Decreased Pathology and Prolonged Survival of Human DC-SIGN Transgenic Mice during Mycobacterial Infection

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Decreased Pathology and Prolonged Survival of Human
DC-SIGN Transgenic Mice during Mycobacterial Infection

Martin Schaefer,¹² Norbert Reiling,²¹ Cornelia Fessler,‡ Johannes Stephani,* Ichiro Taniuchi,⁴⁺ Farahnaz Hatam,§ Ali Oender Yildirim,‖ Heinz Fehrenbach,‖ Kerstin Walter,‡ Juergen Ruland,† Hermann Wagner,* Stefan Ehlers,³⁴‡ and Tim Sparwasser³⁴⁵

Dendritic cell (DC)-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN: CD209) is a C-type lectin that binds ICAM-2,3 and various pathogens such as HIV, helicobacter, and mycobacteria. It has been suggested that Mycobacterium tuberculosis, the causative agent of pulmonary tuberculosis, interacts with DC-SIGN to evade the immune system. To directly analyze the role of human DC-SIGN during mycobacterial infection, we generated conventional transgenic (tg) mice (termed “hSIGN”) using CD209 cDNA under the control of the murine CD11c promoter. Upon mycobacterial infection, DCs from hSIGN mice produced significantly less IL-12p40 and no significant differences were be observed in the secretion levels of IL-10 relative to control DCs. After high dose aerosol infection with the strain M. tuberculosis H37Rv, hSIGN mice showed massive accumulation of DC-SIGN⁺ cells in infected lungs, reduced tissue damage and prolonged survival. Based on our in vivo data, we propose that instead of favoring the immune evasion of mycobacteria, human DC-SIGN may have evolved as a pathogen receptor promoting protection by limiting tuberculosis-induced pathology. The Journal of Immunology, 2008, 180: 6836–6845.

World-wide, it is estimated that every second, another human being is infected with Mycobacterium tuberculosis (Mtb) and with one third of the world’s population latently infected, Mtb is considered one of the most successful pathogens (1, 2). IL-12 producing dendritic cells (DCs) are the most important APC for naive T cells (3), and are therefore essential for mounting cellular immune responses against mycobacteria (4–10). DCs recognize mycobacteria through a variety of germline-encoded pattern recognition receptors including TLRs (reviewed in Ref. 11) and lectins such as DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN: CD209) (12, 13).

TLR2, TLR4, and TLR9 have been shown to be critically involved in mycobacteria-induced cell activation (14–17). TLR2 mediates cellular activation after stimulation with mycobacterial lipoproteins and terminally nonmannosylated lipoarabinomannan (LAM) (18, 19), whereas TLR4 is involved in signaling events induced by a heat-sensitive cell-wall associated mycobacterial factor (16). Bafica et al. (15) demonstrated that TLR9, recognizing bacterial DNA, is essential for mounting a Th1 response against Mtb and cooperates with TLR2 in vivo.

DC-SIGN, a C-type lectin, is a type II transmembrane protein mainly expressed on myeloid DCs and contains a carbohydrate recognition domain important for pathogen binding (20). In primates, the intracellular domain is characterized by the presence of a highly conserved di-leucine/tri-acidic cluster internalization signal and an incomplete tyrosine-based ITAM (21). Several mouse homologues have been cloned (22–24) which all lack the putative internalization and signaling motifs that in primates were under evolutionary (25). Currently, however, the cellular expression pattern has only been elucidated for two of those homologues: CD209a (mDC-SIGN) has an unknown function on plasmacytoid pre-DCs (26), whereas CD209b (SIGNR-1) is present on macrophage sub-populations in spleen and lymph nodes, but not in the lung and has been shown to be unimportant in mouse models of Mtb infection (27, 28).

Binding of mycobacterial mannosylated LAM (ManLAM) to human DC-SIGN induces a block on LPS-induced maturation of human DCs and leads to enhanced release of the anti-inflammatory cytokine IL-10 (12). Based on these findings, it was proposed that

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¹Institute for Medical Microbiology, Immunology, and Hygiene and ²Third Medical Department, Klinikum rechts der Isar, Technische Universität München, Munich; ³Division of Molecular Infection Biology, Research Center Borstel, Borstel; ⁴Experimental Rheumatology, Medical Clinic Rheumatology and Clinical Immunology, Charité Universitätsmedizin Berlin, Berlin; ⁵Clinical Research Group “Chronic Airway Diseases,” Medical Faculty, Philipps University, Marburg; ⁶Molecular Immunology, Christian-Albrechts-University, Kiel, Germany; ⁷Howard Hughes Medical Institute, Molecular Pathogenesis Program, Skirball Institute of Biomedical Medicine, New York University School of Medicine, New York, NY 10016; and ⁸Research Center for Allergy and Immunology, the Institute of Physical and Chemical Research, Yokohama, Japan

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‡Division of Molecular Infection Biology, Research Center Borstel, Borstel; §Experimental Rheumatology, Medical Clinic Rheumatology and Clinical Immunology, Charité Universitätsmedizin Berlin, Berlin; ³Clinical Research Group “Chronic Airway Diseases,” Medical Faculty, Philipps University, Marburg; ⁴Molecular Immunology, Christian-Albrechts-University, Kiel, Germany; ⁵Howard Hughes Medical Institute, Molecular Pathogenesis Program, Skirball Institute of Biomedical Medicine, New York University School of Medicine, New York, NY 10016; and ⁶Research Center for Allergy and Immunology, the Institute of Physical and Chemical Research, Yokohama, Japan

