eight the cells were harvested and the DC populations were analysed by flow cytometry for expression of human DC-SIGN. Cultures obtained in the presence of GMCSF were stained for surface presence of CD11c and human CD209. The levels of detectable human CD209 on the CD11c positive cells were visualized by histograms. Comparing histograms of wild-type and human DC-SIGN transgenic DCs revealed a detectable expression of human DC-SIGN on 60 – 70 % of the $CD11c^+$ myeloid BMDCs from the CD11c-DC-SIGN transgenic line (**Figure 5**). Performing the same analysis on BMDCs from BAC-DC-SIGN transgenic mice expression of the transgene on a subset of approximately 40 % of $CD11c^+$ myeloid BMDCs could be shown (**Figure 5**). Cultures of bone marrow cells treated with Flt3-ligand were analysed for human DC-SIGN by a different staining and gating strategy. To differentiate between pDCs and cDCs, cells were stained for CD11c and CD45R. The pDCs, defined as $CD11c^+/CD45R^+$, were selected and the amount of cell surface human DC-SIGN was compared between control and transgenic cells. Expression of human DC-SIGN on pDCs from the CD11c-DC-SIGN but not from the BAC-DC-SIGN transgenic line was detectable (**Figure 5**).

These findings are remarkable for two reasons. First, the CD11-DC-SIGN and the BAC-DC-SIGN transgenic construct work specifically in the mouse. Second, the expression in the BMDCs of the BAC-DC-SIGN transgenic mice on myeloid but not on plasmacytoid DCs reflects the physiologic situation in men.

4.2.2 Human DC-SIGN expression on *ex vivo* purified splenic cells of naïve mice

Both transgenic lines show expression of the transgene on *in vitro* generated BMDCs. To investigate the role of human DC-SIGN in infection with *M. tuberculosis in vivo* is an important aim of the presented study. The knowledge of expression levels may be important for the interpretation of infection experiments. Therefore, an expression-analysis of human DC-SIGN on *ex vivo* purified splenic cells was performed in both transgenic mouse lines.

To investigate expression of CD209 on DC-subsets *ex vivo*, spleens were prepared and DCs were enriched by gradient centrifugation.

Sex and age matched mice were sacrificed. Spleens were prepared and digested by a combination of Collagenase/DNAse. In one step mononuclear cells were enriched without erythrocytes contamination by a density gradient based on Nycodenz (Ford and Rickwood 1982). These purified cells were stained for subsequent analysis by flow cytometry with antibodies recognizing murine CD11c, CD45R and human CD209 (or corresponding

isotype-control). Focusing primarily on the staining pattern for CD11c and CD45R, four defined populations of splenic cells can be distinguished and further analysed (**Figure 6 A**). The population lacking both CD11c and CD45R contains mainly T cells and macrophages. B cells are characterized by a $CD11c^-/CD45R^+$ phenotype. The two dendritic cell populations found, are the pDCs, expressing CD11c at intermediary levels and CD45R at a B cell comparable level, and the cDCs which are only positive for CD11c.

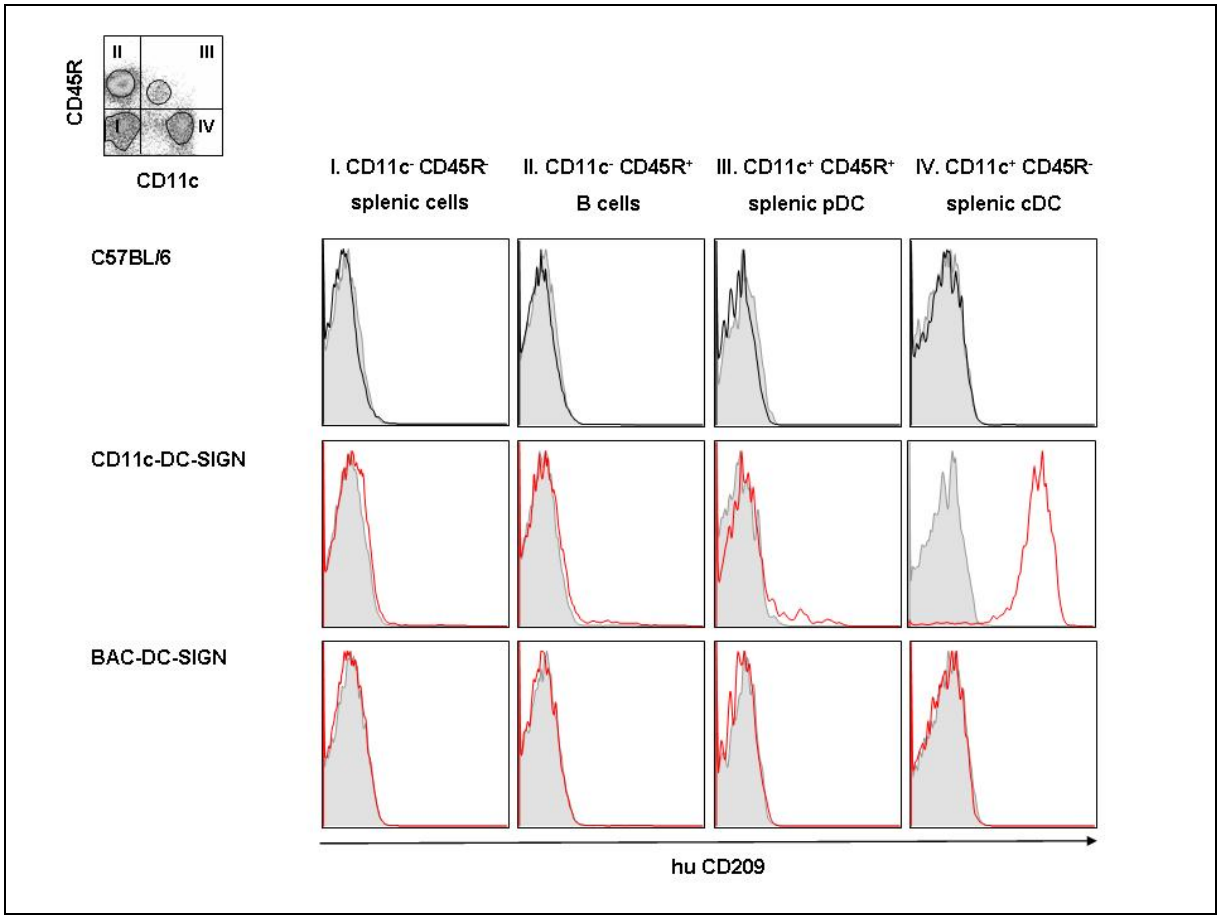

Figure 6: Expression of transgenic human DC-SIGN on splenic cells. Nycodenz gradient enriched splenic cells were stained for CD11c/CD45R and CD209. Histograms demonstrate CD209 expression of electronically gated cells as indicated including a live gate. Filled histograms: isotype control, solid lines: specific CD209 staining.

Detailed analysis of these different cell populations on their expression of transgenic human DC-SIGN was achieved. Nearly 100 % of the splenic cDCs of the CD11c-DC-SIGN transgenic mice express the transgene. Similar to the findings on *in vitro* generated BMDCs (**Figure 5**) splenic pDCs express transgenic human DC-SIGN, but at lower levels than in the splenic cDCs. As expected, neither B cells nor cells double negative for CD11c and CD45R express the transgene. For the BAC-DC-SIGN mouse no transgene expression was detectable on any *ex vivo* isolated splenic cell population.

Ex vivo isolated splenic cDCs and pDCs of naive CD11c-DC-SIGN but not BAC-DC-SIGN transgenic mice express human DC-SIGN.

4.2.3 Human DC-SIGN expression on *ex vivo* purified splenic cells after IL-4 treatment

No expression of human DC-SIGN was detectable on *ex vivo* isolated splenic cells of naïve BAC-DC-SIGN mice. Expression of human DC-SIGN on human monocyte derived dendritic cells has been shown to be IL-4 dependent (Relloso, Puig-Kroger et al. 2002). As the putative human promoter sequence of *CD209* is used to control expression in the BAC-DC-SIGN transgenic line, IL-4 may be needed to achieve detectable levels of human DC-SIGN on the cell surface. Naïve mice are not expected to have high levels of IL-4. For that reason mice of the two transgenic lines and wild-type mice were daily injected intraperitoneally with 2 µg of murine IL-4. After seven days the mice were sacrificed, spleens were removed, digested and enriched for DCs via a Nycodenz gradient (Ford and Rickwood 1982).

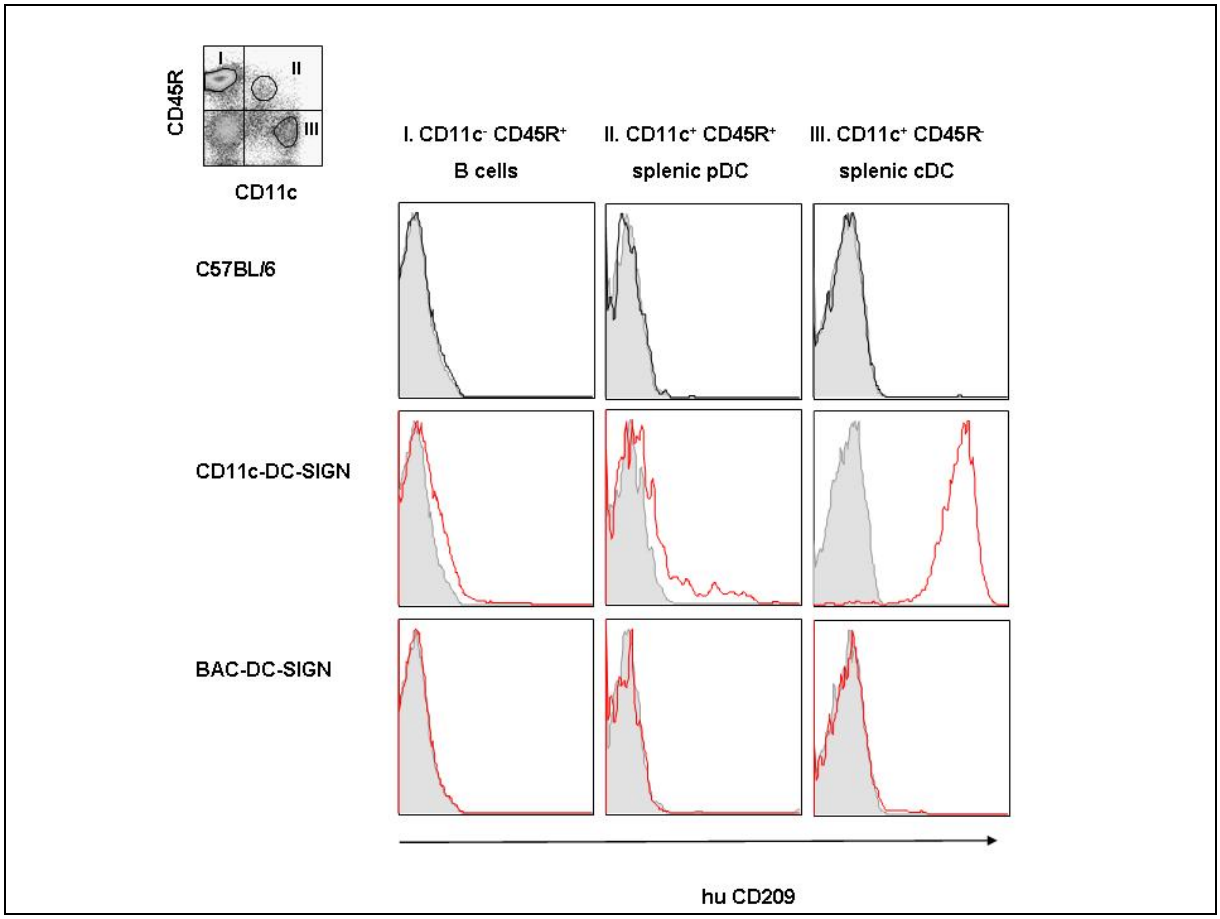

Figure 7: Transgene expression on splenic cells after daily treatment with 2 µg of murine IL-4 for seven days. Daily 2 µg of IL-4 were intraperitoneally applied for one week. Histodenz gradient enriched splenic cells were stained for CD11c/CD45R and CD209. Histograms demonstrate CD209 expression of electronically gated cells as indicated including a live gate. Filled histograms: isotype control, solid lines: specific CD209 staining.

Subsequently isolated cells were stained for analysis by flow cytometry with antibodies recognizing murine CD11c, CD45R and CD209 (or corresponding isotype-control) (**Figure 7**). As shown in naïve mice (**Figure 6**), only *ex vivo* enriched splenic pDCs and cDCs of the

CD11c-DC-SIGN mice express human DC-SIGN after IL-4 application (**Figure 7**). Treatment with murine IL-4 (2 µg per day for seven days) did not induce the expression of human DC-SIGN on splenic cells in BAC-DC-SIGN transgenic mice.

4.2.4 Transgene expression in peritoneal lavage cells

Besides IL-4, also infection with *M. tuberculosis* has been demonstrated to increase levels of human DC-SIGN on the cell surface of target cells (Tailleux, Pham-Thi et al. 2005). In addition, human DC-SIGN has been shown to be slightly expressed on cells, which could be myeloid dendritic cell precursors (McCully, Chau et al. 2005), in the peritoneum of men. Therefore, we decided to analyse peritoneal cells in the two human DC-SIGN mouse models for expression of the transgene after inducing a sterile infection by thioglycolate injection. After performing a peritoneal lavage, cell surface expression of murine CD11c, CD11b and CD209 (and corresponding isotype-control) was analysed by flow cytometry.

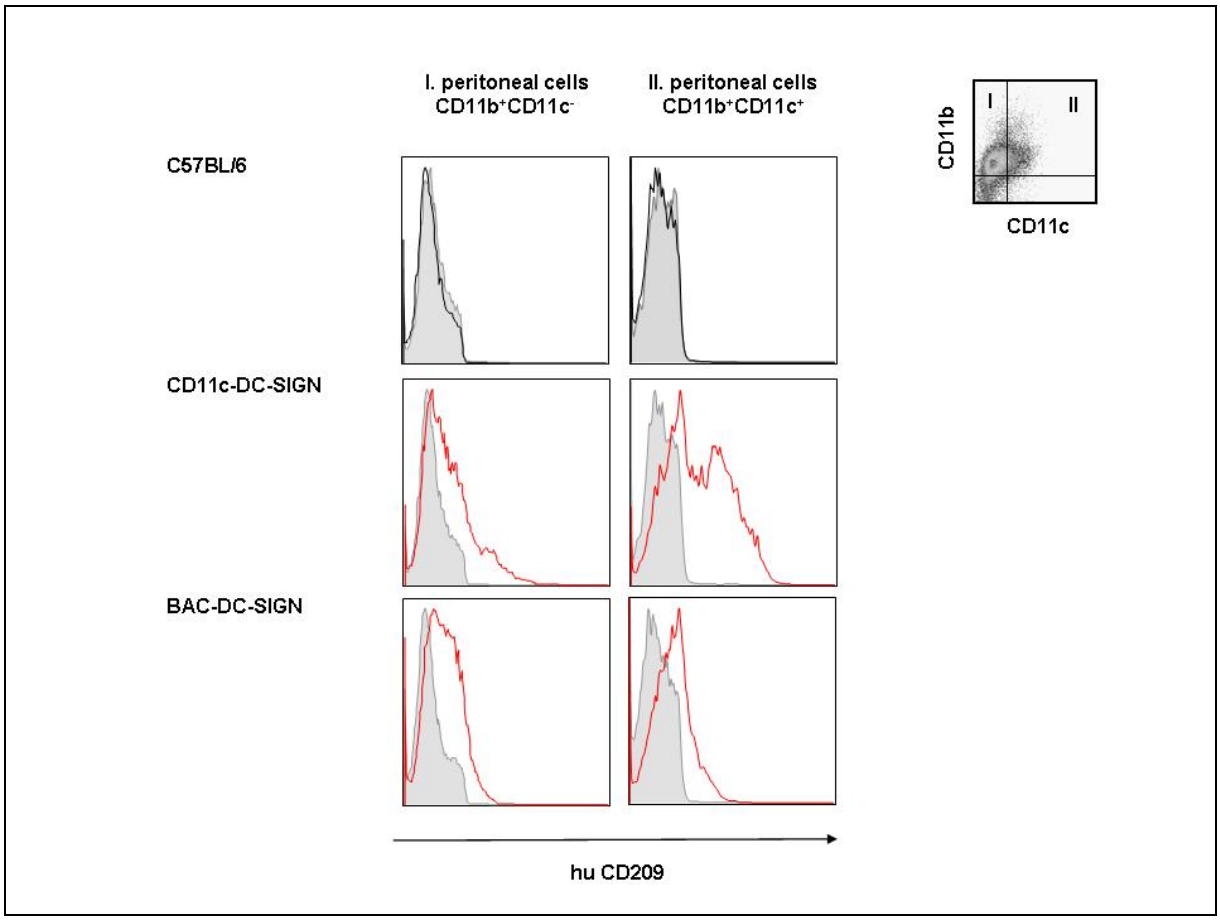

Figure 8: Transgene expression in peritoneal lavage cells. A peritoneal lavage was performed ten days after intraperitoneal application of thioglycolate. Cells were stained for CD11c/CD11b and CD209. Histograms demonstrate CD209 expression of electronically gated cells as indicated including a live gate. Filled histograms: isotype control, solid lines: specific CD209 staining.

Analysis of the lavage cells demonstrated human DC-SIGN to be present on peritoneal lavage cells positive for CD11c and CD11b in the CD11c-DC-SIGN and BAC-DC-SIGN

transgenic mouse (**Figure 8**). On peritoneal cells of the CD11c-DC-SIGN mouse the expression level of the transgenic molecule was higher than in the BAC-DC-SIGN model.

4.2.5 Splenic transgene expression after Flt3-ligand treatment

To expand a possible small human DC-SIGN expressing myeloid DC subset in the spleens of the BAC-DC-SIGN line, one mouse each was subcutaneously injected with $1*10^6$ Flt3-ligand secreting B16 melanoma cells (Shi, Villadangos et al. 1999). Ten days after injection the spleens were harvested and digested. To augment splenic dendritic cells *ex vivo*, an OptiPrep™ gradient was performed. The isolated cells were splitted in two samples. One probe was stained for surface expression of CD11c, CD45R and CD209 whereas the other sample was additionally permeabilized and stained for intracellular expression of CD209 (**Figure 9**).