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mycobacterial cell wall components are able to modulate TLR-signaling by a DC-SIGN-dependent mechanism which may con-tribute to the immune evasion of *Mtb* (21). Genetic epidemiology has yielded conflicting data. A recent study by Vannberg et al. (29) suggested that human single nucleotide polymorphisms (SNPs) leading to decreased DC-SIGN expression are associated with re-duced risk for cavitary tuberculosis disease, while a previous pub-lication demonstrated that SNPs in the CD209 promoter region leading to increased DC-SIGN expression were protective against tuberculosis (30).

Therefore, to address the functional role of human DC-SIGN under experimental in vivo conditions, we generated mice trans-genic for human DC-SIGN (termed “hSIGN”), in which DC-SIGN is expressed under the control of the CD11c promoter, and ana-lyzed these mice in mycobacterial infection models. Interestingly, in contrast to data obtained with human cells, upon in vitro mycobacterial infection DCs from hSIGN mice did not secrete higher amounts of IL-10, and produced significantly less IL-12. In addi-tion, during chronic *Mtb* infection in vivo, hSIGN mice showed reduced tissue damage and survived significantly longer. We con-clude therefore that human DC-SIGN may be an evolutionary con-served molecule in primates, because instead of favoring immune evasion of mycobacteria, it appears to limit the elicited pathology during chronic tuberculosis infection.

### Materials and Methods

#### Constructs

Human DC-SIGN transgenic (tg) mice were generated using a human DC-SIGN cDNA sequence driven by the murine CD11c promoter that was kindly provided by Dr. Thomas Brocker (Ludwig-Maximilians University, Munich, Germany) (31). A 1.3-kb cDNA fragment of human DC-SIGN (CD209) was amplified by PCR using total cDNA from human peripheral blood monocyte-derived dendritic cells as template. The cDNA was cloned into the 3′ of the 5.5-kb CD11c promoter via EcoRI restriction sites. The construct was linearized by a combined NotI/ClaI digest and purified by gel elec-trophoresis for microinjection into fertilized B6D2F1 hybrid oocytes.

#### Mice

C57BL/6 mice were purchased from Harlan Winkelmann. Human DC-SIGN tg founder mice (termed “hSIGN”) were identified by PCR using primers: 5′-AGT CCG AgT 3′ and 360: 5′-ACG CgT CgA CAA AAg ggg gTg AAg TTC TgC TAC g-3′, selection was based on the levels of transgene expression in DCs, and backcrossed for 10 generations on C57BL/6 background. Animals were bred and maintained under SPF conditions at the animal facility of the Institute for Medical Microbiology, Immunology, and Hygiene, Technical University of Munich. Sex- and age-matched tg and littermate control mice were used for all experiments. *Mtb* infection experiments were performed in the biosafety level III facilities at the Di-vision of Molecular Infection Biology, Research Center Borstel. All animal experiments were performed under specific pathogen-free conditions and in accordance with institutional, state, and federal guidelines.

#### Cells

Bone marrow Flt3-ligand- and GM-CSF-derived (GM-DC) bone marrow dendritic cells (BMDCs) were generated as described previously (32, 33). In brief, bone marrow cells were removed from femurs and tibias of mice and cultured in complete RPMI (RPMI 1640, 2 mM l-glutamine, heat-inactivated 10% FCS, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-ME (all from PAA Laboratories GmbH) supplemented with Flt3-ligand (hybridoma from Walter and Eliza Hall Institute) to generate plas-macytoid and conventional DCs or GMCSF (34) to generate conventional DC cultures, BMDCs were harvested on day 8 and plated at a density of 0.5 × 10^6 cells per well. Controls for TLR-mediated DC activation included 1 µM CpG ODN (1826 or 1668 (35, 36) Coley), 1 µg/ml LPS (Sigma- Aldrich), or 0.5 µg/ml Pam3CSK4 (EMC Microcollections GmbH).

#### Mycobacterial infection

*M. bovis* Bacillus Calmette Guérin (BCG; American Type Culture Collection: 27289, DSMZ) and *M. tuberculosis* strains H37Rv, CDC1551 as well as the Beijing strains (354701;420301) were grown at 37°C in Middlebrook 7H9 broth supplemented with Middlebrook OADC-Enrichment (BD Biosciences). Midlog phase cultures were harvested, aliquoted, and frozen at −80°C. Viable cell counts were determined by plating serial dilutions of cultures on Middlebrook 7H10 agar plates (BD Biosciences). For experi-mental in vitro infections, aliquots were diluted in culture media and the preparation was passed six times through a 27-gauge needle to ensure proper dispersion of mycobacteria. Day 9 BMDCs were stimulated for 24 h with *M. bovis* BCG or *Mtb* at different multiplicities of infection. For DC cocultures, day 7 DCs from hSIGN and wild type (WT) mice were harvested, washed, and infected with 50 and 10 multiplicities of infection of *M. bovis* BCG. After overnight culture, DCs were washed, resuspended in complete RPMI 1640, and plated on 96-well plates at a density of 1 × 10^5 DCs/well. CD4 T cells were purified from lymphnodes of C57BL/6 WT mice using magnetic beads (CD4 untouched-kit, Invitrogen) and 2 × 10^5 CD4 T cells were added to 1 × 10^5 DCs/well. After 24 h, supernatants were taken and frozen at −80°C until analysis.

Pulmonary infection was performed using an inhalation exposure system (Glas-Col). Mice were infected with a dose of 100, 1000, or 2000 CFU/lung, confirmed by determining the bacterial load in undiluted homogenates of the entire lung 24 h after infection. Mice were regularly weighed and monitored for additional experiments. CD4 or CD8 T cells of seven infected mice per genotype where pooled into three to four groups. From single cell suspensions, CD4 or CD8 T cells of seven infected mice per genotype where pooled into three to four groups. From single cell suspensions, CD4 or CD8 T cells were enriched by magnetic cell sorting (Miltenyi Biotec) and IFN-γ was detected using a biotinylated anti-IFN-γ (M5/114.15.2, eBioscience), and human CD209 (120507, R&D Systems GmbH). All cells were coated with propidium iodide or ethidium monoazide to exclude nonviable cells (Sigma-Aldrich). Cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences) and CellQuestPro (BD Biosciences). Subsequent data analysis was performed with FlowJo software (Tree Star).

#### Colony enumeration assay

At different time points after infection with *Mtb*, lung, spleen, and liver of sacrificed animals were aseptically removed, weighed, and homogenized in PBS containing a proteinase inhibitor mixture (Roche Diagnostics). For colony enumeration, a 10-fold serial dilution of organ homogenates was 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 CFU per organ. For i.p. infection with *M. bovis* BCG, the spleens were prepared and analyzed as described above.

#### Flow cytometry

To block Fc-receptors, cells were incubated with unlabeled anti-CD16/CD32 (93, eBioscience, Frankfurt, Germany) Ab for 10 min at 4°C. Staining was performed with Abs against murine CD11c (N418, eBioscience), murine CD45R (RA3–6B2, eBioscience), murine MHC class II (MHCII; 7.114.15.2, eBioscience), and human CD20 (120507, R&D Systems GmbH). CD4 or CD8 T cells of seven infected mice per genotype where pooled into three to four groups. From single cell suspensions, CD4 or CD8 T cells were enriched by magnetic cell sorting (Milte-nyi Biotec) and IFN-γ production was detected by an ELISPOT assay kit (AID). In brief, enriched CD4 or CD8 T cells were seeded at an initial concentration of 1 × 10^6 cells/well in anti IFN-γ mAb-coated plates and serially diluted in complete IMDM. Total splenocytes from uninfected WT mice using streptavidin-HRP and AEC (AID) as substrate. Frequency of re-

#### ELISPOT assay for IFN-γ

For Ag-specific stimulation, single-cell suspensions of lungs were pre-

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total number of T cells seeded into the well, a linear regression was performed and the number of spot forming cells per 10000 cells was calculated.

Cytokines and metalloproteinases

Culture supernatants were harvested 24 h after infection with mycobacteria and frozen at -80°C until analysis. IL-10, IL-12p40, IFN-γ, and TNF-α levels were determined by ELISA (Duo Set, R&D Systems). RNA was isolated from lung homogenates of infected mice at the indicated time points, reverse transcribed, and analyzed using quantitative Lightcycler Technology. We monitored matrix metalloproteinase (MMP) activity using a fluorogenic peptide as substrate for MMPs according to the manufacturer’s instructions (Mca-PLGL-Dpa-AR-NH₂, R&D Systems).

Lung histology

Lungs were fixed ex situ with 4% (w/v) phosphate buffered paraformaldehyde via the trachea, removed, and stored in 4% paraformaldehyde. To obtain a representative collection of lung tissue samples, systematic uniform random (SUR) sampling was performed. SUR samples were embedded into paraffin, and 2-μm sections were stained for anti-human DC-SIGN (BD Biosciences) by indirect immunohistochemistry. Standard H&E or Elastica-van-Gieson staining was performed for qualitative morphological analysis of histopathological alterations. One section of each SUR sample was analyzed using a computer-assisted stereology tool box (Visiopharm), and the following parameters were recorded from meander sampled SUR fields of view according to established stereological methods (37): arithmetic mean thickness of airway epithelium, volume of intrabronchial debris per epithelial membrane area, and surface fraction of bronchial elastic membrane gaps relative to total epithelial membrane area. To identify the phenotype of DC-SIGN⁺ cells, four consecutive sections of each block were stained as follows: section 1 by Ziehl-Neelsen staining (TB-Kinyoun staining kit, BD Biosciences), and sections 2 and 3 by indirect immunohistochemistry for anti-human DC-SIGN (Eb-h209, ebioscience) and anti-mouse MHCII (2G9, BD Biosciences), respectively. As murine alveolar epithelial type II cells are also known to express MHCII upon mycobacterial infection (38), section 4 was stained by indirect immunohistochemistry for anti-human surfactant protein B (Chemicon). In addition, double stainings for anti-human DC-SIGN followed by Ziehl-Neelsen staining were performed to reveal whether DC-SIGN⁺ cells were infected with mycobacteria or not.