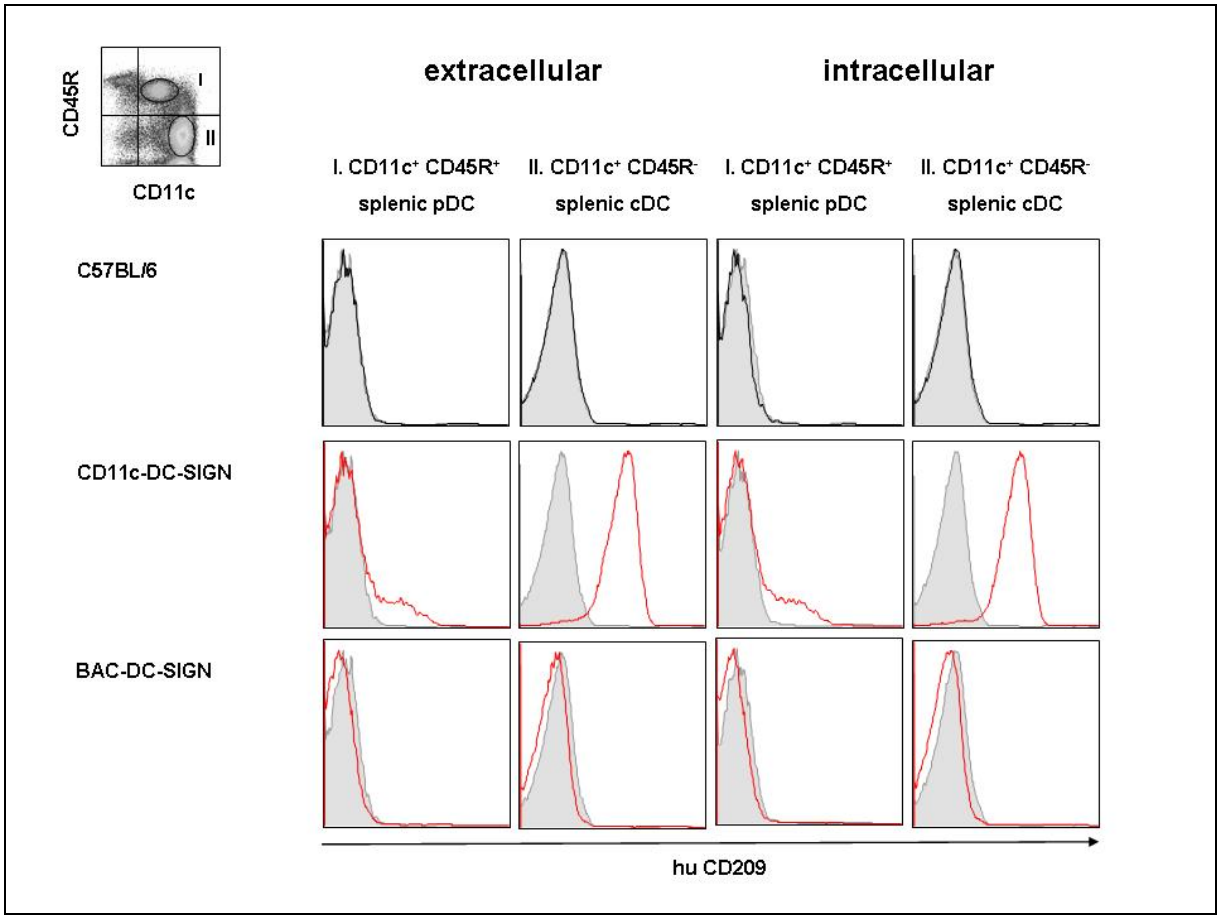

Figure 9: Transgene expression on splenic cells at day 10 after subcutaneous injection of $1*10^6$ Flt3-ligand secreting B16 melanoma cells. At day 10 after subcutaneous injection of $1*10^6$ Flt3-ligand secreting B16 melanoma cells mice were sacrificed. The splenic dendritic cells were enriched by the usage of an OptiPrep™ gradient. Samples were stained for CD11c/CD45R and CD209. Histograms demonstrate CD209 expression of electronically gated cells as indicated including a live gate. Filled histograms: isotype control, solid lines: specific CD209 staining.

Splenic pDCs and cDCs of the CD11c-DC-SIGN transgenic mice express human DC-SIGN after Flt3-ligand treatment (**Figure 9**). Within the cDC population the percentage of transgene

expressing cells is higher than within the pDC population. As permeabilization of the cells does not increase signal intensity, an intracellular localization of human DC-SIGN can be excluded. Even after Flt3-ligand treatment no expression of human DC-SIGN can be detected on splenic DCs in BAC-DC-SIGN transgenic mice, neither on the cell surface nor inside the cells.

4.2.6 Transgene expression in peripheral blood monocytes

In men, a small fraction of peripheral blood monocytes is known to express DC-SIGN (Engering, Van Vliet et al. 2002). To check whether this was true in one of our two transgenic models peripheral blood was collected of each transgenic line. After heparin treatment the mononuclear cells were stained for CD11b, GR-1 and CD209 and finally analysed by flow cytometry.

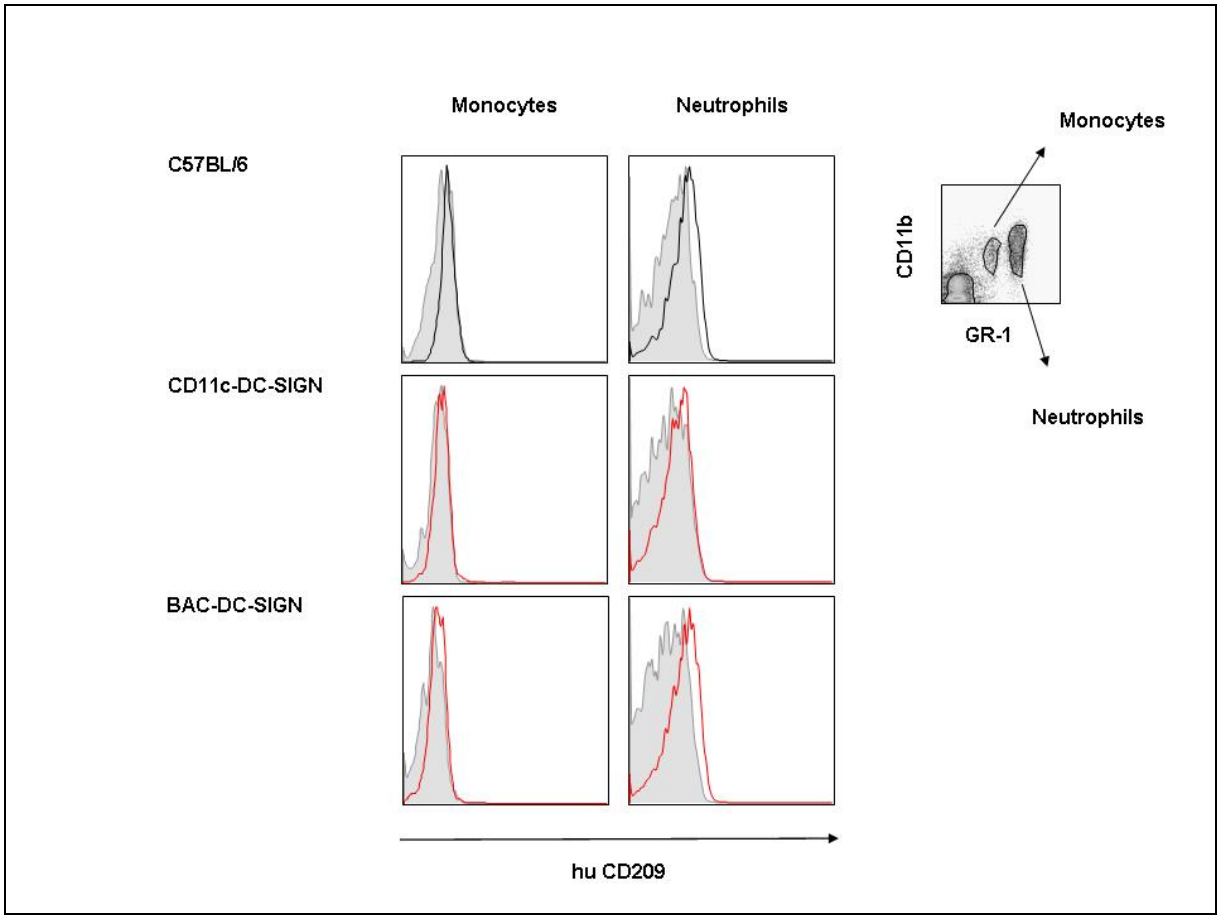

Figure 10: Transgene expression on peripheral blood monocytes. Mice were sacrificed and blood was prepared using a heparin pretreated syringe. Following red blood cell lysation cells were stained for GR-1/CD11b and CD209. Histograms demonstrate CD209 expression of electronically gated cells on monocytes including a live gate. Filled histograms: isotype control, solid lines: specific CD209 staining.

The cell population positive for CD11b and expressing GR-1 on a low level was defined as monocytes. Neutrophils are characterized by high levels of CD11b and GR-1 (Lagasse and Weissman 1996). All peripheral blood monocytes were negative for human DC-SIGN in the

CD11c-DC-SIGN and in the BAC-DC-SIGN model (**Figure 10**). This finding was also true for the neutrophils.

4.2.7 Transgene transcript levels in CD11c-DC-SIGN mice

Besides checking expression of the transgene on the protein level, the mRNA-transcripts of transgenic human DC-SIGN in different organs of the two transgenic mouse models was quantified. Mice were sacrificed and 50 to 100 mg of tissue samples from different organs were rapidly homogenized in TRIzol Reagent. After the sample homogenization, total RNA was prepared following standard protocols.

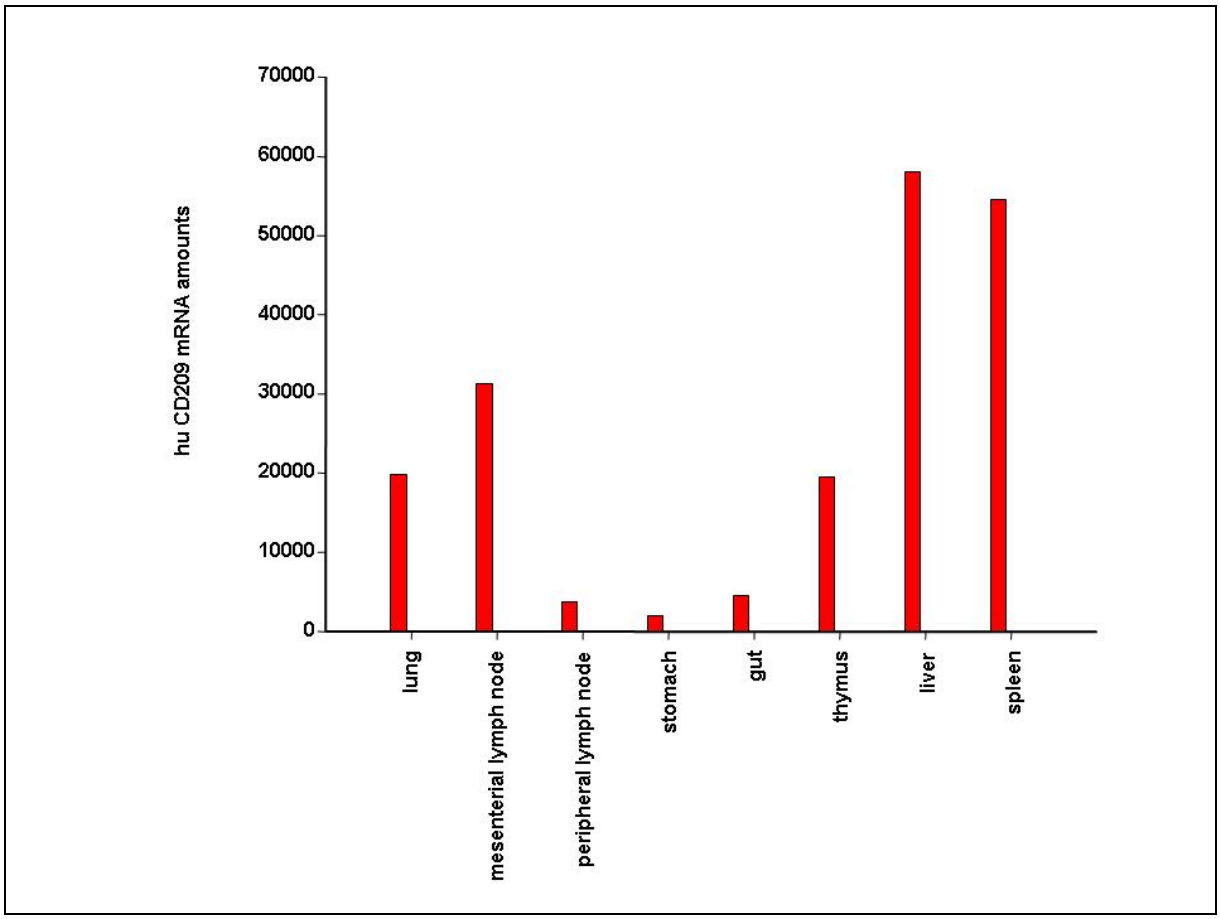

Figure 11: mRNA transcript levels of human DC-SIGN in CD11c-DC-SIGN transgenic mice. Organs were directly homogenized in 1 ml of TRIzol® Reagent. mRNAs were prepared as described. The CD209 mRNA transcript levels were detected by TAQMan Real Time PCR using a probe for human CD209. The demonstrated data are normalized on β-Actin levels.

The aquired mRNA was transcribed into cDNA by RT-PCR. Using a human *CD209* specific probe, the mRNA transcript levels were determined by TAQMan Real Time PCR. As illustrated in **Figure 11**, a very high amount of transgene expression in liver, spleen and mesenterial lymph node can be observed in the CD11c-DC-SIGN transgenic line. Checking the same organs in BAC-DC-SIGN transgenic mice reveals an overall very low expression of the transgene (**Figure 12**).

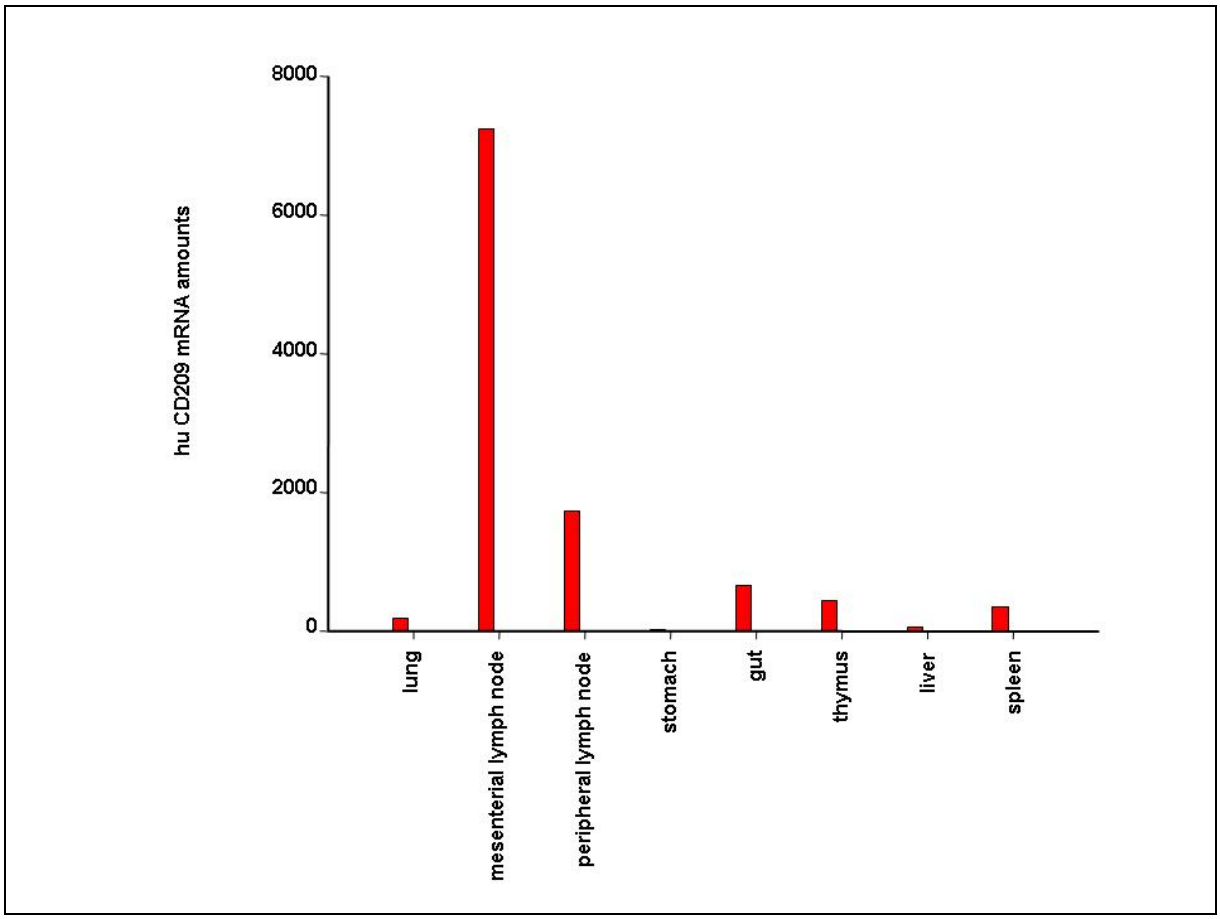

Figure 12: mRNA transcript levels of DC-SIGN in BAC-DC-SIGN transgenic mice. Organs were directly homogenized in 1 ml of TRIzol® Reagent. mRNAs were prepared as described. The CD209 mRNA transcript levels were detected by TAQMan Real Time PCR using a probe for human CD209. The demonstrated data are normalized on β-Actin levels.

The strongest human DC-SIGN signal was detectable in the mesenterial lymph node of the BAC-DC-SIGN model (**Figure 12**).

In the CD11c-DC-SIGN transgenic model expression of the transgene can be found in GMCSF and Flt3-ligand derived bone marrow dendritic cells. *Ex vivo* isolated splenic pDCs and cDCs, no matter whether the animal is in naïve state or has been treated by IL-4 or Flt3-ligand express the transgene. No significant expression of human DC-SIGN was detectable on non DC-like cell populations. On the level of mRNA transcripts it can be concluded that very high amounts of DC-SIGN transcripts are synthesized in liver, spleen and mesenterial lymph nodes.

Using the BAC-DC-SIGN transgenic model we observe a different kind of DC-SIGN expression profile. *In vitro* generated myeloid but not plasmacytoid dendritic cells express the transgene. Neither on the cell surface nor intracellular it is possible to detect the transgene on protein level of different freshly prepared lymphatic organs. Only on peritoneal lavage cells after thioglycolate induced sterile infection transgenic human DC-SIGN is present. Analysing the amount of transgenic human DC-SIGN mRNA in different organs demonstrated an overall very low expression level. The highest amount is detectable in mesenterial lymph nodes.

4.3 Role of human DC-SIGN *in vitro*

4.3.1 Cytokine response by human DC-SIGN transgenic BMDCs to infection with *M. bovis* BCG

Published data using human DCs (Geijtenbeek, Van Vliet et al. 2003), demonstrated a DC-SIGN dependent increase in IL-10 production in response to mycobacterial stimuli *in vitro*. To verify, if DCs of our human DC-SIGN transgenic mice would mirror this effect, GMCSF BMDCs of wild-type and CD11c-DC-SIGN transgenic mice were infected with different doses of *M. bovis* BCG for 24 h. As controls two pure TLR-stimuli (CpG1668 and Pam_3Csk_4) and cell wall components (CWC) of *M. tuberculosis* H37Rv were used.