Results

Generation of hSIGN tg mice

In humans, DC-SIGN is mainly expressed by myeloid DCs in mucosal tissues and can be detected on human blood-derived DCs after in vitro differentiation with IL-4 and GM-CSF as well as on CD11c⁺ myeloid alveolar lavage cells of Mtbo infected patients (21, 39). To drive tissue-specific expression of the human DC-SIGN transgene in murine DCs, we used the CD11c minimal promoter (31) for generating conventional tg mice and introduced the cDNA coding for CD209 via EcoRI restriction sites (Fig. 1A). The construct was injected into the pronuclei of fertilized B6D2F1 hybrid oocytes. Tg mouse lines with high transgene expression were isolated from spleen of wt or hSIGN mice. Nycodenz (67) enriched DCs were stained for CD11c, CD45R, and CD209 markers and analyzed for CD209 expression in the indicated populations. Open histograms, CD209; gray tinted histograms, isotype control. C) CD209 expression on ex vivo enriched splenic cells. DCs were isolated from spleen of wt or hSIGN mice. Nycodenz (67) enriched DCs were stained for CD11c, CD45R, and CD209 markers and analyzed for CD209 expression in the indicated populations. Open histograms, CD209; gray filled histograms, isotype control. Data represent one experiment from three.

Flow cytometric analysis of DC-enriched spleen and LN cells also demonstrated that the human transgene is faithfully expressed in all CD11c⁺CD45R⁻ DCs and is excluded from CD11c⁻ cells (Fig. 1C, not depicted). High transgene expression could be observed in all CD4⁺, CD8⁺, and CD4⁻CD8⁻ conventional DC subsets (not depicted). Expression level in ex vivo isolated splenic CD11c⁺CD45R⁻ plasmacytoid pre-DCs was low (Fig. 1C), which closely resembles the expression pattern of DC-SIGN in conventional human DCs.

For localization of human DC-SIGN tg cells in situ, tissue sections of lymph node, spleen, and lung were analyzed by immunohistochemistry. Cells positive for human DC-SIGN were present in
Reduced IL-12 secretion by hSIGN DCs after mycobacterial infection in vitro

Because triggering of DC-SIGN has been shown to affect TLR4-mediated cellular responses of human DCs leading to increased IL-10 production (12), we infected GM-CSF-derived BMDCs from WT and hSIGN mice with different doses of M. bovis BCG and measured subsequent IL-10 levels after 24 h (Fig. 3 A). TLR-stimuli alone (CpG, LPS, and Pam3CSK4) served as controls. No differences in IL-10 production could be observed between WT and tg DCs at 24 h after BCG infection or earlier time points (Fig. 3A and not depicted). Using the same stimulus, no gross differences were observed with regard to the surface expression of co-stimulatory molecules (CD80, CD86) and DC maturation markers (MHCII) (not depicted). Also, cell wall components or ManLAM from Mtb H37Rv in combination with titrated amounts of LPS did not trigger increased IL-10 secretion from tg DCs (not depicted).

Because IL-12 plays a pivotal role in the control of mycobacterial infection (40, 41), we also measured IL-12p40 formation in response to infection with mycobacteria. A. Five × 10⁶ GM-CSF-BMDC were infected with indicated MOI of M. bovis BCG or TLR ligands. The supernatants were harvested after 24 h and levels of IL-12p40 and IL-10 were detected by ELISA. Mean values (+SD) from duplicates of two individual animals are depicted. One representative experiment of three is shown. Data were analyzed by Student’s t test using www.graphpad.com/quickcalcstest1.cfm. *, p < 0.01; **, p < 0.001; n.s., not significant. B. One × 10⁷ GM-CSF-BMDC were infected with indicated multiplicities of infection (MOI) with different strains of Mtb. Supernatants were harvested after 24 h and levels of IL-12p40 and TNF-α were detected by ELISA. Mean values (+SD) from triplicates depicted. Data from one representative experiment of three are shown. Data were analyzed by Student’s t test using www.graphpad.com/quickcalcstest1.cfm. *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant.