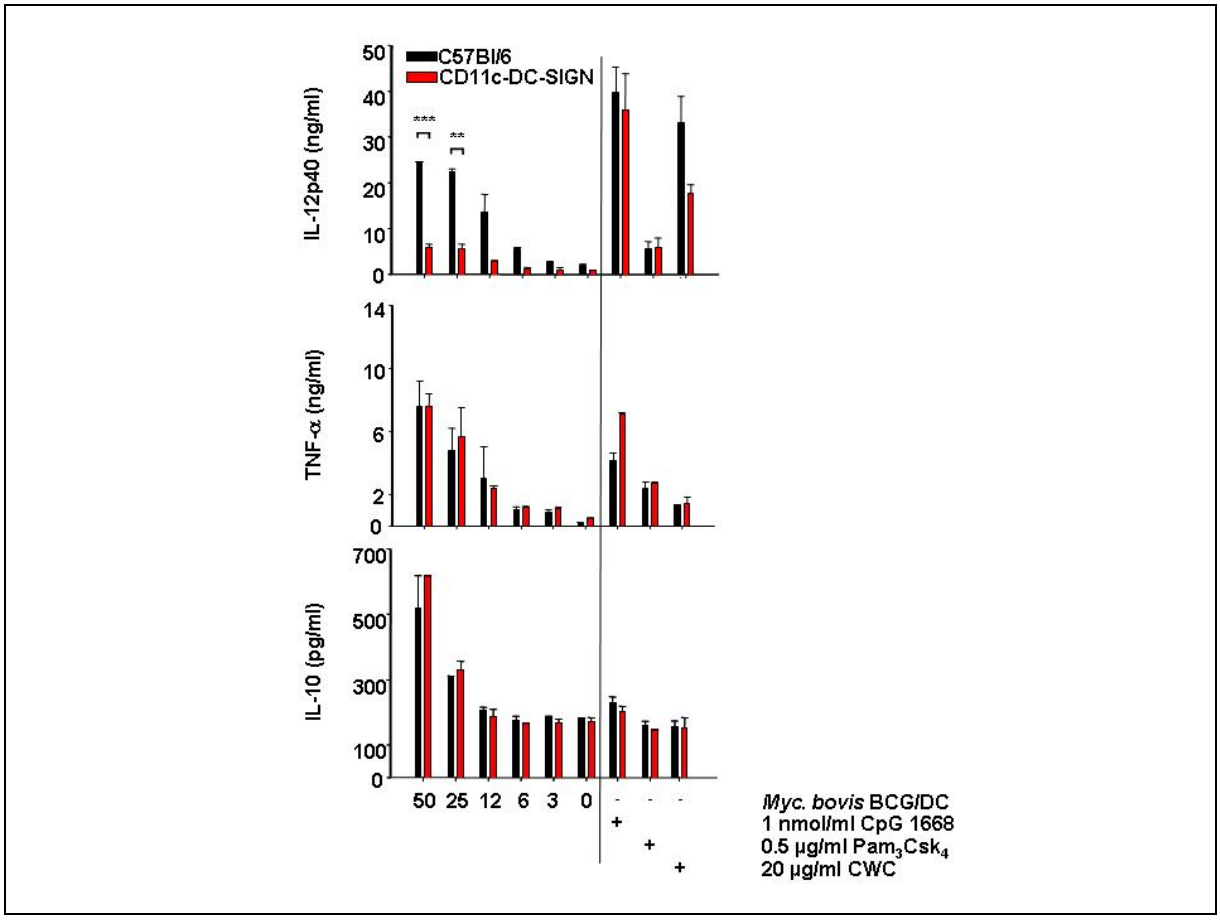

Figure 13: Cytokine response of DC-SIGN transgenic GMCSF-BMDCs to infection with *M. bovis* BCG. $5*10^4$ GMCSF d8 BMDCs per well of a 96-well plate were stimulated for 24 h. Data represent duplicate values of two individual mice each. Each points indicates the mean +SD. **, p<0,01; ***, p<0,001.

The supernatants were analysed by ELISA for IL-10, TNF-α and IL-12p40 production. Production of all three cytokines after infection with *M. bovis* BCG is clearly dose dependent. In contrast to the published *in vitro* data using human DCs, demonstrating a DC-SIGN dependent enhancement in IL-10 secretion after costimulation of human DCs with LPS and the mycobacterial cell wall component ManLAM (Geijtenbeek, van Vliet et al. 2003), no DC-SIGN dependent increase in the production of IL-10 by BMDCs, infected with *M. bovis*

BCG, can be observed (**Figure 13**). In addition there is no difference in the production of TNF-α between wild-type and human DC-SIGN transgenic dendritic cells. But human DC-SIGN transgenic BMDCs show a significant reduction in IL-12p40 production by 30–50 % (dose dependent) in response to infection with *M. bovis* BCG (**Figure 13**). Using pure TLR-stimuli for TLR2 (Pam_3Csk_4) or TLR9 (CpG) similar cytokine responses in wild-type and DC-SIGN transgenic BMDCs were observed. Stimulation with cell wall components (CWC) of *M. tuberculosis* H37Rv resulted in cytokine profiles reflecting the outcome after infection with *M. bovis* BCG. The cytokine production of BAC-DC-SIGN transgenic BMDCs was comparable to wild-type BMDCs (data not shown).

Also the release of the bioactive IL-12p70, a heterodimer of IL-12p35 and IL-12p40 (Gubler, Chua et al. 1991), by DC-SIGN transgenic BMDCs is decreased in response to infection with *M. bovis* BCG (**Figure 14**).

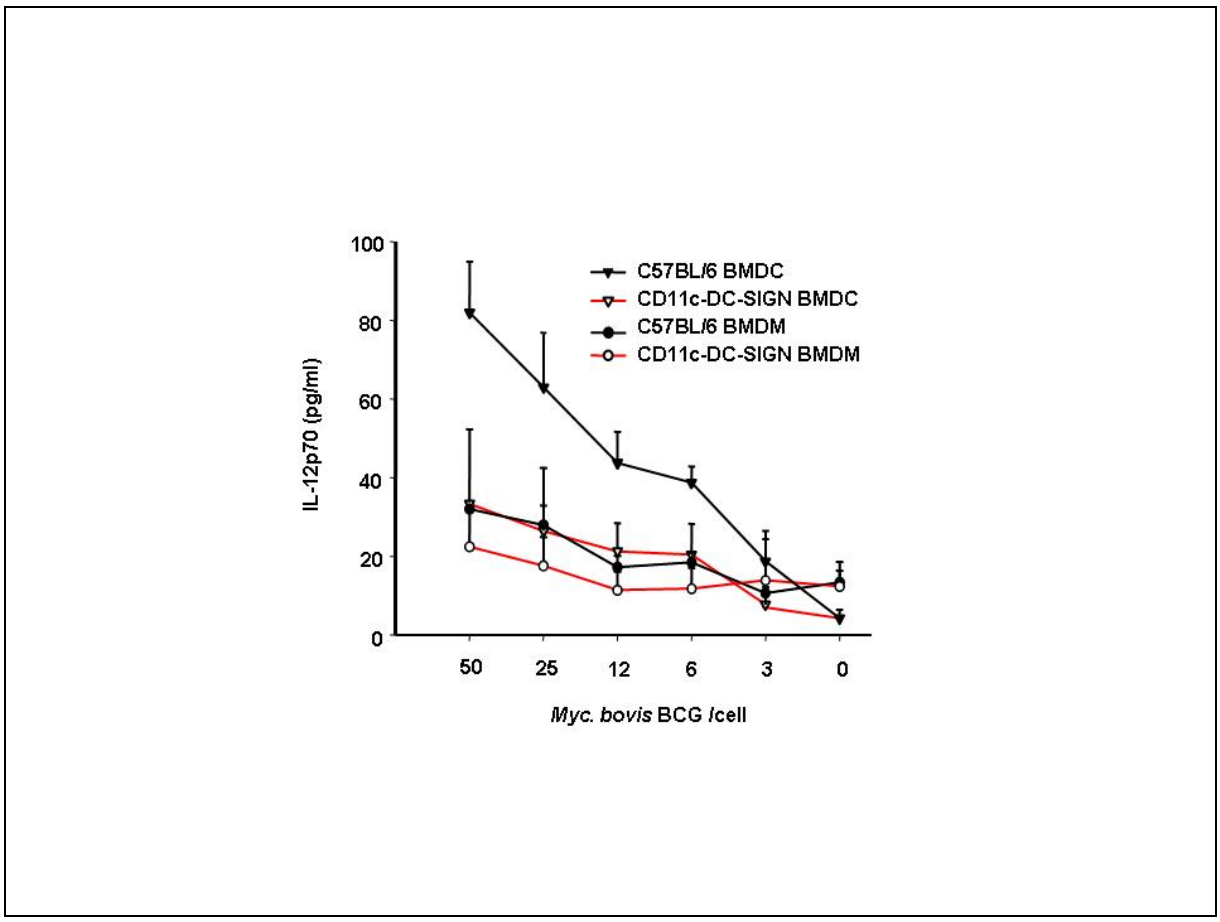

Figure 14: Release of IL-12p70 of DC-SIGN transgenic BMDCs and BMDMs after infection with *M. bovis* BCG. $5*10^4$ BMDCs or BMDMs per well of a 96-well plate were stimulated for 24 h. Data represent duplicate values of two individual mice each. Each points indicates the mean +SD.

Macrophage cultures generated from the same bone marrows produced low amounts of IL-12p70. However, no difference could be observed between wild-type and human DC-SIGN transgenic BMDMs.

4.3.2 Cytokine response by human DC-SIGN transgenic BMDCs infected with *M. tuberculosis*

After analysing the DC-SIGN dependent effect on cytokine production by murine BMDCs infected with *M. bovis* BCG, the cytokine response of BMDCs to infection with different virulent strains of *M. tuberculosis* was studied in cooperation with the group of Prof. Ehlers (Research Center Borstel, Borstel, Germany). Murine BMDCs of wild-type and CD11c-DC-SIGN transgenic animals were infected for 24 hours with five different strains of *M. tuberculosis*. The first strain H37Rv is the major strain of *M. tuberculosis* used in laboratories worldwide. Secondly, the mycobacterial strain CDC 1551, which was isolated during an outbreak of tuberculosis close to the Kentucky-Tennessee (USA) border (Valway, Sanchez et al. 1998), was used. For this strain the induction of higher amounts of cytokines, compared with the common lab strain H37Rv, was demonstrated (Manca, Tsenova et al. 1999). The third and fourth used bacterial strains belong to the so called Beijing-family. The Beijing family was initially identified to be responsible for severe course of tuberculosis in the Far East (van Soolingen, Qian et al. 1995). They were designated as Beijing family since a common precursor of these strains was thought to derive from the area around Beijing. A patient suffering from tuberculosis in Afghanistan was the source of the strain Beijing 3547/01 whereas Beijing 4203/01 was isolated from samples of a Vietnamese. At last a new isolate from Ghana named Ghana TB2 8256/02 was used for infection of BMDCs. The strain from Ghana has so far not been analysed in more detail. An MOI of 3 was used for infection with all these mycobacterial strains. After 24 hours the supernatants were analysed for their amount of induced IL-12p40 cytokine production. The amount of secreted IL-12p40 is dependent on the strain used for infection. From the strains used for this experiment, H37Rv is the least efficient inducer of cytokine production (20 ng/ml). Using the *M. tuberculosis* CDC1551 strain for infection, wild-type BMDCs produce around 45 ng/ml of IL-12p40. The two members of the Beijing family and the isolate from Ghana are the most potent IL-12p40 inducers. In response to these pathogens BMDCs from control mice release up to 60 ng/ml of IL-12p40, as seen in **Figure 15**.

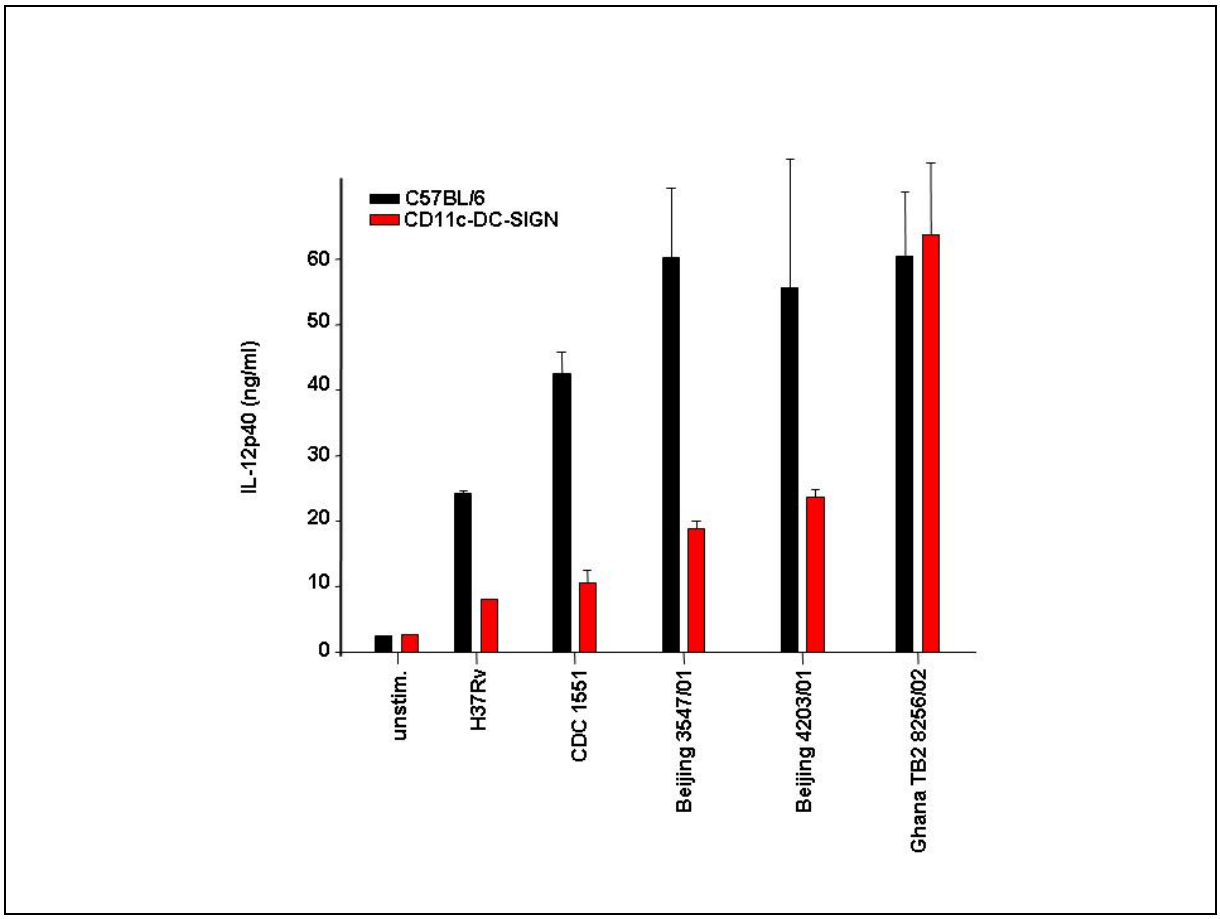

Figure 15: Cytokine response of DC-SIGN transgenic GMCSF-BMDCs to infection with different strains of *M. tuberculosis* 2.5*10^5 GMCSF d8 BMDCs per well of a 24-well plate were infected with different strains of *M. tuberculosis* (MOI:3). The supernatants were harvested 16 h later.

In the case of infection with H37Rv, CDC1551 and the two Beijing strains of *M. tuberculosis* a reduction in IL-12p40 production can be observed for human DC-SIGN bearing cells. Only infection with the new isolate from Ghana, termed Ghana TB2 8256/02 leads to similar IL-12p40 production by control and human DC-SIGN transgenic BMDCs. At the moment the reason for this difference is still unclear.

4.3.3 Uptake of *M. bovis* BCG by BMDC

Several receptors are involved in recognition, binding and uptake of mycobacteria by APCs. Lack of these receptors can decrease the capability of APCs to phagocytose the bacilli and/or impair the cellular response towards a mycobacterial infection, as it has been shown for TLR2 (Heldwein, Liang et al. 2003) and the complement receptor 3 (Rooyakkers and Stokes 2005). We determined whether the presence of the transgenic human DC-SIGN induces an increased uptake of mycobacteria. For that reason an assay analysing binding and uptake of *M. bovis* BCG by control and human DC-SIGN transgenic BMDCs was performed. BMDCs were infected for two hours with *M. bovis* BCG at an MOI of 10 at 37°C or 4°C. One half of the infected cells were additionally treated with trypsin-EDTA, in order to remove unbound mycobacteria and to determine the percentage of ingested bacilli.

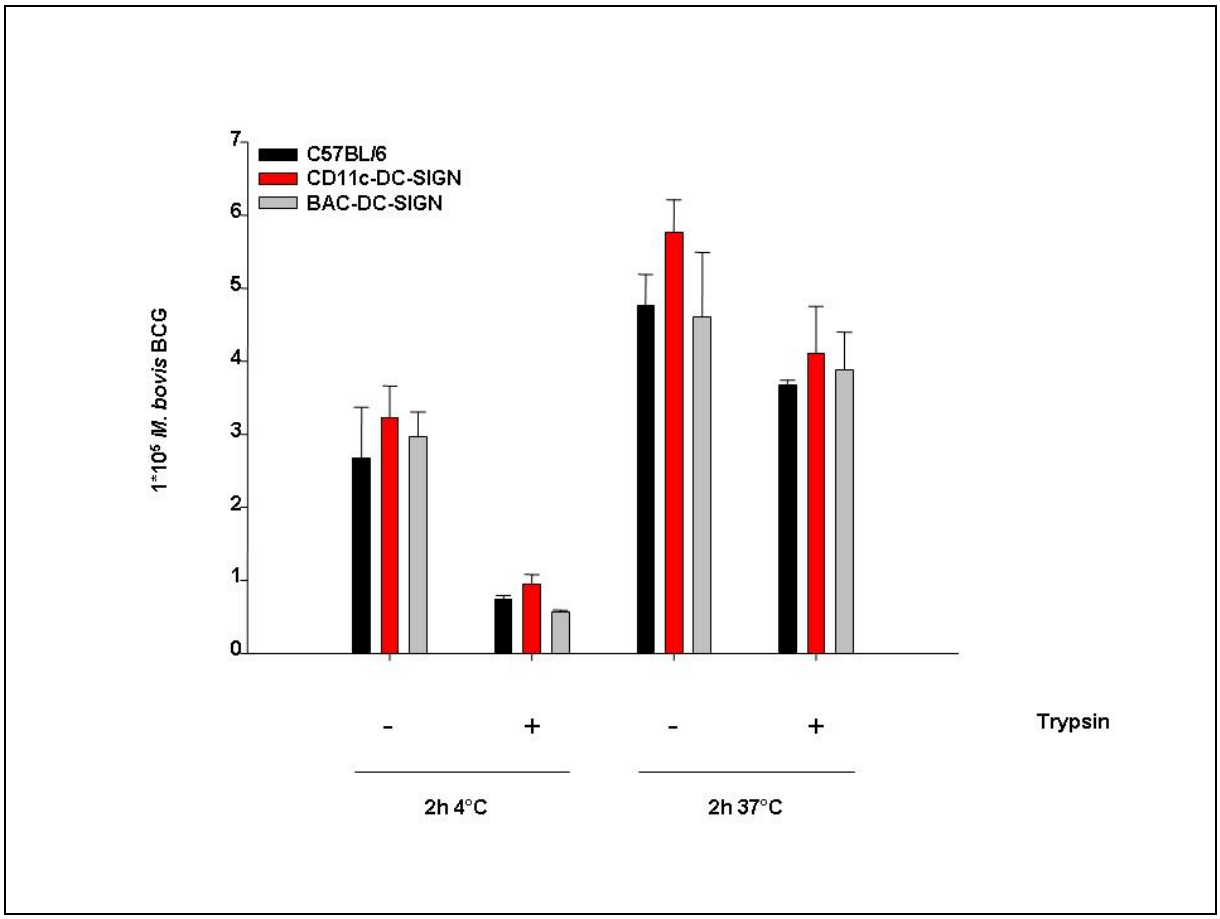

Figure 16: Binding and Uptake of *M. bovis* BCG by GMCSF-BMDCs. $1*10^6$ BMDCs were infected with *M. bovis* BCG at an MOI of 10 for the indicated time and temperature. After infection cells were washed twice with PBS. All samples were lysed by the addition of 1 ml of H_2O (0.1%SDS). Finally samples were plated in tenfold serial dilutions on Middlebrook 7H10 agar plates containing Middlebrook OADC-Enrichment. After 21 days the number of clones was counted.

At 4°C only a small portion of bacteria seemed to be taken up (represented by the trypsinized sample). The difference between the untreated and the trypsinized probes at 4°C represents the steady-state binding of mycobacteria to the cell surface of wild-type, CD11c-DC-SIGN or BAC-DC-SIGN transgenic dendritic cells. BMDCs of all three lines bind similar quantities of *M. bovis* BCG at steady-state. A similar effect was observed after shifting cells to 37°C. Nearly 80 % of the cell surface bound mycobacteria have been phagocytosed by the dendritic cells after 2h at 37°C. There is no evidence, that the presence of the transgene influences mycobacterial uptake and binding by dendritic cells(**Figure 16**).

4.3.4 Activation of MAPK in *M. bovis* BCG infected BMDCs

It was postulated that after mycobacterial infection of human dendritic cells a DC-SIGN dependent inhibition of TLR signalling is induced (Geijtenbeek, van Vliet et al. 2003). For that reason the activation of effectors downstream of TLRs in BMDCs from *M. bovis* BCG infected wild-type and DC-SIGN transgenic mouse lines were verified. Members of the mitogen-activated protein kinases (MAPK) family are crucial mediators for TLR induced signalling pathways.

BMDCs were infected for different periods with *M. bovis* BCG, lysed and analysed by SDS-PAGE and subsequent Western Blot for phosphorylation of the MAPKs p38, Erk and JNK. For monitoring DC-SIGN independent stimulation, one set of samples was stimulated for 40 minutes with the the TLR9-stimulus CpG. To demonstrate equal protein loading per lane, the nitrocellulose membrane was also reprobed for total amount of each MAPK and the constitutively expressed protein β-Actin.

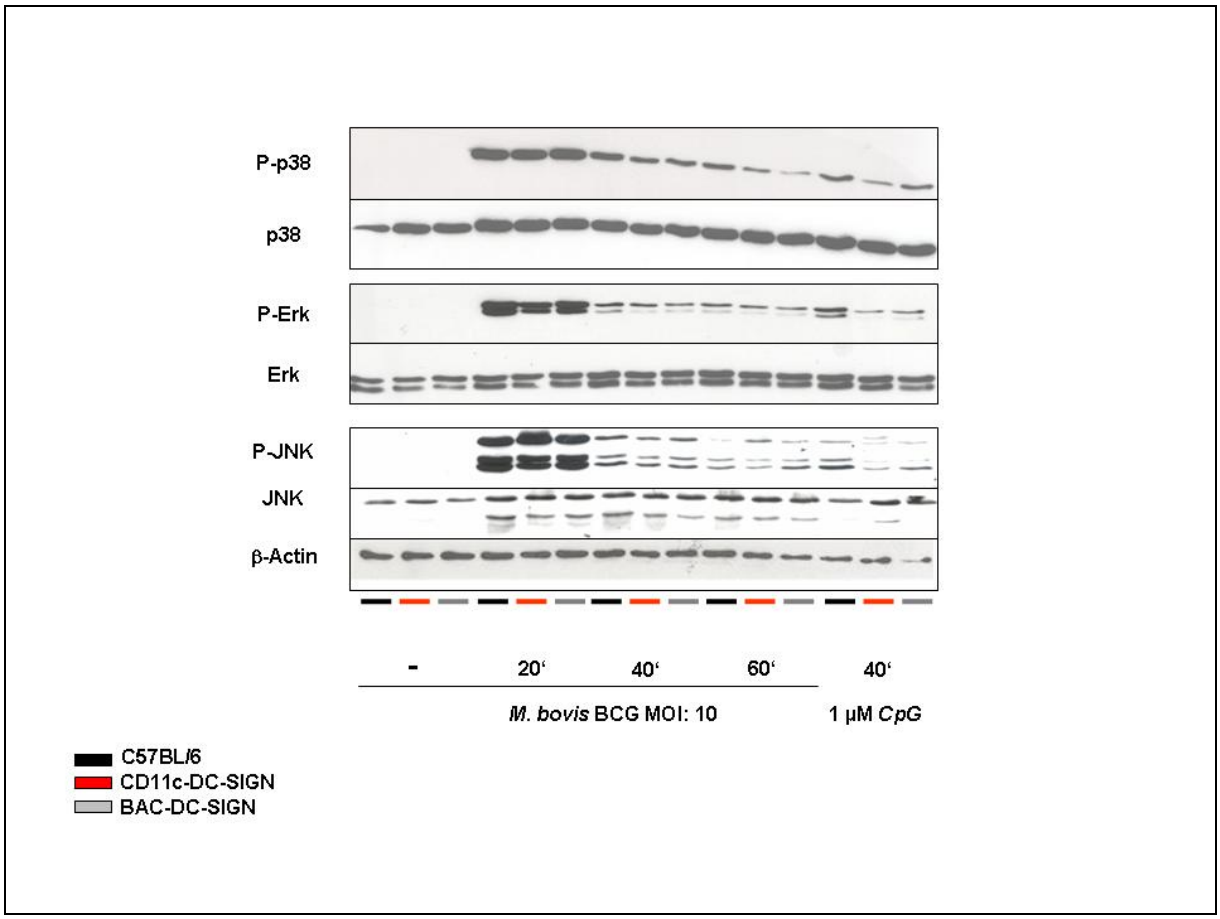

Figure 17: MAPK-activation in *M. bovis* BCG infected BMDCs. $1*10^6$ BMDCs were infected for the indicated times with *M. bovi*s BCG at an MOI of 10. Lysates were prepared as described and checked for activation of MAPK.

20 minutes after the infection of BMDCs with *M. bovis* BCG a significant activation of all three MAPKs (p38, Erk and JNK) was detectable. Following initial induction the activation of the kinases is quickly reduced almost to background levels at 40 and 60 minutes. Because signal intensity in lysates of wild-type and DC-SIGN transgenic BMDCs was similar, the phosphorylation level of the analysed MAPKs did not seem to be affected by the presence of human DC-SIGN. Stimulation by the pure TLR9 ligand CpG resulted in a similar activation of all three MAPKs in wild-type and human DC-SIGN transgenic BMDCs. Concerning the bands representing total amounts of MAPKs and β-Actin equal protein loads can be ensured for all samples (**Figure 17**).

4.3.5 IκBα activation in *M. bovis* BCG-infected BMDCs

Besides MAPKs also IκBα plays a role in mediating TLR-signalling to the nucleus *via* final activation of NF-κB (Ghosh and Baltimore 1990). Phosphorylation of IκBα induces a fast degradation of the molecule and results in translocation of NF-κB into the nucleus. As IκBα is resynthesized quickly these experiments were performed in the presence of cycloheximide, an inhibitor of protein synthesis. BMDCs were infected for different time periods with *M. bovis* BCG, lysed and analysed by Western Blot for signals representing phosphorylated isoforms of IκBα. After detection of phosphorylated IκBα the western blot membrane was reproped for the constitutively expressed protein β-Actin to verify equal protein loads.

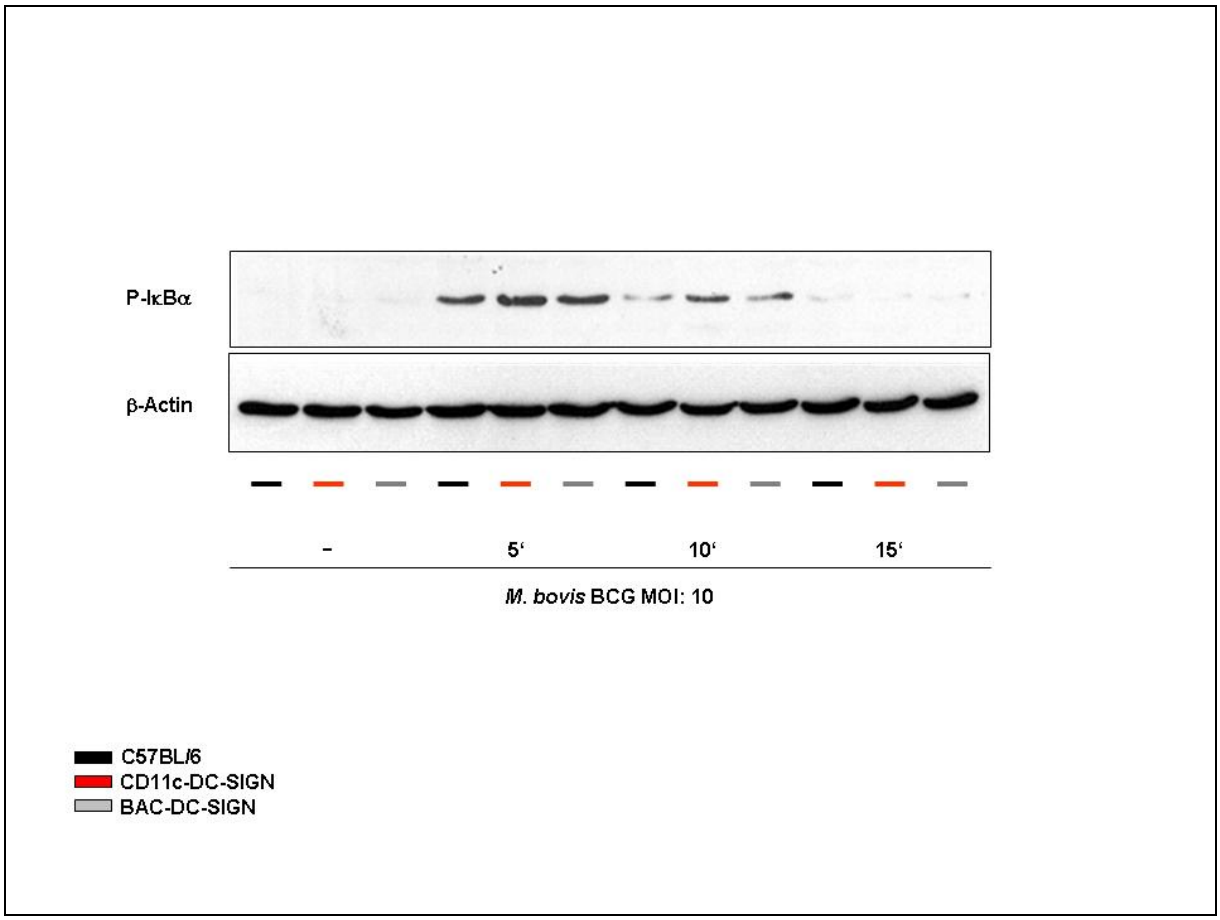

Figure 18: IκBα activation in *M. bovis* BCG infected BMDCs. $1*10^6$ BMDCs were infected for the indicated times with *M. bovis* BCG at an MOI of 10. Lysates were prepared as described and checked for activation of IκBα.

Phosphorylated IκBα can be detected already five minutes after infection with *M. bovis* BCG. Ten minutes after infection the signal intensity is dramatically reduced and reached the level of unstimulated cells as early as five minutes later. For non of the investigated time points any difference in activation of IκBα in response to mycobacterial infection between wild-type and human DC-SIGN transgenic BMDCs could be confirmed.

4.3.6 NF-κB activation in DCs after mycobacterial stimulation

The induction of the proinflammatory cytokine IL-12p40 is mainly dependent on activation of the transcription factor NF-κB (Murphy, Cleveland et al. 1995). Therefore a reduced secretion of IL-12p40 after infection with *M. bovis* BCG and *M. tuberculosis* H37Rv, as measured for BMDCs expressing DC-SIGN, could be due to an impaired activation and nuclear translocation of NF-κB. To address the question of a direct DC-SIGN impact on the NF-κB activation, electrophoretic mobility shift assays (EMSA) were performed. BMDCs were stimulated for 40 minutes with two different suboptimal doses of the TLR4-stimulus LPS alone and in combination with cell wall components of *M. tuberculosis* H37Rv (CWC). Nuclear extracts were prepared and analysed for activation of NF-κB by EMSA. As loading control we verified NF-Y, a constitutively expressed transcription factor.

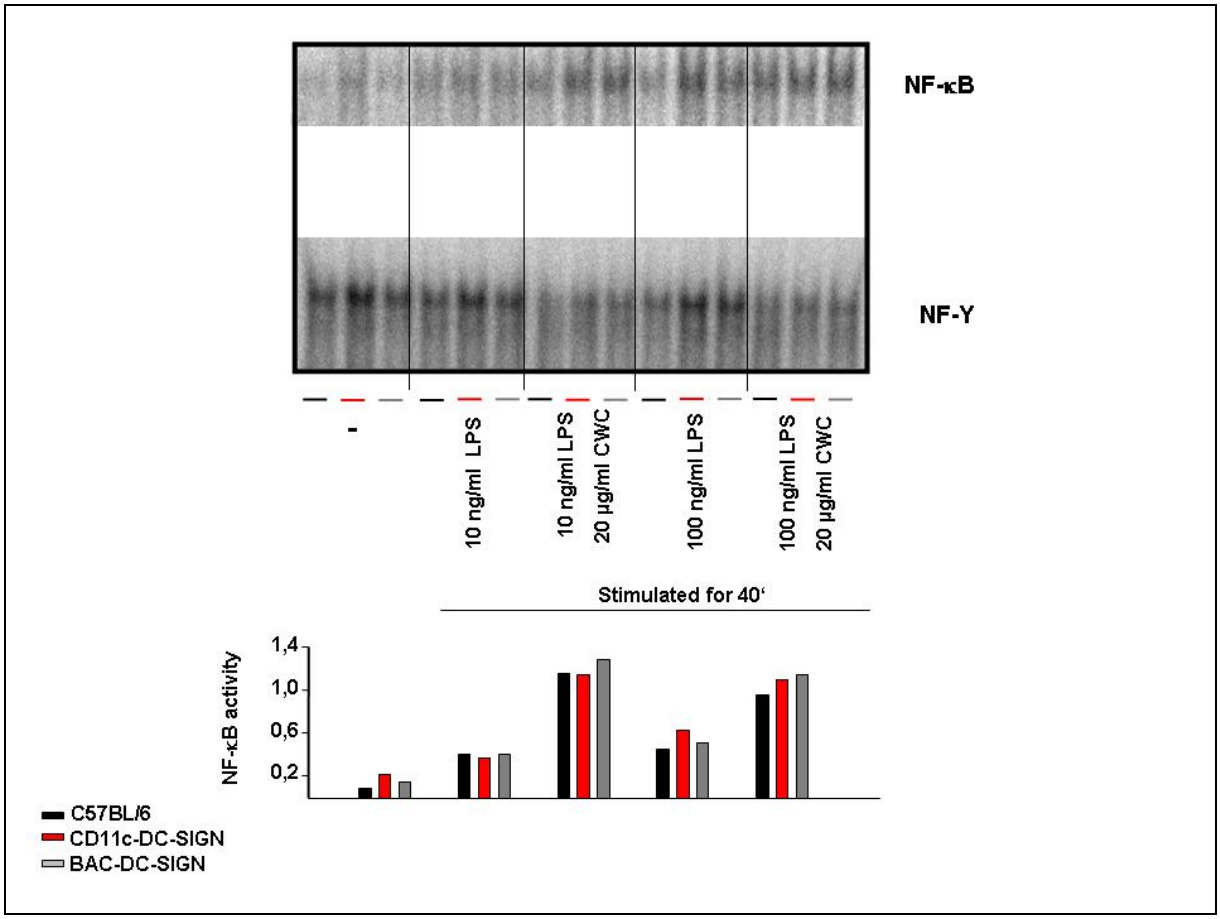

Figure 19: Activation of NF-κB in BMDCs. 5*10^6 BMDCs were stimulated for 40 minutes. Nuclear cell extracts were prepared as described and checked for activation of NF-κB by EMSA. For normalization purpose induction of NF-Y, a constitutive transcription factor was detected.

To quantify the detected signals a densiometric analysis was conducted. Each of the supoptimal doses of LPS only slightly induces activation of NF-κB. Additional stimulation with cell wall preparations of *M. tuberculosis* elevates the signal up to a level three times as high as LPS alone. After stimulation for 40 minutes there is no difference in the activation of

NF-κB between wild-type and human DC-SIGN transgenic BMDCs. This is true for stimulation with LPS alone and in combination with mycobacterial cell wall components.

For the *in vitro* situation it can be concluded, that no difference for the production of TNF-α and IL-10 but a 30-50 % reduction in IL-12p40 by human DC-SIGN transgenic BMDCs can be observed after infection with pathogenic mycobacteria. MyD88-dependent signalling pathways downstream of TLRs do not seem to be affected.

4.4 Role of transgenic human DC-SIGN *in vivo*

4.4.1 Bacterial burden in the spleen at d14 after infection with *M. bovis* BCG

So far not much is known about the *in vivo* role of human DC-SIGN in infection with *M. tuberculosis*. Initially the bacterial burden in the spleen of *M. bovis* BCG infected animals was analysed during the early phase of infection. Five mice per group were infected with $1*10^7$ CFU *M. bovis* BCG by i.p. injection. At day 14 after infection the animals were sacrificed and the spleens were removed and homogenized in PBS. For colony enumeration, 10-fold serial dilutions of the homogenates were plated.

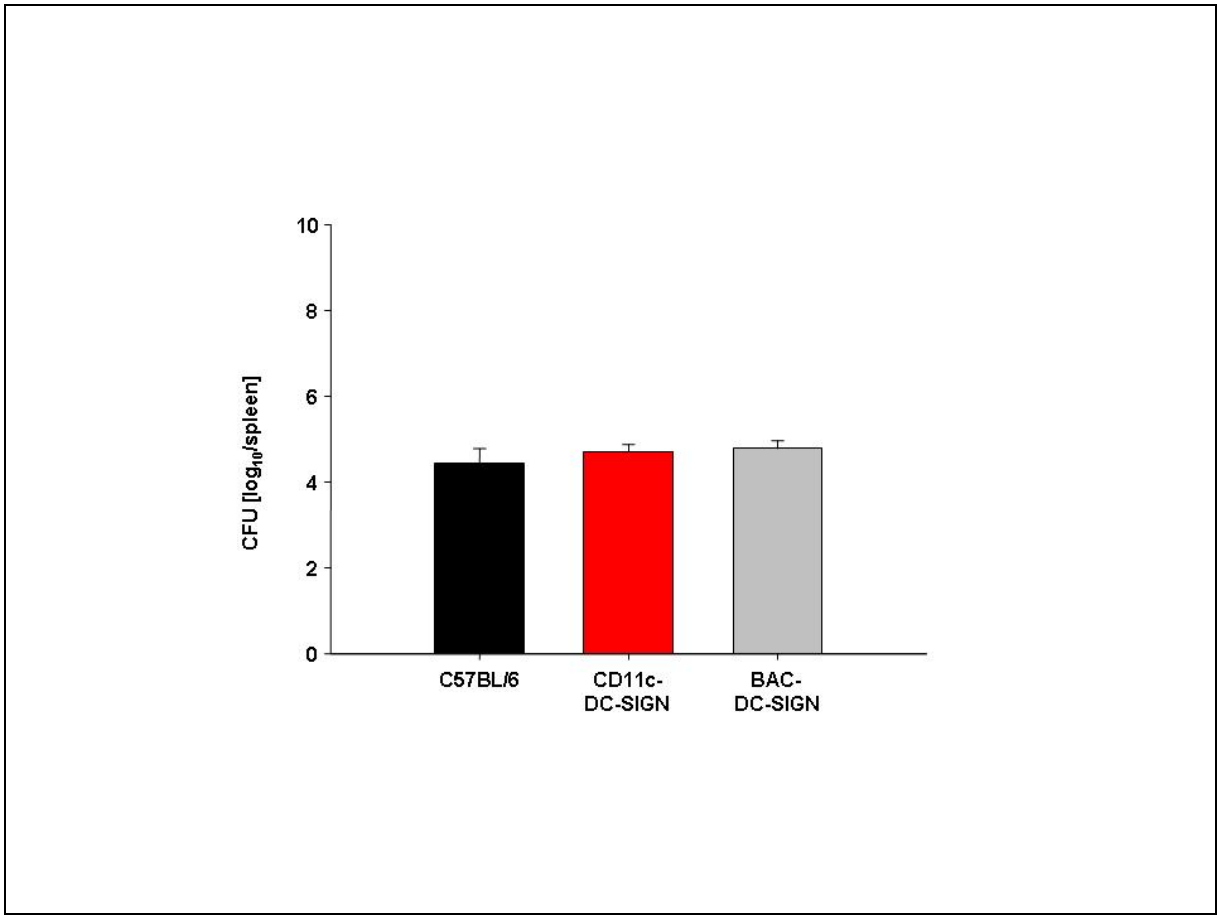

Figure 20: Bacterial burden in the spleen at d14 after *M. bovis* BCG infection. Mice were infected with $1*10^7$ CFU *M. bovis* BCG i.p.. At d14 animals were sacrificed and spleens were prepared. Data represent means +SD of 5 mice per group.