FIGURE 3. Cytokine production of human DC-SIGN tg DCs in response to infection with mycobacteria. A, Five × 10⁶ GM-CSF-BMDC were infected with indicated MOI of M. bovis BCG or TLR ligands. The supernatants were harvested after 24 h and levels of IL-12p40 and IL-10 were detected by ELISA. Mean values (+SD) from duplicates of two individual animals are depicted. One representative experiment of three is shown. Data were analyzed by Student’s t test using www.graphpad.com/quickcalcstest1.cfm. *, p < 0.01; **, p < 0.001; n.s., not significant. B, One × 10⁷ GM-CSF-BMDC were infected with indicated multiplicities of infection (MOI) with different strains of Mtb. Supernatants were harvested after 24 h and levels of IL-12p40 and TNF-α were detected by ELISA. Mean values (+SD) from triplicates depicted. Data from one representative experiment of three are shown. Data were analyzed by Student’s t test using www.graphpad.com/quickcalcstest1.cfm. *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant.
(Fig. 3A). Also, incubation with other pathogens not recognized via DC-SIGN (e.g., \textit{L. monocytogenes}) did not lead to differential IL-12 production between WT and tg DCs (not depicted). In addition to total IL-12/IL-23p40, we also measured bioactive IL-12p70 in supernatants from BCG-infected WT and hSIGN BMDCs. Although harder to detect, we observed a similar reduction of bioactive IL-12p70 production in BCG-infected hSIGN BMDCs compared with WT cells (data not shown). Importantly, marine bone marrow-derived macrophages from hSIGN mice lacking hDC-SIGN expression displayed similar cytokine profiles to macrophages from WT mice (data not shown). Next, we wanted to analyze whether reduced IL-12 production by tg DCs may affect the priming of naive T cells during DC-T cell cocultures. We infected DCs from WT and hSIGN mice with different doses of \textit{M. bovis} BCG and added naive CD4 T cells to the cultures 14 h after infection. Supernatants of cocultures collected at 38 h after infection demonstrated decreased IL12p40 levels from infected DCs as well as decreased IFN-\gamma secretion by T cells primed with hDC-SIGN tg DCs compared with T cells cocultured with WT DCs (Table I).

To test whether reduction of IL-12p40 levels was also observed using pathologically relevant mycobacterial strains, we performed similar experiments with different strains of \textit{Mtb} including the widely used lab strain \textit{Mtb} H37Rv as well as \textit{Mtb} CDC 1551, which was isolated during an outbreak of TB close to the Kentucky-Tennessee border (42). In addition, two clinical \textit{Mtb} isolates of the Beijing genotype (43), from TB patients from Afghanistan (3547/01) and Vietnam (4203/01), were included in this study. Supernatants of infected DCs were analyzed for IL-12p40 and TNF-\alpha production (Fig. 3B). Again, released IL-12p40 upon stimulation with all \textit{Mtb} isolates was significantly reduced in hSIGN DCs. However, the release of TNF-\alpha, another key cytokine in controlling mycobacterial infection (44), was similar between WT and human DC-SIGN tg DCs (Fig. 3B).

To address the question of whether TLR signaling pathways involved in IL-12 production were affected by the human transgene, we examined the influence of human DC-SIGN on the activation of MAPK known to be critically involved in the release of cytokines upon TLR ligation (45). BMDCs expressing the human transgene did not show differences in phosphorylation and activation of JNK-, ERK 1/2-, and p38 MAPK after infection with mycobacteria (data not shown). Furthermore, no differences in IkB\alpha degradation and NFkB activation during acute BCG infection of DCs (5–40 min) could be detected (not depicted). In summary, while putative DC-SIGN-mediated signaling pathways await further elucidation, in DCs from hSIGN mice the main cytokine driving Th1 cellular immune responses is significantly reduced after stimulation with both BCG and \textit{Mtb}. This humanized mouse model now allowed us to study the in vivo relevance of DC-SIGN during mycobacterial infection.

\section*{No differences in mycobacterial load between WT and hSIGN infected mice}

To investigate the course of \textit{Mtb} infection in the humanized mouse model, the mycobacterial load was analyzed after aerosol infection of WT and hSIGN mice with 100 CFU \textit{Mtb}. B. Mycobacterial load in the lung, liver, and spleen was determined on day 21 and 42 days after aerosol infection with 2000 CFU \textit{Mtb}.

\begin{table}[h]
\centering
\caption{Cytokine release by BCG infected DCs cocultured with naive CD4\textsuperscript{+} WT T cells\textsuperscript{a}}
\begin{tabular}{|c|c|c|c|c|}
\hline
 & WT DCs &  & hSIGN DCs & \\
 & IL12 & IFN-\gamma & IL12 & IFN-\gamma \\
MOI & (ng/ml) & (ng/ml) & (ng/ml) & (ng/ml) \\
\hline
50 & 22.11 \pm 3.20 & 0.16 \pm 0.06 & 4.75 \pm 1.93 & 0.04 \pm 0.02 \\
10 & 19.88 \pm 3.32 & 0.11 \pm 0.03 & 5.97 \pm 2.22 & 0.06 \pm 0.02 \\
0 & 4.47 \pm 0.80 & 0.01 \pm 0.00 & 3.86 \pm 1.22 & 0.00 \pm 0.00 \\
\hline
\end{tabular}
\textsuperscript{a}One \times 10^5 BMDCs were infected with indicated multiplicities of infection (MOI) of \textit{M. bovis} BCG. After 14 h, 2 \times 10^5 naive CD4\textsuperscript{+} T-cells were added to the cultures. The supernatants were harvested 38 h after infection and levels of IL-12p40 and IFN-\gamma were detected by ELISA. Results are expressed as means of triplicate values \pm SD.
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Course of mycobacterial infection in hSIGN mice. C57BL/6 and hSIGN mice were infected with mycobacteria by aerosol. \textbf{A}, CFU amounts were determined in the lungs at indicated time points after infection with 100 CFU \textit{Mtb}. \textbf{B}, Mycobacterial load in the lung, liver, and spleen was determined on day 21 and 42 days after aerosol infection with 2000 CFU \textit{Mtb}.}
\end{figure}
To analyze whether the priming of T cells would differ between WT and hSIGN mice, we incubated APCs with purified CD4+ and CD8+ cells from lymph nodes or lungs of Mtb-infected mice in the presence of Mtb-derived peptides (ESAT6, Mtb32) and measured IFN-γ production by ELISPOT. The total number of CD4+ IFN-γ-producing cells in either lymph node or lung did not differ between WT and hSIGN mice, in line with our finding that the total amount of IFN-γ mRNA and protein in Mtb-infected mouse lungs was similar in WT and hSIGN mice. In contrast, the number of Mtb-specific, CD8+ IFN-γ-producing cells was significantly reduced in the lungs of hSIGN mice, particularly on day 21 after infection (Fig. 5). A similar trend was seen in CD8+ cells isolated from lymph nodes, however this difference did not reach statistical significance (data not shown). Because the total number of CD3/CD28-stimulated IFN-γ-producing CD8 cells was similar in WT and tg mice (data not shown), these data suggest that the priming of Mtb-specific CD8 cells is impaired or delayed in hSIGN mice.