In the spleens of infected wild-type animals around $3*10^4$ CFU *M. bovis* BCG were detectable after 14 days of infection. The bacterial burden found in the spleens of the CD11c-DC-SIGN ($5*10^4$) as in the BAC-DC-SIGN transgenic animals was increased ($6*10^4$). In fact the amount of living bacilli still detectable in the spleens of the human DC-SIGN

transgenic animals was nearly twice as much as in the C57BL/6 mice. But this difference was calculated as not to be highly significant according to student's t-test.

4.4.2 Bacterial burden in the lung at d41 after infection with *M. tuberculosis*

In cooperation with the group of Prof. Ehlers (Research Center Borstel, Borstel, Germany) the bacterial burden in the lungs of *M. tuberculosis* infected mice was analysed.

Five mice per group were aerogenically infected with 2000 CFU *M. tuberculosis*. One day after infection the dose of mycobacteria was checked in the lungs of control animals. At day 41 animals were sacrificed and the lungs were examined for the bacterial burden.

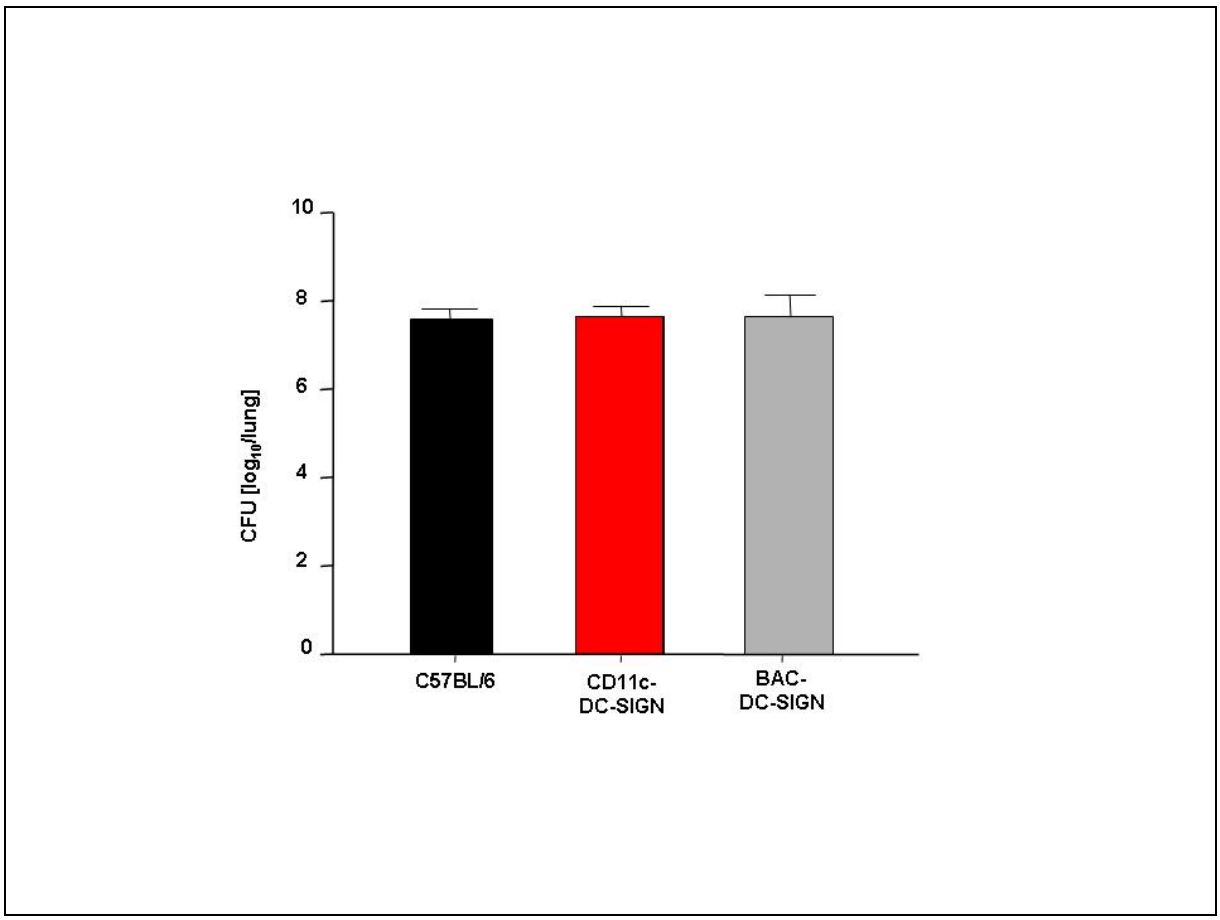

Figure 21: Bacterial burden in the lung at d41 after *M. tuberculosis* infection. Mice were aerogenically infected with 2000 CFU *M. tuberculosis*. At d41 animals were sacrificed and the lungs were prepared. Data represent means + SD of 5 mice per group.

At day 41 the initial 2000 CFUs *M. tuberculosis* used for infection were increased up to $1*10^8$ per lung of infected wild-type animals. An equal increase of bacterial burden was detected in the spleen of CD11c-DC-SIGN as well as BAC-DC-SIGN transgenic mice.

4.4.3 Survival after low dose *M. tuberculosis* infection

Infection with *M. tuberculosis* is typically characterized by its chronic course. At day 14 or 41 only the acute phase is monitored, therefore the long term outcome after aerosolic infection with *M. tuberculosis* was analysed.

For monitoring the course of infection in control and CD11c-DC-SIGN transgenic mice, ten mice each were infected with a low dose (100 CFU) of *M. tuberculosis*. Mice were checked daily for bodyweight. Mice that lost 25 % of their original weight during course of infection were sacrificed.

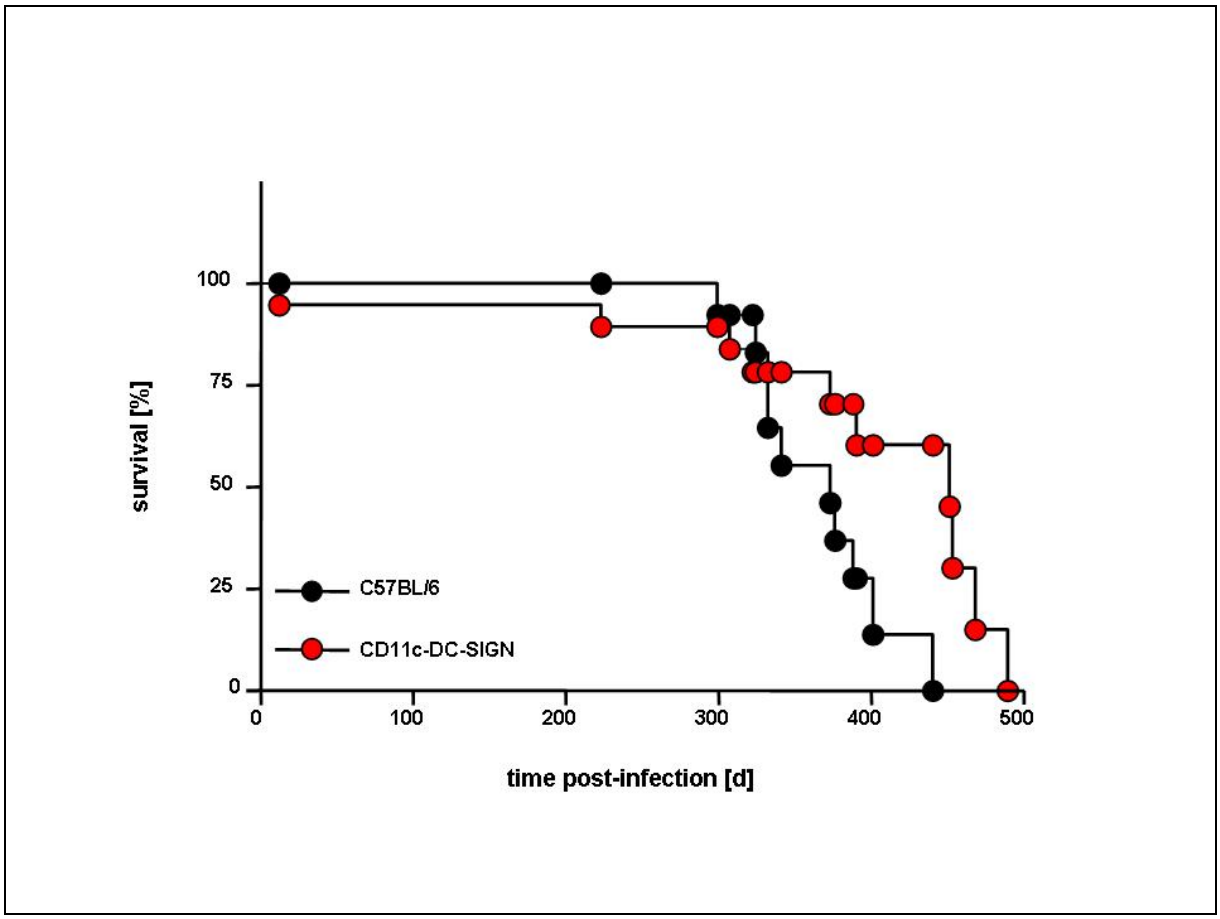

Figure 22: Low-dose aerosol infection of CD11c-DC-SIGN mice with *M. tuberculosis*. Control and CD11c-DC-SIGN mice were aerogenically infected with 100 CFU *M. tuberculosis*. Ten mice each were infected and monitored for survival.

Until approximately day 330 wild-type and human DC-SIGN transgenic mice demonstrate a similar survival rate. 50 % of all wild-type animals died until day 370. In contrast to the control animals 50 % of the infected CD11c-DC-SIGN animals survived until day 390. After day 390 mice bearing DC-SIGN died at a lower rate than wild type mice. The prolonged survival of human DC-SIGN transgenic mice was not significant.

4.4.4 Survival after high dose *M. tuberculosis* infection

To clarify the prolonged survival and to induce full activation of immune response the experiment was repeated using a high dose (2000 CFU) of *M. tuberculosis* for infection. 10 mice of each mouse strain were infected aerogenically and monitored.

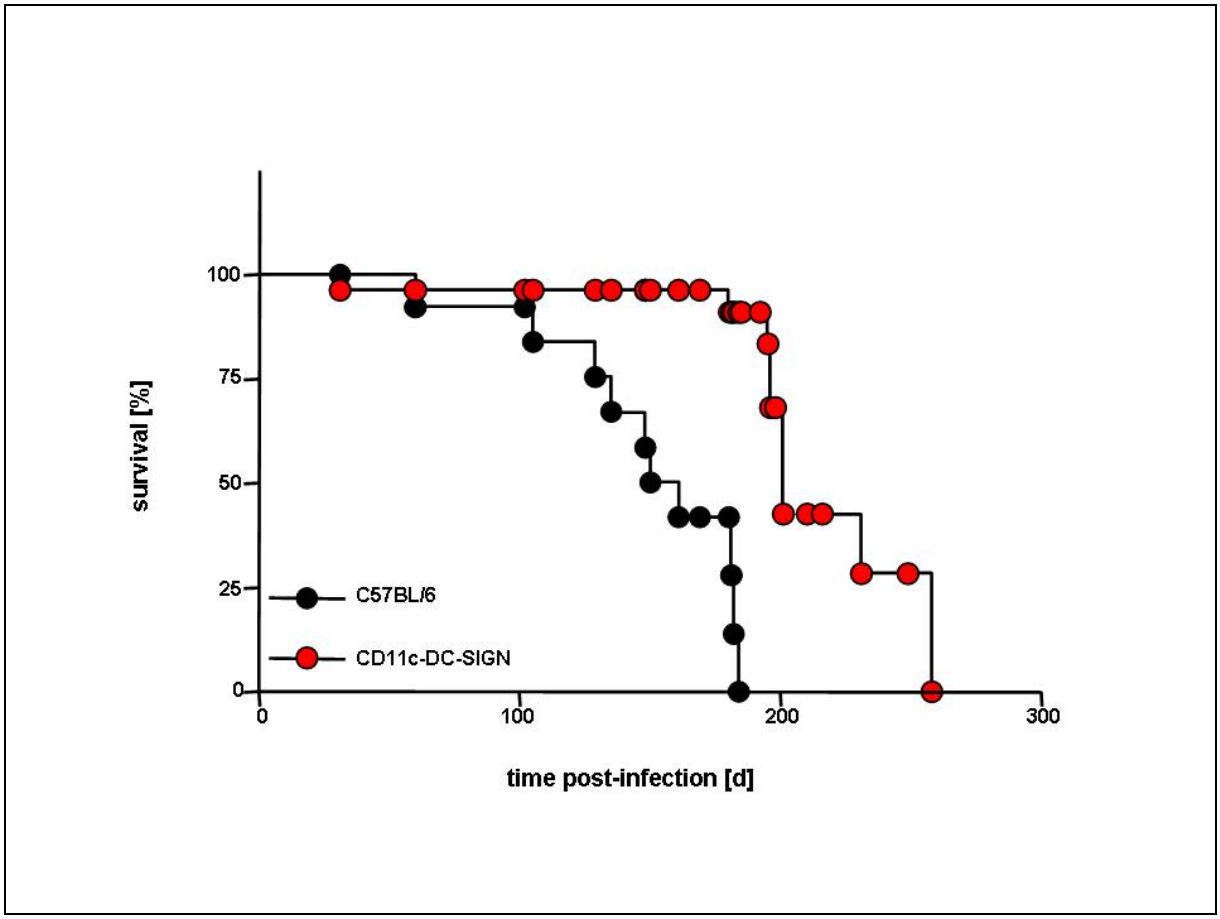

Figure 23: High-dose aerosol infection of CD11c-DC-SIGN mice with *M. tuberculosis*. Control and CD11c-DC-SIGN mice were aerogenically infected with 2000 CFU *M. tuberculosis*. Ten mice each were infected and monitored for survival.

Until day 100 no difference in survival of infected C57BL/6 and CD11c-DC-SGN mice was observed. At day 150 around 50 % of the infected wild-type mice had succumbed to the infection with *M. tuberculosis*. In comparison CD11c-DC-SIGN mice demonstrated a prolonged average survival rate of about 200 days. No control mouse survived day 190 after infection whereas the last CD11c-DC-SIGN transgenic mouse died at day 270. Thereby a highly significant (Log Rank-Test $p<0.001$) prolonged survival of human DC-SIGN bearing mice in contrast to wild-type animals could be observed (**Figure 23**).

Finally the survival of wild type and BAC-DC-SIGN transgenic mice was compared in the same experimental setting. The average survival rate of BAC-DC-SIGN transgenic animals was calculated to be 220 days and the last BAC-DC-SIGN mouse died around day 295. So the significantly prolonged survival of CD11c-DC-SIGN transgenic mice infected with

M. tuberculosis was even more pronounced when using BAC-DC-SIGN transgenic animals (Log Rank-Test p<0.001) (**Figure 24**).

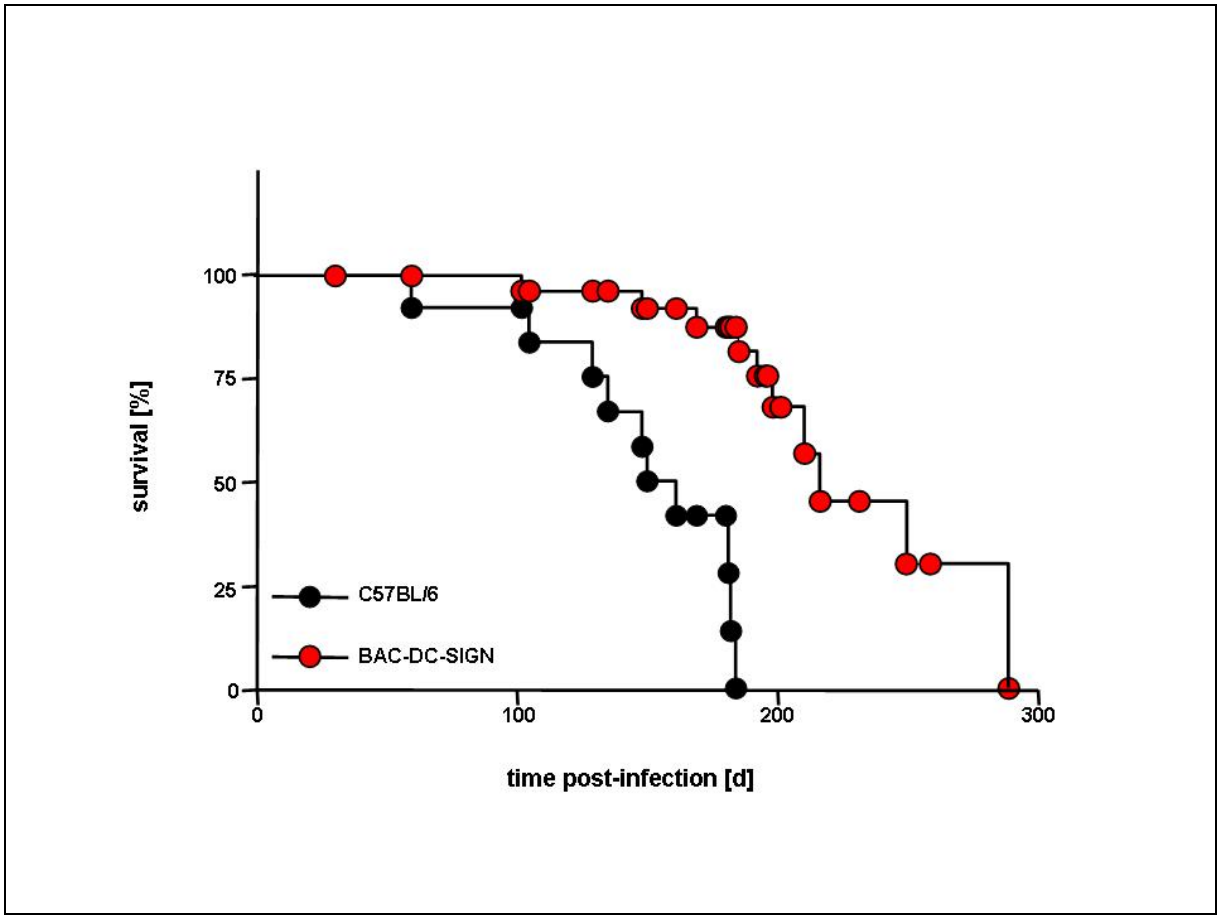

Figure 24: High-dose aerosol infection of BAC-DC-SIGN mice with *M. tuberculosis*. Control and BAC-DC-SIGN mice were aerogenically infected with 2000 CFU *M. tuberculosis*. Ten mice each were infected and monitored for survival.

Analysis of the obtained *in vivo* data demonstrated, that during the acute phase of the infection by mycobacteria the bacterial burden of the affected organs is not affected by the presence of human DC-SIGN in any of the mouse models.

But monitoring survival of mice during the chronic phase of infection with *M. tuberculosis* revealed a prolonged survival of both DC-SIGN transgenic models compared to wild-type animals. Infecting mice with a high dose of *M. tuberculosis* amplifies this prolonged survival, resulting in a highly significant prolonged survival of human DC-SIGN transgenic animals.

4.4.5 Survival of wild-type and human DC-SIGN transgenic mice after infection with *C. albicans*

Another pathogen known to bind to human DC-SIGN is *C. albicans* (Cambi, Gijzen et al. 2003). As human DC-SIGN transgenic mice succumbed later than wild-type mice after infection with *M. tuberculosis* we wanted to determine, whether this effect is specific for infection with *M. tuberculosis* or a general phenomenon in transgenic animals. Therefore,

seven mice were infected intravenously with $1*10^5$ CFUs *C. albicans*. In the case of the CD11c-DC-SIGN transgenic animals the strain background was changed to Balb/c (6 time backcrossed) as they are known to be more susceptible during a Th2 cell mediated immune response (Liew, Millott et al. 1990). For the BAC-DC-SIGN model no Balb/c strain animals are available. Mice were monitored and checked for survival every 12 hours.

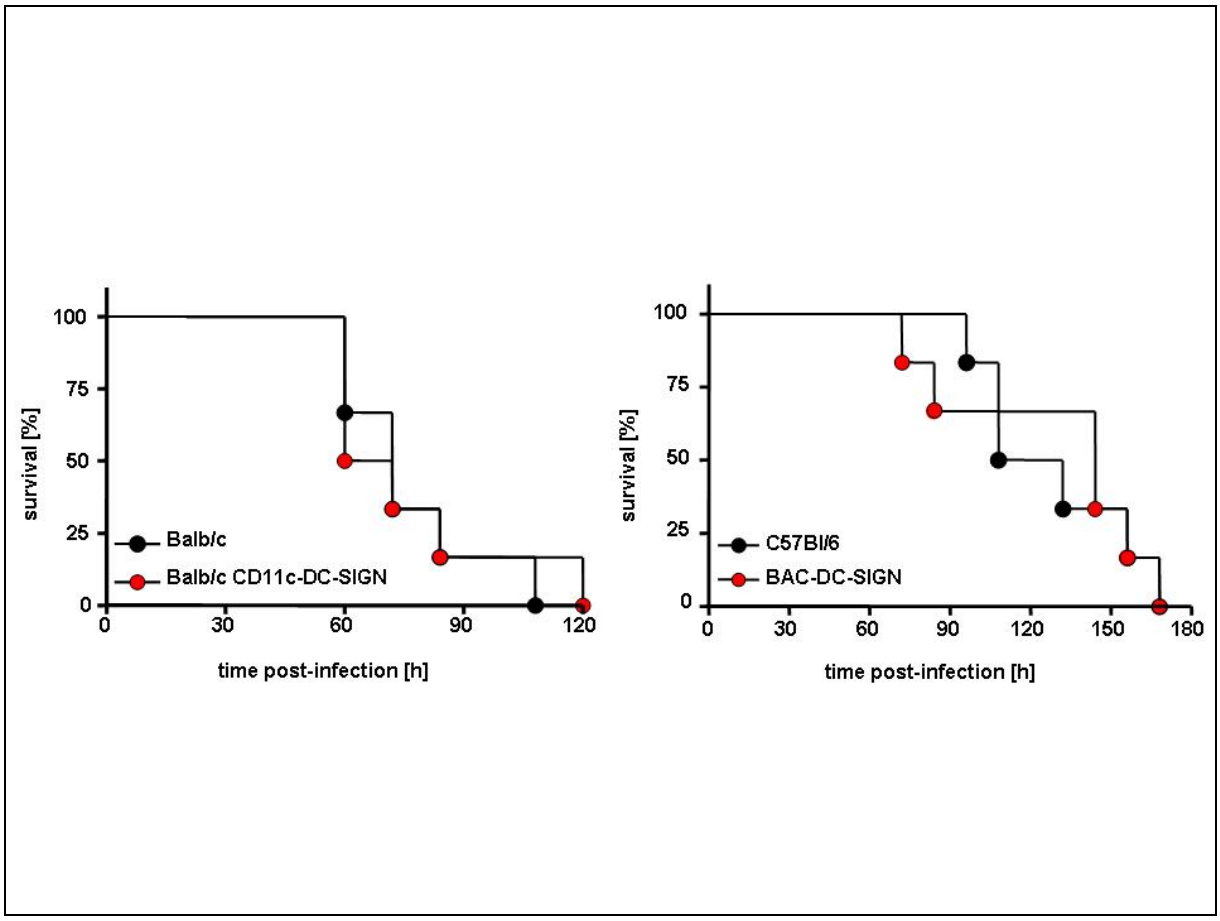

Figure 25: Infection of mice with *C. albicans*. Control and human DC-SIGN transgenic mice were intravenously infected with $1X10^5$ CFU *C. albicans*. Six mice each were infected and monitored for survival.

In general no mouse of the Balb/c background survived longer than five days. The survival rates of CD11c-DC-SIGN transgenic mice and littermate controls are nearly identical. All mice on C57BL/6 background survived until 72 hours after infection. 168 h after infection the last C57BL/6 and BAC-DC-SIGN mouse died. The survival rates of wild-type and BAC-DC-SIGN transgenic animals are not distinguishable.

4.4.6 Percentage of regulatory T cells in mediastinal lymph nodes at day 41 after high dose *M. tuberculosis* infection

Regulatory T cells, defined as FoxP3 expressing $CD4^+/CD25^+$ double positive cells, have been shown to be expanded in patients suffering from *M. tuberculosis* (Tailleux, Pham-Thi et al. 2005). As regulatory T cells have been shown to suppress inflammation (Hori, Carvalho et al. 2002) and a DC-SIGN mediated immune suppression was postulated, we analysed

mediastinal lymph nodes at day 41 after infection of mice with 1000 CFUs *M. tuberculosis* for presence of regulatory T cells. Mice were sacrificed and mediastinal lymp nodes were prepared. After generation of single cell suspensions the T cells were stained by anti murine CD4 and CD25 antibodies. To detect the population of regulatory T cells, the probes were permeabilized and intracellulary stained for FoxP3. By flow cytometry the samples were analysed.

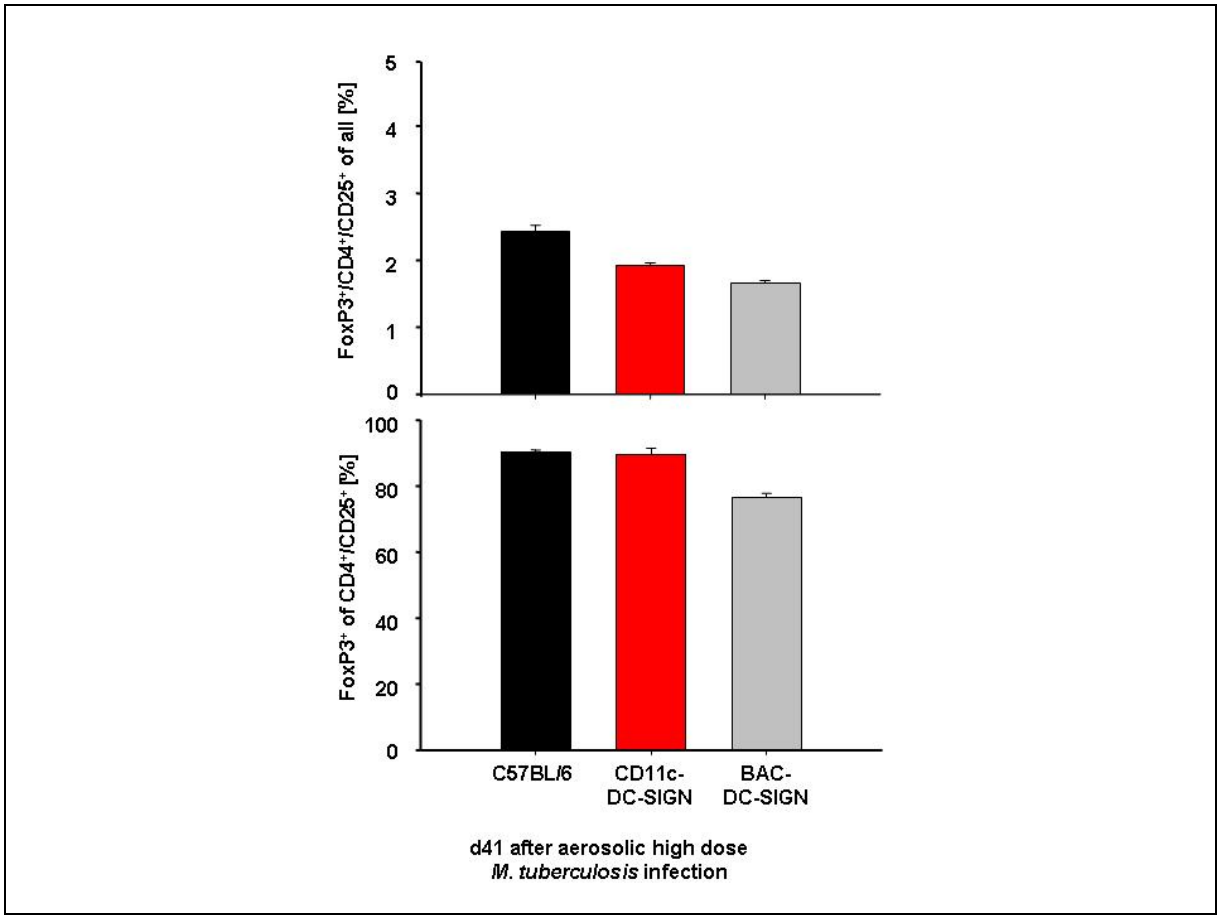

Figure 26: Percentage of regulatory T cells in mediastinal lymph nodes at d41 after infection with M. tuberculosis. Mice were aerogenically infected with 1000 CFU *M. tuberculosis*. At d41 animals were sacrificed and the mediastinal lymph nodes were prepared. Data represent means + SD of 5 mice per group.

Around 90 % of $CD4^+/CD25^+$ T cells in the mediastinal lymph nodes of infected wild-type and CD11c-DC-SIGN mice were positive for FoxP3 whereas only 77 % are found in the BAC-DC-SIGN animals. Also the total percentage of regulatory T cells is slightly reduced from 2.4 % in the wild-type to 1.9 % in the CD11c-DC-SIGN and 1.6 % in the BAC-DC-SIGN transgenic animals.

5 Discussion

5.1 Mouse models for human DC-SIGN

Mice are the experimental tool of choice to test *in vitro* and *in vivo* the immunologic function of molecules involved in recognition of and or response to different pathogens.

Humanized mice, mimicking human physiologic systems after genetic manipulation, have been shown to be important tools to analyse human infectious diseases. The transgenic expression of a human receptor important for the uptake, binding and infection of a pathogen has been successfully demonstrated for infection with *Listeria monocytogenes* (Lecuit, Vandormael-Pournin et al. 2001) and human coronavirus 229E (Lassnig, Sanchez et al. 2005). The model for listeriosis described by Lecuit and colleagues used transgenic mice that express human E-cadherin, the receptor interacting with the surface protein internalin of *L. monocytogenes*, under the control of the *iFABP* promoter. Using this model an internalin mediated invasion of enterocytes and crossing of the intestinal barrier via human E-cadherin was demonstrated. In the case of generating a mouse model of human coronavirus 229E infection mice carry the specific human receptor human aminopeptidase N (APN).

The most common inbred strains of mice are only slightly susceptible to infection with *M. tuberculosis* (Medina and North 1998). In men it has been shown, that not only macrophages but also DCs are targeted by *M. tuberculosis*. The C-type lectin DC-SIGN was identified as to be the main receptor of *M. tuberculosis* on human DCs (Tailleux, Schwartz et al. 2003). *In vitro* studies using human dendritic cells demonstrated, that simultaneous ligation of human DC-SIGN by ManLAM of *M. tuberculosis* and stimulation with the TLR4-ligand LPS induce secretion of the anti-inflammatory cytokine IL-10 and inhibition of DC maturation (Geijtenbeek, van Vliet et al. 2003). Therefore, it has been hypothesized that during mycobacterial infection human DC-SIGN mediates immune evasion by inhibiting TLR-signalling. Most of the receptors and signalling pathways involved in pathogen recognition are conserved between mice and men, as it is the case for the highly conserved TLR-signalling. But none of the human DC-SIGN mouse homologues is expressed on myeloid DCs in mice. In addition, all of these homologues lack cytoplasmatic motifs that may be relevant in human DC-SIGN mediated endocytosis or signalling events (van Kooyk and Geijtenbeek 2003). Altogether these facts underline the importance for a human DC-SIGN transgenic mouse model to get further insight into the possible role of human DC-SIGN during infection with *M. tuberculosis in vitro* and *in vivo*.

Two different human DC-SIGN transgenic mouse models were presented in this study (**Figure 4**). First a conventional transgenic model was generated, by using the minimal murine CD11c promoter (Brocker, Riedinger et al. 1997), which has been shown to be highly specific for DCs in mice, controlling the expression of the cDNA of human DC-SIGN. An alternative attempt was the design of a transgenic mouse model by BAC-technology.

Both constructs demonstrated an expression of the transgene on *in vitro* generated BMDCs using GMCSF (**Figure 5**). Dendritic cell cultures of plasmacytoid phenotype, derived from bone marrow cells in the presence of Flt3-ligand, express the transgene in the CD11c-DC-SIGN but not in the BAC-DC-SIGN transgenic mouse model (**Figure 5**).

Expression of transgenic human DC-SIGN *ex vivo* is detectable on splenic cDCs and pDCs (**Figure 6**), in the CD11c-DC-SIGN model. By flow cytometry an expression of transgenic human DC-SIGN is hard to detect *ex vivo* on any cell type checked in the BAC-DC-SIGN model. Expression of human DC-SIGN was shown to be dependent on IL-4 (Relloso, Puig-Kroger et al. 2002). In naïve mice IL-4 levels are low. Even after intraperitoneal injection of 2 µg murine IL-4 for seven days no expression was detectable on splenic DC-populations in BAC-DC-SIGN transgenic mice in contrast to CD11c-DC-SIGN transgenic animals (**Figure 7**). We analysed expression of transgenic human DC-SIGN in thioglycolate-elicited peritoneal cells in both models for two reasons. First, in men DC-SIGN expression has been shown on putative myeloid dendritic cell precursors (McCully, Chau et al. 2005) of the peritoneum. Second, infection with *M. tuberculosis* was reported to induce DC-SIGN expression (Tailleux, Pham-Thi et al. 2005) in human target cells. In the CD11c-DC-SIGN transgenic mouse strain peritoneal cells express human DC-SIGN, whereas only a small subset of myeloid cells of the peritoneum seems to express human DC-SIGN in the BAC-DC-SIGN model (**Figure 8**).

A comparison of human CD209 mRNA-levels in a broad range of organs of the two different transgenic mice revealed an overall lower expression of human CD209 in the tissues of the BAC-DC-SIGN transgenic mice (**Figure 11** and **Figure 12**).

The *in vitro* expression pattern of human DC-SIGN in GMCSF induced conventional DC cultures but not on pDCs in the BAC-DC-SIGN model reflects the situation found in humans. Therefore, this model may be more physiologic than the CD11c-DC-SIGN model. In the CD11c-DC-SIGN model the expression of human DC-SIGN is driven by the murine CD11c promoter active also in pDCs. As a consequence, transgenic human DC-SIGN in the CD11c-DC-SIGN mouse model is also expressed on plasmacytoid DCs, a DC subset not known to express DC-SIGN in men (Jameson, Baribaud et al. 2002). It is very difficult in the BAC-DC-SIGN model, to identify a population of transgene expressing cells *ex vivo*. The facts that only in cultures of dendritic cells treated with GMCSF, a cytokine known to be a key regulator of inflammation (Sheridan and Metcalf 1972), and in cells of the peritoneum, treated with thioglycolate to induce a sterile inflammation (Werb and Gordon 1975), DC-SIGN is detectable in the BAC-DC-SIGN model suggest factors induced during an infection to be involved in expression of human DC-SIGN. These factors might act on the level of mRNA transcription or translation. Unpublished data from our collaborator Dr. Roland Rad, who analyses human DC-SIGN transgenic mice in a septic model using *H. pylori*, demonstrated mRNA of human DC-SIGN to be upregulated in the stomach of BAC-DC-SIGN transgenic mice after infection. A further hint for a regulation of human DC-SIGN expression on the transcriptional level during inflammation was the description of a possible binding site for NF-κB in the promoter core region of human DC-SIGN (Liu, Yu et al. 2003). Activated NF-κB is known to play an important role in inflammation by inducing transcription of proinflammatory genes (Baldwin 1996). The reason for IL-4 not to induce expression of

human DC-SIGN in the BAC-DC-SIGN model may be a too low dosage used, the selected route of application and the usage of murine IL-4. Even if murine and human IL-4 receptors are highly conserved (Nelms, Keegan et al. 1999) and signalling of human IL-4 seems to function in a murine B-cell line (Deutsch, Koettnitz et al. 1995), not much is known about the crossreactivity between human and murine IL-4 response elements that may induce DC-SIGN expression. Also the purified fragment of the BAC containing the human DC-SIGN gene locus, covering up to 22 kb sequence upstream of the first human DC-SIGN exon, may not include all genomic elements influencing the initiation of transcription. In case of the murine CD4 locus distal enhancer elements have been described up to 24 kb upstream of the transcriptional start site (Ellmeier, Sawada et al. 1999). But also other still unknown mechanisms can be involved in regulating human DC-SIGN expression. These may be absent, present at lower levels or differentially regulated in mice in comparison to humans.

5.2 The balance between IL-10 and IL-12 after infection with *M. tuberculosis*

Optimal protection towards infection with different pathogens has been associated for a long time to the development of an adequate T helper cell response. Mainly two subtypes of Th cells have been defined. Th1 cells are effective in fighting intracellular pathogens, whereas type 2 effector Th cells play a crucial role in eliminating extracellular pathogens by inducing antibody production. DCs control the proper polarization of a Th cell response by the release of different cytokines and the expression of costimulatory molecules on their cell surface. IL-12 released by DCs was demonstrated to be an efficient promoter for a Th1 response (Macatonia, Hosken et al. 1995). The antagonistic acting IL-10 inhibits the production of IL-12 and favours the development of type 2 Th cells (Fiorentino, Bond et al. 1989).

Several studies confirmed the importance of IL-12 for the effective immune response to infection with *M. tuberculosis*. The initial control of an infection is correlated with the presence of IFN-γ producing CD4 T^+ cells (Orme, Roberts et al. 1993), which itself is induced *via* IL-12 released from infected APCs (Cooper, Magram et al. 1997). As shown by Cooper and colleagues the inability of IL-12p40 deficient mice to control mycobacterial infection is linked to a lack in IFN-γ producing T cells.

Also IL-10 is secreted during infection with *M. tuberculosis* (Barnes, Chatterjee et al. 1992). A study from 2002 demonstrated, that IL-10 deficient DCs infected with *M. bovis* BCG show in wild-type animals an improved migration to the draining lymph node and induction of IFN-γ producing T cells in response to mycobacterial antigen. This points to a role of autocrine IL-10 in suppressing immune responses by DCs to mycobacterial infection via limiting DC migration and IL-12 production (Demangel, Bertolino et al. 2002).