**Delayed priming of Mtb-specific CD8+ T cells**

To further investigate the functional role of hDC-SIGN in vivo, we characterized the localization of hDC-SIGN+ cells in the lung after infection with Mtb in our model system. To decipher the properties of hSIGN+ cells in the lungs of infected animals, we analyzed lung sections by Ziehl-Neelsen staining for identification of Mtb. III, Numbers 1–4 in (I and II) identify DC-SIGN+/MHCIi+ cells; small letters a–d in (II and IV) identify SpB+/MHCIi+ cells; asterisks in (I and III) identify areas of Mtb infected cells. B, Mycobacterial load of 1 × 10^6 GM-CSF-BMDC (±200 U/ml IFN-γ) on day 7 after infection with a MOI of 1 Mtb.

**FIGURE 5.** IFN-γ T cell responses in Mtb-infected mice. Seven mice per group were infected with 1000 CFU M. tuberculosis H37Rv via the aerosol route. A, At day 21 and 42 post infection the frequency of IFN-γ-producing CD4+ T cells in lungs was determined by ELISPOT. Results are expressed as mean of spot forming cells per 1 × 10^5 cells ± SD. B, At day 21 and 42 post infection, the frequency of IFN-γ-producing CD8+ T cells in lungs was determined by ELISPOT. Results are expressed as mean of spot forming cells per 1 × 10^5 cells ± SD. Data were analyzed by Student’s t test using www.graphpad.com/quickcalcs/ttest1.cfm. *p < 0.05; **p < 0.01; ***p < 0.001.

**FIGURE 6.** Properties of hSIGN+ cells after infection with Mtb. A, Consecutive sections of lungs fixed at day 42 after infection of mice with 1000 CFU Mtb were stained with Abs against human DC-SIGN (I), mouse MHCII (II), and human surfactant protein B (IV) in addition to Ziehl-Neelsen staining for identification of Mtb. III, Numbers 1–4 in (I and II) identify DC-SIGN+/MHCIi+ cells; small letters a–d in (II and IV) identify SpB+/MHCIi+ cells; asterisks in (I and III) identify areas of Mtb infected cells. B, Mycobacterial load of 1 × 10^6 GM-CSF-BMDC (±200 U/ml IFN-γ) on day 7 after infection with a MOI of 1 Mtb.

Prompt influx of DC-SIGN+ cells into the lungs of Mtb-infected animals and reduced tissue damage in hSIGN mice

To further investigate the functional role of hDC-SIGN+ cells in the lung after infection with Mtb in our model system. To decipher the properties of hSIGN+ cells in the lungs of infected animals, we analyzed lung sections by Ziehl-Neelsen staining for identification of Mtb and by immunohistochemistry for expression of DC-SIGN, MHC-II, and surfactant protein B (Fig. 6A). Our data demonstrated DC-SIGN+ cells in the lungs of infected animals to be of DC phenotype. Interestingly, despite the described role as an uptake receptor for mycobacteria, we could not detect a preference of Mtb to be located in DC-SIGN+ cells. In vitro infection of WT and hSIGN BMDCs with Mtb also showed equal bacterial burden at day 7 after infection (Fig. 6B). On day 42 and 148 after aerosol infection with 1000 CFU Mtb, formalin-fixed lungs, prepared from control and hSIGN animals, were stained for hDC-SIGN+ and analyzed in parallel for cellular recruitment to the site of infection and granuloma formation (Fig. 7, A–J). The amount of hDC-SIGN+ cells in the lungs of hSIGN mice was drastically increased after infection (Fig. 7, B and D). Human DC-SIGN+ cells in the lungs of infected mice were located throughout the gas exchange region in alveolar septal walls and capillaries, and were regularly observed between epithelial cells in all airway generations. In contrast, inside granulomas only few hDC-SIGN+ cells were present and no differences between WT and hSIGN mice were observed with regard to granuloma development. In fact, the amount and size of lymphocyte infiltrations (arrow heads) were comparable at day 42 (Fig. 7, E and F). However, in contrast to hSIGN mice, on
day 148 the lungs of WT mice showed massive damage to the lung parenchyma and distal airways as indicated by a marked increase in necrotic tissue and cell debris occluding distal and mid-level bronchi (Fig. 7, G and H). In addition, Elastica-van-Gieson staining revealed an increase in elastic membrane gaps of airways after 148 days of infection in WT mice but not hSIGN mice (Fig. 7, I and J). Stereological analysis of histological sections, which were collected by systematic uniform random sampling to assure that the fields of view analyzed were representative of the whole organ, revealed a marked increase in elastic membrane breaks (p = 0.01), a significantly enhanced accumulation of cellular debris within bronchi and bronchioli (p < 0.001), and a decrease in airway epithelium thickness (p < 0.02) between day 42 and 148 after Mtb infection in WT but not hSIGN mice (Fig. 8). Critical mediators of tissue reorganization and, if dysregulated, destruction are MMPs. We therefore monitored MMP activity using a fluorogenic peptide as substrate for MMP-1, MMP-2, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13, MMP-14, MMP-15, MMP-16, and Cathepsin D and E. Although a time dependent increase in MMP activity in lung homogenates after Mtb infection was observed, there was no difference between WT and DC-SIGN tg mice after day 148 (data not shown). Analysis of the bacterial burden in the lungs of these mice did not reveal a significant difference between WT and DC-SIGN tg mice (WT: 7.707 ± 0.274 CFU (log10/lung), DC-SIGN tg 7.182 ± 0.275 CFU (log 10/lung); Mean ± SD, p = 0.0571; Mann-Whitney U Test).

Prolonged survival of hSIGN mice during chronic Mtb infection

The observations that the presence of human DC-SIGN: 1) limits the production of the proinflammatory cytokine IL-12 by BMDCs after infection with Mtb in vitro and 2) has a beneficial effect on the pathology elicited by Mtb infection, raised the question whether DC-SIGN would have an impact on long term survival of Mtb infected mice tg for human DC-SIGN. Until approximately day

FIGURE 7. Granuloma formation and human DC-SIGN expression in the lungs of Mtb-infected hSIGN mice. C57BL/6 and hSIGN mice were infected with 1000 CFU of Mtb. Lung sections were analyzed for granuloma formation and expression of human DC-SIGN after 42 and 148 days of infection. Sections were stained with anti-human DC-SIGN anti-body (A–D), H&E (E–H) or Elastica-van-Gieson (I and J) staining and analyzed by microscopy. Shown is one representative result of five mice per group. Arrow heads, lymphocyte aggregates; arrows, elastic membrane breaks.

FIGURE 8. Quantitative lung histopathology of C57BL/6 and hSIGN mice infected with 1000 CFU of Mtb. Elastica-van-Gieson stained lung sections of mice sacrificed on day 42 and 148 after infection (n = 4–5 per group) were obtained by systematic uniform random sampling and analyzed by computer-assisted stereology tool box (computer-assisted stereology tool box-grid, Visiopharm) for (upper panel) arithmetic mean thickness of airway epithelium, (middle panel) volume of intrabronchial cell and tissue debris per airway epithelial basal membrane area, and (lower panel) fraction of gaps in the elastic membrane relative to total area of the airway wall inner elastic membrane. Statistical analysis was performed using two-way ANOVA followed by post hoc multiple pairwise comparisons (Holm-Sidak method). The p values >0.05 are indicated.
Statistical analysis of the survival curve was performed using log-rank test. Mtb. Ten mice per group were monitored and moribund mice were sacrificed. and hSIGN mice were aerogenically infected with 100 (A) and 2000 (B) CFU Mtb. Ten mice per group were monitored and moribund mice were sacrificed. Statistical analysis of the survival curve was performed using log-rank test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant.

Fig. 9. Survival of hSIGN mice after infection with Mtb. C57BL/6 and hSIGN mice were aerogenically infected with 100 (A) and 2000 (B) CFU Mtb. Ten mice per group were monitored and moribund mice were sacrificed. Statistical analysis of the survival curve was performed using log-rank test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant.

Discussion

Animal models of low-dose aerosol infection with Mtb are believed to reflect the typical infection that occurs when humans inhale only a few virulent bacteria aerosolized in the course of an infected individual’s coughing (48, 49). However, the use of mice as model organisms for the study of immune responses during infectious diseases carries the risk that important host-pathogen interactions may be overlooked or misinterpreted, for example if the corresponding pathogen receptor is not expressed in mice. Humanized mice, which mimic human physiological systems after genetic manipulation, are therefore becoming important tools in the analysis of infectious human diseases (50, 51).

Based on in vitro studies using human DCs it has been hypothesized that in mycobacterial infection human DC-SIGN mediates immune evasion by inhibiting TLR-signaling (12). Whereas TLR-signaling is highly conserved between mice and men, none of the DC-SIGN mouse homologues are expressed on GM-CSF bone marrow-derived myeloid DCs in mice (our unpublished data). In addition, all mouse homologues lack the putative signaling and internalization motifs that are highly conserved in primates (21). To resolve these conflicting issues and gain further insight into a possible role of human DC-SIGN after infection with Mtb in vivo, we have generated a hSIGN tg mouse model. Immunohistochemical stainings of lung sections from naive hSIGN mice showed expression of human DC-SIGN on cells of DC-like morphology demonstrating the expression of hDC-SIGN on murine DCs. In addition, hDC-SIGN cell surface expression could be identified directly on ex vivo isolated cells and on DCs from in vitro-derived GM-CSF or Flt3L bone marrow cells from hSIGN mice. Taken together, the expression of hDC-SIGN on murine DCs demonstrates that CD11c-driven expression of human DC-SIGN is an appropriate model system to characterize the functional role of hDC-SIGN in murine DCs in vitro and in vivo.