In vitro infection of BMDCs from wild-type and human DC-SIGN transgenic animals with *M. bovis* BCG or different strains of *M. tuberculosis* demonstrated a significantly reduced level of IL-12p40 production by transgenic DCs. Also the release of the bioactive IL-12p70 was diminished in CD11c-DC-SIGN transgenic DCs in response to infection with *M. bovis* BCG (**Figure 14**). After infection with *M. bovis* BCG at an MOI of 50 the amount of secreted

IL-10 was slightly increased by human DC-SIGN transgenic DCs. These differences in cytokine production between control and human DC-SIGN transgenic BMDCs were only found using CD11c-DC-SIGN transgenic BMDCs (**Figure 13**). The cytokine production of BAC-DC-SIGN transgenic BMDCs mimics the results of wild-type cells. A significantly lower amount of cells carrying the transgenic molecule on the cell surface of BMDCs from the BAC-DC-SIGN line compared to the CD11c-DC-SIGN model may explain this finding. Cultures of the BAC-DC-SIGN model contain more than 50 % of dendritic cells which are negative for human DC-SIGN. As a consequence effects on IL-12 production by DC-SIGN transgenic dendritic cells can be masked by an unaffected cytokine response of DC-SIGN negative cells. Attempts to enrich the DC-SIGN$^+$ cells from BAC-DC-SIGN BMDC cultures by magnetic activated cell sorting did not succeed. The procedure of the sorting seemed to induce a state of anergy in the dendritic cells. Sorted cells did not produce significant amounts of cytokines at all, independent of the stimulus used. Lower MOIs of infection induced similar amounts of IL-10 in wild-type and human DC-SIGN transgenic DCs. The production of TNF-α, known to be produced by APCs in response to mycobacterial infection (Henderson, Watkins et al. 1997), was similar in control and human DC-SIGN transgenic cells. Stimulation with pure TLR-stimuli induced similar cytokine levels in wild-type and human DC-SIGN transgenic cells, confirming that TLR signalling in the absence of DC-SIGN ligands was not affected. In contrast to the *in vitro* data using human dendritic cells (Geijtenbeek, van Vliet et al. 2003) a significant human DC-SIGN dependent increase in IL-10 production was not detectable after mycobacterial stimulation. But it can not be excluded that a subtle increase by human DC-SIGN transgenic cells in IL-10 production is undetectable. These amounts could act locally in an autocrine-manner with physiologic relevance.

Instead of an increased production of a Th2 cytokine (IL-10) a significantly reduced production of a Th1 cytokine (IL-12) was detectable.

To our surprise one *M. tuberculosis* isolate from Ghana, called Ghana TB2 8256/02, did not induce a reduced production of IL-12 in the human DC-SIGN transgenic DCs (**Figure 15**). Just recently there were hints, that the binding and recognition of *M. tuberculosis* to human DC-SIGN is not only mediated via the ManLam of the bacterial cell wall, but also dependent on the accessibility of ManLam in combination with the binding of additional mannosylated ligands (Pitarque, Herrmann et al. 2005). If any of the ligands is mutated or not present in the cell wall of the *M. tuberculosis* isolate from Ghana, this could possibly explain the differences. Currently, the cell wall composition of this isolate is analysed in more detail to answer this intriguing question.

5.3 Presence of DC-SIGN does not dramatically change uptake of *M. bovis* BCG by BMDCs

Beside human DC-SIGN several other receptors have been proven to take part in recognition, binding and uptake of mycobacteria. Nearly all of these receptors have also been shown to be involved in signalling events, for example complement receptor 3

(Thornton and Ross 1996) and TLR2 (Means, Wang et al. 1999). After *M. bovis* BCG infection mouse macrophages lacking TLR2 are not affected in the uptake and the ability to suppress intracellular bacterial growth. But they produce significantly reduced levels of proinflammatory cytokines (Heldwein, Liang et al. 2003) in response to mycobacterial infection. The opposite is true for complete loss of complement receptor 3 on APCs. As a consequence a reduced surface binding and uptake but no effect on the production of reactive oxygen and nitrogen has been described (Rooyakkers and Stokes 2005).

By antibody mediated blocking of human DC-SIGN on human DCs human DC-SIGN has been shown to be responsible for specific binding of *M. tuberculosis* (Tailleux, Schwartz et al. 2003). Therefore binding and uptake of *M. bovis* BCG was monitored to check, whether this finding is also true for murine DCs expressing transgenic human DC-SIGN on their cell surface. BMDCs derived of the two DC-SIGN transgenic mouse models bind and phagocytose *M. bovis* BCG to a similar extent as wild-type BMDCs (**Figure 16**). Reasons for the contrary results may be higher expression levels of receptors involved in binding of mycobacteria like TLR2 or the mannose receptor on the cell surface of *in vitro* generated murine BMDCs. Furthermore the density of the DC-SIGN specific ligands in the membrane of *M. bovis* BCG may be lower than on the cell surface of *M. tuberculosis*.

Therefore a human DC-SIGN dependent change in binding and uptake as the explanation for the reduced IL-12 production by mycobacteria-infected BMDCs can be excluded. Presence of transgenic human DC-SIGN might influence signalling pathways induced after mycobacterial recognition. As a consequence, we next checked activation of such pathways in DCs infected with mycobacteria.

5.4 Normal Activation of MAPKs, Iκbα and NF-κB in DC-SIGN transgenic DCs after *M. bovis* BCG infection

Maturation of human DCs by LPS, a major stimulus for TLR4, could be blocked by simultaneous ligation of human DC-SIGN *via* mycobacterial components (Geijtenbeek, van Vliet et al. 2003), indicating that TLR4 mediated signals may be inhibited by ligation of DC-SIGN. Signalling cascades downstream of many TLRs, including TLR4, induce the expression of proinflammatory cytokines by nuclear translocation of NF-κB (Akira and Takeda 2004). Two further TLRs have been shown to be involved in recognition of mycobacteria. A study published by our own laboratory demonstrated TLR9 to be involved in recognition of *M. bovis* BCG by DCs in concert with TLR2 and TLR4 (Meyenn, Schaefer et al. 2006).

As cultures of DC-SIGN transgenic BMDCs infected with mycobacteria produce less IL-12, a proinflammatory cytokine induced by NF-κB (Murphy, Cleveland et al. 1995), we analysed pathways downstream of TLR activation in wild-type and DC-SIGN transgenic DCs after mycobacterial infection.

Recognition of pathogens by TLRs induces activation of the MAPKs Erk1/2, JNK and p38. Activation of all three MAPKs was similar in wild-type and human DC-SIGN transgenic

BMDCs infected with *M. bovis* BCG (**Figure 17**). We observed in some experiments a slight decrease in phosphorylation of the kinase Erk1/2. These finding would fit to data published by Knutson et al. in 1998, showing that LAM (including ManLAM) from *M. tuberculosis* induces dephosphorylation of Erk2 by activating the Src homology 2-containing tyrosine phosphatase-1 (SHP-1) in human monocytes (Knutson, Hmama et al. 1998). By RNAse protection assay they could also demonstrate an inhibitory effect of LAM on TNF-α and IL-12 mRNA production. ManLam, which has later been shown to be the key ligand of mycobacteria for DC-SIGN on human DCs (Geijtenbeek, van Vliet et al. 2003), therefore may induce recruitment and activation of SHP-1 to silence Erk activation and induction of proinflammatory cytokines by binding to human DC-SIGN.

In contrast to the findings of the study presented here a recent publication demonstrated that ligation of human DC-SIGN leads to Erk1/2-phosphorylation and enhances production of LPS induced IL-10 (Caparros, Munoz et al. 2006). Capparos et al. also observed a possible coprecipitation of human DC-SIGN and the tyrosine kinases Lyn and Syk. These findings would fit very well into the hypothesized role of human DC-SIGN. Especially the possible interaction of human DC-SIGN and Syk, known to be involved in Dectin-1 dependent IL-10 production (Rogers, Slack et al. 2005), was discussed. But the described results of Caparos et al. were obtained by antibody mediated human DC-SIGN crosslinking. So far no natural ligand of human DC-SIGN was proven to induce the described effects. In addition, antibody ligation of human DC-SIGN has been reported to block DC-SIGN function (Geijtenbeek, van Vliet et al. 2003). Since T cells have not been shown yet to express human DC-SIGN the use of Jurkat cells, a human T cell clone, instead of the human monocyte like THP-1 cells makes the interpretation of these data difficult. Therefore, cell specific factors can not be excluded to be important for physiologic human DC-SIGN function and this study may need to be confirmed in a more physiologic setting.

Also in several other studies an increase in Erk activation was associated with reduced secretion of proinflammatory cytokines like IL-12 and increased levels of IL-10. In human monocytes infected with *M. tuberculosis* inhibition of Erk1/2 activation leads to an increase in IL12p40 and bioactive IL-12p70 production (Yang, Lee et al. 2005). In CpG-ODN treated murine macrophages the activation of Erk was shown to be controlled by phospatidylinositol 3-kinase, leading to more IL-10 and less IL-12p70 production (Saegusa, Yotsumoto et al. 2006).

Taken together, possible pathways leading to DC-SIGN mediated Erk activation or deactivation may await further elucidation.

IκBα mediates nuclear translocation of NF-κB downstream of MAPKs (Ghosh and Baltimore 1990). In the publication by Gosh et al. IκBα has been shown to be phosphorylated and inactivated in the presence of protin kinase C and haem-regulated eIF 2 kinase. As a result of these modifications, NF-κB is released and translocated to the nucleus.

In our experiments activation of IκBα was equal in wild-type and human DC-SIGN transgenic BMDCs (**Figure 18**).

Consequently an assay addressing the question of a possible impact of human DC-SIGN on NF-κB activation was performed. However, after stimulation with mycobacterial cell wall components in combination with a suboptimal dose of LPS there is no difference between wild-type and human DC-SIGN bearing BMDCs on the level of NF-κB activation (**Figure 19**).

In BMDCs of the human DC-SIGN transgenic models presented in this study a significant decrease in IL-12p40 and IL-12p70 but no effect on production of IL-10 or TNF-α was detectable after infection with mycobacteria. A significant impact of human DC-SIGN downstream of TLR-signalling, as hypothesized at the beginning of this study, was not detectable.

Several TLRs (2, 4 and 9) are involved in recognition of mycobacteria. Therefore, an inhibition induced by the ligation of transgenic human DC-SIGN downstream of one of the receptors may be masked by signalling events mediated by the non-affected receptors. This is very likely, as even the complete loss of TLR4-signalling has only subtle effects on the cytokine production of macrophages infected with mycobacteria (Reiling, Holscher et al. 2002).

Also the involvement of glykogen synthase 3, can be discussed. In human monocytes inhibition of this protein, after stimulation with TLR agonists, has been shown to result in an increase in IL-10 secretion and diminished production of proinflammatory cytokines (Martin, Rehani et al. 2005). However, here changes in NF-κB translocation were observed, therefore the mechanism may not be relevant in our model system.

Most of the TLRs use the adaptor protein MyD88 to activate MAPKs and NF-κB. TLR4, involved in mycobacterial recognition, is in addition able to use a MyD88-independent pathway that leads to activation of IRF-3 via TRIF and delayed activation of Nf-κB (Kawai, Adachi et al. 1999). Activated IRF-3 has been shown to induce expression of IFN-inducible genes (Kawai, Takeuchi et al. 2001). Also the maturation of DCs after LPS treatment seems to be regulated by the MyD88-indepentent pathway *via* TLR4 (Kaisho, Takeuchi et al. 2001).Therefore, human DC-SIGN ligation may also inhibit the MyD88-independent pathway, as ligation of human DC-SIGN by mycobacterial ManLAM blocks maturation of LPS treated human DCs (Geijtenbeek, van Vliet et al. 2003).

We tried to address this question by using reporter assays, based on HEK293 cells expressing human TLR4 and an IRF-3 dependent reporter construct. But we could not detect any influence of human DC-SIGN on IFN-induction (data not shown).

Besides TLRs also different TLR-independent pathways recognizing pathogen-associated molecular pattern have been described in recent studies (Gross, Gewies et al. 2006; Ishii, Coban et al. 2006). Therefore, even as yet unknown TLR-independent pathways recognizing mycobacteria may be targeted by human DC-SIGN.

Mice and men share about 99% of their genes reflecting the fact that mice are known to mimic human biology in a variety of examples. TLR-signalling is highly conserved between both species. This high degree of homology is demonstrated by the fact that e.g. a murine cell line expressing human TLR4 and the co-factor MD-2 can be stimulated using LPS

(Akashi, Nagai et al. 2001). This high degree of similarity makes a human DC-SIGN transgenic mouse model an interesting tool to analyse the function of this molecule. However, after 65 million years of separate evolution between mice and men (Mestas and Hughes 2004) unknown differences still may complicate the analysis of the function of transgenic human DC-SIGN in the mouse.

5.5 DC-SIGN transgenic mice infected with mycobacteria do not show increased bacterial burden but are less susceptible than control mice

It was shown in mice that a complete loss of the IL-12p40 gene leads to a dramatic reduction in the development of IFN-γ producing T-cells during mycobacterial infection. As a consequence, these animals lose control of bacterial burden (Cooper, Magram et al. 1997). Even the opposite situation characterized by a significant increase in IL-12 as observed in the WSX 1 deficient mice after infection with *M. tuberculosis* (Holscher, Holscher et al. 2005) leads to an accelerated death caused by a very strong inflammatory response. WSX 1 is a subunit of the IL-27 receptor. Holscher et al. demonstrated *in vitro*, that IL-27 induces activation of STAT-3 and inhibits IL-12 and TNF-α production in peritoneal macrophages. Mice deficient in IL-27 signalling due to a loss of the required receptor develop *in vivo* a more protective immunity against *M. tuberculosis* infection than wild-type mice. This protective immunity is characterized by an increased proinflammatory cytokine production (TNF-α and IL-12), increased numbers of IFN-γ producing $CD4^+$ T cells and reduced bacterial loads. These obvious advantages for the WSX 1 deficient animals were overshadowed by a diminished survival as consequence of an increased chronic inflammation. Neither the loss of IL-12 nor an excessive inflammation induced by uncontrolled production of this cytokine is of advantage in fighting mycobacterial infection.

Our *in vitro* data demonstrated human DC-SIGN transgenic DCs to produce significantly reduced IL-12 levels after infection with mycobacteria in comparison to wild-type cells. After *in vivo* infection with mycobacteria both human DC-SIGN transgenic models were able to control the bacterial burden during the acute phase of the infection similarly to control mice. But both human DC-SIGN transgenic mice succumbed significantly later to the infection than wild-type animals.

The *in vitro* data of the BAC-DC-SIGN transgenic DCs did not demonstrate a decrease in production of IL-12 after mycobacterial infection. But as an expression of DC-SIGN has been described to be induced during mycobacterial infection in men (Tailleux, Pham-Thi et al. 2005) the expression of the transgenic human DC-SIGN in the BAC-DC-SIGN model may be induced after infection with *M. tuberculosis* and thereby might contribute to the observed effects *in vivo.*

C. albicans is another known human DC-SIGN binding pathogen (Cambi, Gijzen et al. 2003). Neither CD11c-DC-SIGN nor BAC-DC-SIGN transgenic mice demonstrate a change in susceptibility to infection with *C. albicans* in comparison to littermate controls (**Figure 25**). For

that reason we can rule out the DC-SIGN transgenic mice to be more resistant to infections in general. Also the fact that recognition of *C. albicans* is mainly mediated by the PRR Dectin-1 (Brown, Taylor et al. 2002) would explain the similar susceptibility of control and human DC-SIGN transgenic animals.

Humans infected with *M. tuberculosis* show an expansion of $CD4^+/CD25^+$ T cells expressing FoxP3 at the site of infection (Guyot-Revol, Innes et al. 2005). After infection with *M. tuberculosis* human DC-SIGN bearing mice survived longer. Downregulation of the inflammatory reaction followed by less massive organ destruction was proposed as the reason. A possible enlarged population of regulatory T cells, acting anti-inflammatory, could explain this observation. But the overall amount of regulatory T cells is slightly reduced in mediastinal lymph nodes, which strengthens the hypothesis of a less severe inflammation in the human DC-SIGN transgenic animals.

Combining the *in vitro* and *in vivo* data, presence of human DC-SIGN may be of benefit for the host in reducing the inflammatory response after infection with *M. tuberculosis* (reduced level of IL-12, equal levels of IL-10) and thereby inducing less severe lesions and granuloma formation.

Preliminary histological analysis of the lungs from CD11c-DC-SIGN mice at day 148 after infection demonstrated a significantly decreased amount of tissue damage within the bronchiolar membranes in comparison to wild-type animals (data not shown), indicating a less severe immunopathology in DC-SIGN transgenic mice infected with *M. tuberculosis*.

The matrix metalloproteinases (MMPs) may link the decrease in IL-12 production and a reduced pathology after infection with *M. tuberculosis*. During immune response to an infection these proteolytic enzymes break down barriers to allow influx of effector cells, killing of the pathogens and final remodelling of the extracellular matrix (ECM). But if a pathogen induces an excessive inflammation the activity of this enzymes can also cause severe tissue damages (Brinckerhoff and Matrisian 2002). This tissue damages may fascilitate dissemination and persistence of the pathogen. In mice infected with *M. tuberculosis* an increase of MMP2 and MMP9 levels can be detected in infected tissues (Rivera-Marrero, Schuyler et al. 2000). In the same study human monocytic THP-1 cells were shown to induce MMP9 secretion after stimulation with *M. tuberculosis* derived LAM. In humans a correlation between higher levels of circulating MMP9 and severity of the disease in patients suffering from tuberculosis was described (Hrabec, Strek et al. 2002). The activity of MMPs is partly regulated by cytokines. Action of IL-10 has been shown to inhibit MMPs (Mertz, DeWitt et al. 1994) and elevation of MMP-9 levels could be induced in murine monocytes by IL-12 treatment (Abraham, Shapiro et al. 2002). Besides cytokines also specific inhibitors, called tissue inhibitors of metalloproteases (TIMP), are involved in regulation of MMP activity (Brew, Dinakarpandian et al. 2000). Therefore, changes in cytokine production (decrease in IL-12) or modulation of TIMP activity by DC-SIGN transgenic DCs after mycobacterial infection may dampen MMP activity and subsequently prevent tissue damage. Further immunohistological studies of the affected organs are currently under investigation to get further insights into the pathology.

After infection with *M. tuberculosis* the decrease in IL-12 production by DC-SIGN transgenic DCs does not seem to result in uncontrolled mycobacterial growth as described in mice deficient in IL-12 (Cooper, Magram et al. 1997). This may be a result of human DC-SIGN function in dendritic cells of the transgenic mice. Human DCs have been shown to phagocytose mycobacteria. After mycobacterial infection the *M. tuberculosis* containing vacuole in human DCs has been characterized to be arrested in maturation similar to the mycobacteria containing vacuoles in macrophages. But in addition also intracellular trafficking to and from this vacuole is blocked (Tailleux, Neyrolles et al. 2003). The cytoplasmatic tail of human DC-SIGN contains a possible di-leucine internalization motif, shown to be involved in uptake of Dengue Virus (Lozach, Burleigh et al. 2005). As a consequence, the trafficking of mycobacteria in human DCs may depend on DC-SIGN mediated uptake. In the study by Tailleux et al. it was shown that the bacilli neither are killed nor divide in this trafficking blocked compartment. A DC-SIGN mediated uptake of mycobacteria into an intracellular compartment that inhibits further growth of the pathogen may balance less effective immune responses, due to a decrease in IL-12 production after DC-SIGN ligation. Living mycobacteria are known to facilitate presentation of specific antigens to T cells (Neyrolles, Gould et al. 2001). Persistence of living mycobacteria in such vacuoles might therefore also help DC-SIGN transgenic DCs to present mycobacterial antigens to T cells more efficiently and might contribute to an improved resistance of the DC-SIGN transgenic mice towards mycobacterial infection.