The priming of a Th response into Th1 or Th2 differentiation by DCs is mediated via the expression of different costimulatory molecules on the cell surface and the release of cytokines (52). Particularly, the production of IL-12 has been shown to be important for DCs to drive a Th1 response by CD4+ T cells (53). Several independent studies have demonstrated the importance of IL-12 for the induction of an effective immune response after infection with Mtb. The initial control of an infection is correlated with the presence of IFN-γ-producing CD4+ T cells (54), which is induced by IL-12 released from infected APCs (40). As shown by Cooper and colleagues, the inability of IL-12p40-deficient mice to control mycobacterial infection is linked to a deficiency in IFN-γ-producing T cells. Another soluble factor secreted during infection with Mtb is IL-10 (55). Adoptively transferred IL-10-deficient DCs infected with M. bovis BCG show an improved migration to the draining lymph node in WT animals and enhanced induction of IFN-γ-producing T cells in response to mycobacterial Ag, indicating that during mycobacterial infection, immune responses by DCs can be suppressed by autocrine IL-10 via limiting DC migration and IL-12 production (56). In the current study, we observed a significantly reduced level of IL-12p40 production by myeloid BMDC from human DC-SIGN tg animals compared with WT mice after infection with M. bovis BCG or different strains of Mtb. In contrast to the in vitro data using human dendritic cells (12), a significant human DC-SIGN dependent increase in IL-10 production was not detectable after mycobacterial stimulation, which may be due to intrinsic dissimilarities between murine and human DCs. Differences to the previously reported results could also be a consequence of the lack of interaction of human DC-SIGN with murine adaptor or signaling molecules. First preliminary data suggest, however, that in transgenic DCs, human DC-SIGN triggers similar signaling pathways as detected in human DCs after DC-SIGN ligation. Those studies are currently under investigation and will shed light on the mechanism of action of human DC-SIGN. The fact that Mtb-infected hSIGN mice do not differ from WT mice with regard to IL-12p40 expression levels in the lung suggests that in vivo during chronic infection hDC-SIGN independent compensatory mechanisms inducing IL-12 and hDC-SIGN+ producers of IL-12 may be present. It is equally possible that even though the overall IL-12p40 levels do not differ between infected WT and hSIGN mice, regional differences do exist, for example at the granuloma fringes where newly recruited hDC-SIGN bearing cells are found. These more subtle and compartmentalized variations of cytokine levels may have escaped detection by mRNA or protein measurements in total lung homogenates, but may still have profound consequences for the regulation of DC-dependent immune responses. Interestingly, however, using IFN-γ production as a read-out for Ag-specific effector function, we found CD8 cells...
from hSIGN mice to be significantly less active than CD8 cells from WT mice, possibly suggesting that DC-SIGN may be a hitherto undervalued factor for the priming of CD8 T cells in tuberculosis. Although IFN-γ secretion by CD8 cells certainly contributes to macrophage activation and tissue damage, lysis of incompetent infected target cells by Ag-specific CD8 cells may also be beneficial, because the released bacteria are then taken up by activated macrophages within the granuloma, and presumably contained or destroyed (57, 58). The role of CD8 T cells in Mtb infection is most often viewed as a protective one especially during the late phase of experimental infection (59–63), however cytotoxic cells have also been implicated in the pathogenesis of disease because of their potential to cause tissue damage (64), possibly explaining the reduced tissue damage in hSIGN mice. We did not directly measure levels of CD8-mediated cytotoxicity in the lungs of Mtb-infected hSIGN mice, but instead used Mtb-specific IFN-γ secretion as a surrogate marker for CD8 T cell activity. Because hSIGN mice exhibited significantly less structural damage to the lung during Mtb infection than WT mice, it is tempting to speculate that this may have been caused by the recorded dampened CD8 response. To what extent this modulated CD8 response is also causally linked to the longer survival of hSIGN mice needs further detailed examination of cytotoxic effector functions of CD8 cells late during pulmonary Mtb infection.

The role of IL-12 in murine mycobacterial infections is probably more complex than previously thought: On the one hand it has been demonstrated 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 cannot control the mycobacterial growth and die early after infection (40). The opposite situation is characterized by a significant increase in IL-12 as observed in WSX 1-deficient mice after infection (40). The human DC-SIGN tg model presented in this study demonstrates that human DC-SIGN may have evolved primarily act as a vehicle for lesion formation in the lungs of infected mice in vivo. The current study did not show a difference in mycobacterial loads between hSIGN and WT mice, which may indicate that DC-SIGN does not primarily act as a vehicle for Mtb to evade the immune system. Instead, our data suggest that human DC-SIGN may have evolved as a receptor, which enables the host to survive longer by limiting the tissue-damaging inflammatory response. The precise mechanism by which DC-SIGN modulates excessive inflammation may provide important clues for developing strategies to fight tuberculosis and to improve vaccinations.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