It is difficult to decide whether human DC-SIGN or pathogenic mycobacteria have evolved separately or whether the present situation reflects the result of a classical co-evolution typical for host-parasite relationships. Studies comparing genes of the *CD209* family in primates demonstrated highly conserved sequences (Bashirova, Wu et al. 2003). Therefore it is likely that infection with the slow-growing pathogenic mycobacteria enforced primates to develop mechanism like human DC-SIGN to keep control of the invader. In contrast, for rodents living more in close contact to the fast-growing soil-dwelling non pathogenic mycobacteria there was no such need. A further genetic proof of DC-SIGN to be important in human infection with *M. tuberculosis* was the finding that the two -871G and -336A promoter variants of CD209 are associated to a lower risk in developing tuberculosis (Barreiro, Neyrolles et al. 2006). For their analysis the authors of this publication compared the CD209 promotor genotypes of around 350 healthy and 350 infected individuals. Further investigation on human chromosomes from Europe, Asia and Africa (ca. 80 cases each) revealed that the -871G and -336A forms are more common in Eurasian groups than in African populations. This could be due to a prolonged evolutionary selection for several hundred years in Europe. In Africa contact with the pathogen was not initiated before colonization by Europeans. As also other pathogens are known to bind to human DC-SIGN their influence may even have speeded up this development. This is supported by the recently described correlation between the severity of a dengue virus infection and the -336A form of the CD209 promoter (Sakuntabhai, Turbpaiboon et al. 2005). Among patients suffering from dengue fever the -336A form is significantly increased. Besides the relevance of a special CD209 promoter variant for the outcome of an infection the study by Sakuntabhai and colleagues suggested a possible mechanism. They could demonstrate the - 336A isoform of the CD209 promoter to

be involved in binding the transcription factor SP1 *in vitro*. The -336A form additionally increases *in vitro* the CD209 promoter activity. Human DC-SIGN expression is induced in alveolar macrophages of patients suffering from tuberculosis (Tailleux, Pham-Thi et al. 2005). However, a higher level of human DC-SIGN surface expression after mycobacterial infection may be a benefit for the host by facilitating uptake, presentation and efficient T cell activation. For the infection with dengue virus the opposite is true. High levels of human DC-SIGN seem to increase susceptibility of dendritic cells and subsequent outbreak of dengue fever. In the case of parental HIV infection a reduced susceptibility was associated to the -336A form of the CD209 promoter sequence. In the case of HIV and Dengue virus infection no association could be made to the -871G form of the human DC-SIGN promoter. These studies checked different ethnics, with different size of groups for cases and controls. Even if the results indicate opposite effects of human DC-SIGN in different diseases they demonstrate the *in vivo* importance of human DC-SIGN. The exact role of the described promoter polymorphisms of CD209 associated to severity of infection with known pathogens binding to human DC-SIGN has to be elucidated in more detail. The role of human DC-SIGN expression may differ from pathogen to pathogen. As in the case of Dengue Virus infection higher level of human DC-SIGN expression (-336A form) increases the risk of suffering from Dengue Fever in the case of *M. tuberculosis* and HIV the opposite is true. Other mechanisms beside expression levels like cell type specific presence in the membrane or secondary mechanisms induced after infection may play a role in infection.

Human DC-SIGN may take over the role of a key player in balancing the immune response after infection with *M. tuberculosis.* A too strong inflammatory reaction leading on the one hand to a fast clearance of pathogens but on the other hand to severe tissue damages and subsequent death might be slightly dampened by human DC-SIGN. This reduced, but not abolished, pro-inflammatory response would allow the pathogen to survive and reach a chronic but controlled state. The advantage would be the formation of less severe organ destructions enabling the host to develop a stronger resistance towards the mycobacterial infection.

5.6 C-type lectins, immune evasion or protection

Human DC-SIGN is known to bind a broad range of pathogens beside of virulent mycobacteria. Several of them are known to induce chronic infections and are speculated to evade the immune system via DC-SIGN.

In the case of HIV, it has been shown, that human DC-SIGN on DCs is responsible for the efficient binding and transfer of the virions to target $CD4^+$ T cells (Arrighi, Pion et al. 2004). Another virus which targets human DC-SIGN and L-SIGN (liver homologue of DC-SIGN) positive cells is hepatitis C virus which targets this molecules to escape lysosomal degradation (Ludwig, Lekkerkerker et al. 2004).

A mouse homologue of human DC-SIGN, named SIGNR1, which is expressed on marginal zone macrophages and peritoneal macrophages, has been shown to be important for

binding, recognition and uptake of *Streptococcus pneumoniae*. In fact mice lacking SIGNR1 are impaired in clearing an infection with *S. pneumoniae* and succumb to a sublethal *S. pneumoniae* infection (Lanoue, Clatworthy et al. 2004). These data gave the first hint of a SIGN family member to play a role in protection in response to an infection by a specific pathogen.

Dectin-1, another member of c-type lectins, was identified as being the most important receptor for yeast or the yeast-derived particle zymosan on mouse macrophages and dendritic cells (Brown, Taylor et al. 2002). The induction of inflammatory cytokine release *via* Dectin-1 after stimulation with zymosan is mediated in cooperation with TLR2 (Gantner, Simmons et al. 2003). Also a cooperation between the two c-type lectins Dectin-1 and SIGNR1 has been described in the recognition of yeast by a subset of peritoneal macrophages (Taylor, Brown et al. 2004). These data demonstrate that a cooperation of Dectin-1 with other receptors (TLR2, SIGNR1) is limited on special cell subsets. Even on the field of signalling different cell types influence the outcome of an interaction between Dectin-1 and Syk, underlining the complexity of inflammatory responses. In a subpopulation of macrophages Dectin-1 needs Syk-signalling to induce the respiratory burst (Underhill, Rossnagle et al. 2005), whereas the activation of Syk in DCs by Dectin-1 is crucial for the release of IL-2 and IL-10 upon yeast infection (Rogers, Slack et al. 2005). Together with the group of Dr. Jürgen Ruland we could show Card9 to act as the mediator between Syk-activation and IL-10 secretion in dendritic cells during fungal infection (Gross, Gewies et al. 2006). The recruitment and activation of Syk by Dectin-1 is mediated by the phosphorylation of a single tyrosine in the incomplete cytoplasmatic ITAM-motif (Rogers, Slack et al. 2005). An incomplete ITAM is also present in human DC-SIGN. Therefore, Syk mediated signalling may be a mechanism by which human DC-SIGN induces an increased IL-10 production and limits the release of IL-12 upon ligation by *M. tuberculosis.*

A first direct hint of a pathogen binding to DCs via human DC-SIGN and a thereby mediated possible shift towards a type 2 T helper cell response was demonstrated for *Helicobacter pylori* (Appelmelk, van Die et al. 2003; Bergman, Engering et al. 2004). The ligation of human DC-SIGN by *H. pylori* on gastric DCs induces an increase in IL-10 production and blockage of Th1 cell induction (Bergman, Engering et al. 2004). Whether this is of benefit for the host or represents the initial hypothesized evasion strategy via human DC-SIGN has not been elucidated in this infection model.

A recent publication ruled out human DC-SIGN to be abundant in lesions of lepromatous leprosy, after infection with *Mycobacterium leprae,* associated to a Th2 cytokine profile (Soilleux, Sarno et al. 2006). Regarding these publications the proposed role of human DC-SIGN as a factor dampening a Th1 and favouring a type 2 T helper cell response, to protect the host, after infection with *M. tuberculosis* becomes very likely.

5.7 Concluding remarks

The study presented here introduced two different transgenic mouse models for human DC-SIGN. A conventional model using the minimal murine CD11c promoter to drive expression of human DC-SIGN cDNA was generated first. An alternative approach was the design of a BAC-DC-SIGN model, using a commercial BAC containing the complete human CD209 gene locus. Both models demonstrated expression of the transgene on *in vitro* generated GMCSF-cultures of BMDCs. FLT3-L derived BMDCs from the CD11c-DC-SIGN but not from the BAC-DC-SIGN transgenic animals express the transgene. Splenic cDCs and pDCs show expression of the transgene in the CD11c-DC-SIGN transgenic line. Presence of the transgene was hard to detect on several organs of naïve BAC-DC-SIGN mice. Only after sterile infection and analysis of peritoneal cells, a small myeloid like cell population could be reported to express the transgene. *In vitro* infection of BMDCs with *M. bovis* BCG or *M. tuberculosis* revealed an impaired production of IL-12p40 and equal or slightly increased levels of IL-10 by human DC-SIGN (CD11c-DC-SIGN) bearing cells. For MAPKs, IκBα and NFκB-activation no impact of human DC-SIGN was detectable. The hypothesized role of human DC-SIGN as being a vehicle for *M. tuberculosis* to evade the immune system could not be proven. Infection *in vivo* with a high dose of *M. tuberculosis* demonstrated a significantly prolonged survival of our human DC-SIGN transgenic mouse models. In contrast to what has been postulated before, human DC-SIGN could have been evolved as a receptor, which enables the host to survive longer by controlling bacterial growth and limiting a tissue damaging inflammatory response. In future projects the precise mechanism explaining this effect has to be analysed in more detail. This may give important clues to strategies fighting tuberculosis and developing more efficient vaccinations in men.

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8 Curriculum vitae

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9 Deutsche Zusammenfassung

M. tuberculosis, der Erreger der Tuberkulose, stellt mit über zwei Milliarden infizierter Menschen weltweit einen der effektivsten bakteriellen Pathogene der Menschheit dar. Von diesen zwei Milliarden Infizierten entwickeln jedoch nur ca. 10 % sofort eine akute Tuberkulose. Die restlichen 90 % der infizierten Personen scheinen in der Lage zu sein, die Infektion komplett auszuheilen beziehungsweise soweit kontrollieren zu können, dass zunächst keine symptomatische Erkrankung zu Tage tritt. Bei der letzteren Gruppe kann die Infektion jedoch, häufig nach einer Schwächung des Immunsystems (zum Beispiel durch HIV), reaktiviert werden.

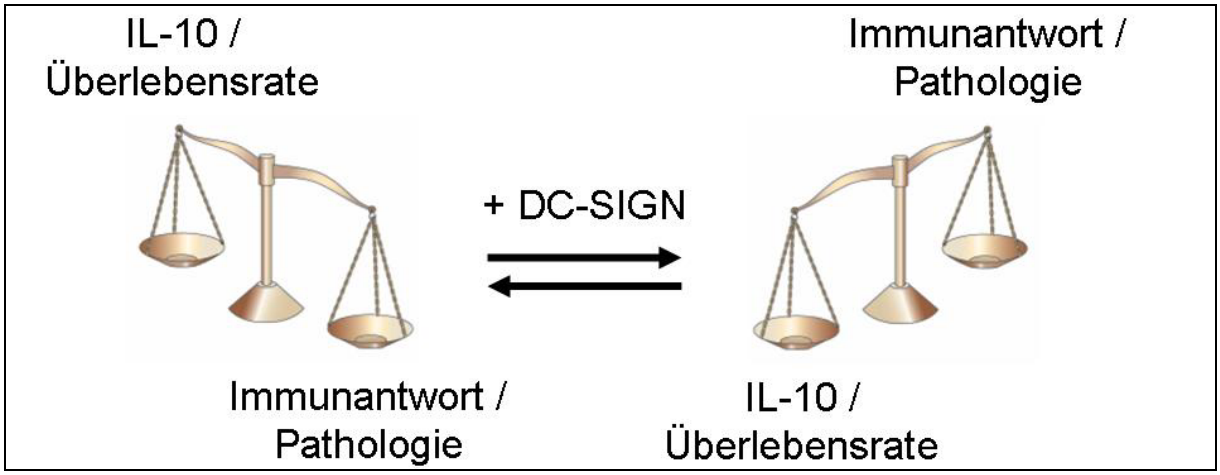
Nach wie vor gibt es eine Vielzahl ungelöster Fragen. Bis heute ist nicht genau bekannt, welche genauen Faktoren entscheidend sind ob und wie schwer ein Mensch an Tuberkulose erkrankt. Darüber hinaus steht der Menschheit auch knapp 125 Jahre nach der ersten wissenschaftlichen Beschreibung des Erregers der Tuberkulose, durch Robert Koch im Jahr 1882, kein wirkungsvoller Impfstoff zur Verfügung. Die Verwendung des nah verwandten Impfstammes *M. bovis* BCG scheitert an einem effektiven Schutz Erwachsener vor einer Lungen-Tuberkulose, der häufigsten Form der Erkrankung. Entgegen der allgemeinen Erwartung entwickeln Individuen, die für Tuberkulose empfänglich sind, während der akuten Erkrankung eine *M. tubculosis*-spezifische Immunität. Charakteristisch ist die Entwicklung einer Typ-1-T-Helferzellen-(Th1)-Antwort in Form von IFN-γ produzierenden $CD4^+$ T-Zellen. Für die Ausbildung der Th1-Antwort ist hauptsächlich die Sekretion des Zytokins IL-12 durch antigenpräsentierende dendritische Zellen (DZ) verantwortlich. Diese Immunantwort führt jedoch letztendlich nicht zu einem Schutz vor der Infektion. Zahlreiche Rezeptormoleküle auf der Oberfläche von DZs sind an der Erkennung als auch an der Aufnahme von *M. tuberculosis* beteiligt. Den Rezeptoren der PRR (engl. *pattern recognition receptors*) – Familie, die Mikroorganismen charakteristische Motive erkennen, kommt dabei eine besondere Rolle zu. Zu dieser Familie von Rezeptoren, die während der mycobakteriellen Infektion eine sehr wichtige Funktion haben, zählen die Toll-like Rezeptoren (TLR). Aktivierung von TLRs (2, 4 und 9) auf DZs durch *M. tuberculosis* führt zur Aktivierung dieser Zellen gefolgt von der Freisetzung von Zytokinen und der Expressionssteigerung kostimulatorischer Moleküle auf der Zelloberfläche. Im Jahre 2003 wurde gezeigt, dass das C-Typ-Lektin DC-SIGN (CD209) jedoch als eigentlicher Hauptrezeptor für *M. tuberculosis* auf humanen DZs fungiert. Eine nachfolgende Studie mit *in vitro* generierten humanen myeloiden DZs zeigte, dass eine gleichzeitige Stimulation von TLR4, über bakterielle Lipopolysaccharide (LPS), und DC-SIGN, über Bestandteile der Zellwand von *M. tuberculosis* (ManLAM), sowohl die Reifung der DZs inhibiert als auch die Synthese des anti-inflammatorisch wirkenden Zytokins IL-10 induziert. Beide Effekte wären klassische Indizien für eine Typ-2-T-Helferzellen-Antwort. Basierend auf diesen Erkenntnissen wurde spekuliert, dass die simultane Interaktion von *M. tuberculosis* mit TLRs und DC-SIGN an der Oberfläche von DZs es dem Pathogen ermöglicht durch Blockade der TLR-vermittelten

Zellaktivierung das Immunsystem zu umgehen. Dies könnte den mangelnden Schutz bei an Tuberkulose erkrankten Individuen, trotz Ausbildung einer Th1-Antwort, erklären.

Auch in Mäusen ist hauptsächlich die Lunge von einer Tuberkulose betroffen und verschiedene Mausmutanten mit Defekten in verschiedenen infektionsrelevanten Genen stehen zur Verfügung. Keines der fünf Mausmoleküle, die Homologien zu humanem DC-SIGN aufweisen, besitzt ein mögliches Signalmotiv des humanen DC-SIGN und wird auf myeloiden DZs expremiert. Daher haben wir in der vorliegenden Arbeit zwei verschiedene transgene Mausmodelle für humanes DC-SIGN vorgestellt, um die Rolle von humanem DC-SIGN in der mycobakteriellen Infektion *in vitro* und *in vivo* genauer untersuchen zu können.

Zuerst haben wir ein konventionell transgenes Mausmodell von humanem DC-SIGN erzeugt. Dazu wurde die Minimalsequenz des Promoters von murinem CD11c, einem Oberflächenmolekül mit hoher Spezifität für DZs in der Maus, gefolgt von der cDNA-Sequenz von humanem DC-SIGN in das Mausgenom integriert. Auf *in vitro* generierten DZs dieser Mäuse konnte das transgene Molekül detektiert werden. Auch *ex vivo* analysierte DZs der Milzen dieser, als CD11c-DC-SIGN bezeichneten, Mäuse wiesen eine deutliche Expression von humanem DC-SIGN auf. Als zweite Strategie zur Herstellung einer humanen DC-SIGN transgenen Mauslinie wählten wir die BAC-Technologie. Dazu wurde ein 70-kb großes DNA-Fragment, das den kompletten Genlocus von humanem DC-SIGN enthält, aus einem kommerziell erwerblichen BAC präpariert. Auch dieses Fragment wurde durch Mikroinjektion in den Pronukleus einer befruchteten C57BL/6 Mauseizelle transferiert. Diese Mauslinie wurde als BAC-DC-SIGN bezeichnet. Im Gegensatz zu den CD11c-DC-SIGN-Mäusen, ist das transgene Molekül auf *in vitro* generierten DZs aber nicht auf *ex vivo* analysierten Milz-DZs der BAC-DC-SIGN-Mäuse zu finden. Nur nach einer sterilen Infektion konnte auf myeloiden Peritonealzellen humanes DC-SIGN *ex vivo* in der BAC-DC-SIGN Maus nachgewiesen werden. Nach Infektion von DZs mit *M. tuberculosis in vitro* wurde die Produktion von Zytokinen bestimmt. Im Überstand von DZs der CD11c-DC-SIGN Linie wurde im Vergleich zu DZs von Mäusen des Wildtyps ein deutlich reduzierter Spiegel an IL-12p40 gemessen. Die Menge an sezerniertem IL-10 war nach Infektion mit Mycobakterien bei transgenen und wildtyp-Zellen ähnlich. Somit haben wir nicht eine DC-SIGN-abhängige Zunahme des Th2-Zytokins IL-10 aber eine Abnahme des Th1-Zytokins IL-12 nachweisen können. DZs der BAC-DC-SIGN-Mäuse produzierten nach Infektion durch Mycobakterien ähnliche Mengen an Zytokinen, wie DZs aus Mäusen des Wildtyp. Eine mögliche Ursache könnte sein, dass *in vitro* DZ-Kulturen der BAC-DC-SIGN Tiere im Vergleich zu *in vitro* kultivierten CD11c-DC-SIGN DZs prozentual weniger DC-SIGN positive Zellen enthielten. Um zu analysieren, an welcher Stelle DC-SIGN möglicherweise TLR-vermittelte Signalkaskaden stören könnte wurde die Aktivierung von Mitogen-aktivierten-Protein-Kinasen (MAPK), IκBα und NF-κB nach Infektion von DZs *in vitro* bestimmt. Die Aktivierung aller drei Faktoren zeigte keinerlei signifikante Unterschiede nach Infektion mit Mycobakterien zwischen wildtyp-DZs und DZs, die transgenes humanes DC-SIGN auf ihrer Oberfläche aufwiesen. Nach Aerosol-Infektion mit *M. tuberculosis in vivo* überlebten transgene Mäuse beider Modelle für humanes DC-SIGN deutlich länger als Kontrolltiere.

Die ursprünglich vermutete Rolle von humanem DC-SIGN, durch *M. tuberculosis* zur Umgehung des Immunsystems missbraucht zu werden, konnte nicht bestätigt werden. Durch die Untersuchungen mit den hier vorgestellten transgenen Mausmodellen für humanes DC-SIGN erscheinen die bisherigen Erkenntnisse in einem neuen Licht. Nun scheint sich humanes DC-SIGN vielmehr als Rezeptor entwickelt zu haben, der dem Wirt durch Kontrolle des bakteriellen Wachstums und durch Begrenzung einer zu starken und damit organschädigenden Immunantworte ein längeres Überleben ermöglicht. Aktuell werden im Labor von Herrn Dr. Sparwasser und Kooperationspartnern weitere Untersuchungen durchgeführt, um den genauen Wirkmechanismus von DC-SIGN während einer Infektion mit *M. tuberculosis* aufzuklären.



Modell der Rolle von humanem DCIGN in der Infektion mit *M. tuberculosis*.