In vivo targeting of human DC-SIGN drastically enhances CD8$^+$ T-cell-mediated protective immunity

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Abbreviations used in this paper: BMDC, bone marrow-derived DC; cDC, conventional DC; DC-SIGN, DC-specific-ICAM3-grabbing-non-integrin; MMR, macrophage mannose receptor

Abstract

Vaccination is one of the oldest yet still most effective methods to prevent infectious diseases. Yet, eradication of intracellular pathogens and treatment of certain diseases like cancer requiring efficient cytotoxic immune responses remain a medical challenge. In mice, a successful approach to induce strong CTL reactions is to target antigens to DCs using specific antibodies against surface receptors in combination with adjuvants. A major drawback for translating this strategy into one for the clinic is the lack of analogous targets in human DCs. DC-SIGN (DC-specific-ICAM3-grabbing-non-integrin/CD209) is a C-type lectin receptor with potent endocytic capacity and a highly restricted expression on human immature DCs. Therefore, DC-SIGN represents an ideal candidate for DC targeting. Using transgenic mice that express human DC-SIGN under the control of the murine CD11c promoter (hSIGN mice), we explored the efficacy of anti-DC-SIGN antibodies to target antigens to DCs and induce protective immune responses in vivo. We show that anti-DC-SIGN antibodies conjugated to OVA induced strong and persistent antigen-specific $CD4^+$ and $CD8^+$ T-cell responses, which efficiently protected from infection with OVA-expressing *Listeria monocytogenes*. Thus, we propose DC targeting via DC-SIGN as a promising strategy for novel vaccination protocols against intracellular pathogens.

Introduction

The development of effective vaccines against intracellular infectious agents is one of the biggest challenges in science and an urgent unmet medical need. In addition, other highly prevalent diseases like cancer strongly require new vaccination strategies that promote effective and long lasting cytotoxic $CD8^+$ T-cell (CTL) responses.

With the discovery of dendritic cells (DCs) in 1973 by the Nobel Prize laureate Ralph Steinman, a new window of opportunities opened in vaccine development. Consequently, for many years efforts have been made to develop vaccines based on ex vivo generated DCs carrying specific antigens from tumours or pathogens [1]. Although these studies showed promising results in mice and in a number of clinical studies, the production of antigen-bearing cellular DC vaccines is a labour intensive and costly procedure [1]. Therefore, it hardly represents an option to vaccinate against infectious diseases like tuberculosis, malaria or HIV, which mainly affect developing countries. More recently, a new strategy has been explored to target defined antigens to DCs by using antibodies or glycosylated molecules that bind specific surface receptors directly in vivo [2-4]. Many of the receptors studied for DC targeting purposes belong to the family of C-Type lectin receptors (CLRs) such as DEC-205 (CD205) [2, 5, 6], macrophage mannose receptor (MMR) [7] and Clec9A [6, 8], which represent antigen uptake receptors that internalise antigen for loading MHC molecules. Amongst the above mentioned surface receptors, the best-studied example for antigen delivery to DCs is DEC-205. DEC-205 is an endocytic CLR found on the surface of a subgroup of conventional DCs (cDCs) present in the T-cell areas of LNs [9] with a high capacity to cross-present antigens to $CD8^+$ T cells [5, 10]. Antibodies against DEC-205 conjugated to OVA have been shown to induce potent $CD8^+$ and $CD4^+$ T-cell responses, when used in combination with adjuvants [5, 11]. Moreover, this vaccination strategy has successfully provided protective antigen-specific immune responses against tumours [2] and HIV antigens [6, 12, 13]. Some of these conjugated antibodies, such as anti-MMR [7] and

anti-DEC-205 (CDX-1401; Celldex Therapeutics) have even entered clinical trials. Yet, in humans, the expression pattern of DEC-205 and the MMR is less DC-restricted than in mice [1, 7, 14]. Therefore, targeting these receptors in humans carries a risk of undesired side effects and high antibody doses might be required to achieve adequate immune responses.

DC-SIGN is a C-type lectin receptor initially described as a surface molecule present on cDCs that mediates innate recognition of various pathogens [15-17]. One advantage of DC-SIGN over other receptors for delivering antigens in vivo is the specificity of its expression. DC-SIGN is mainly found on human immature DCs and at low levels on mature DCs and macrophages [15, 16], as well as on a small subset of blood plasmacytoid DCs (pDCs) [16, 18]. Targeting DC-SIGN via anti-DC-SIGN antibodies [19] or Lewis oligosaccharides [3, 4, 20] conjugated to a model antigen, has been shown to induce potent T-cell proliferative responses both in vivo and in vitro, and also inhibited tumour growth in a humanised mouse model [4, 21]. However, little is known on the efficacy of DC-SIGN targeting vaccines to induce protective responses against pathogens.

Listeria monocytogenes, a Gram-positive bacterium, is one of the best-characterised models to study vaccines against intracellular pathogens, since clearance of this bacterium is strongly dependent on $CD8^+$ T-cell responses [22]. Therefore, we used *L. monocytogenes* expressing OVA (LM-OVA) as a model organism to test the efficacy of an OVA conjugated anti-human-DC-SIGN (αDC-SIGN:OVA) antibody vaccine. Using the previously described hSIGN mouse [23] we here show that targeting antigens to DCs via DC-SIGN induces a robust antigen-specific response in vivo that leads to rapid clearance of *L. monocytogenes* infection. Thus, for the first time, this study provides in vivo evidence that anti-DC-SIGN conjugated antibodies can be used as a vaccination strategy against intracellular pathogens.

Results

In vitro targeting of antigen to DCs via DC-SIGN induces efficient T-cell proliferation

Delivery of antigens to DCs via specific antibodies against surface receptors can induce antigen-specific T-cell proliferation. In particular, DC-SIGN targeting has been shown to induce efficient cross-presentation by human DCs [19, 24]. To determine whether antigen presentation can also be induced by murine DCs expressing the human DC-SIGN receptor [23], bone marrow derived DCs (BMDCs) were prepared from WT or hSIGN mice and pulsed with soluble OVA protein, anti-DC-SIGN (clone AZN-D1) or isotype control antibodies conjugated to OVA (αDC-SIGN:OVA and isotype:OVA, respectively). The efficiency of antibody conjugation as well as the specificity of AZN-D1 binding to human DC-SIGN was evaluated by western blot as well as silver staining and binding of the antibody to Raji cells transfected with the human DC-SIGN receptor (Supporting Information Fig.1 A,B). The capacity of antibody-targeted WT and hSIGN BMDCs to present OVA was evaluated by co-culturing them with $CD4^+$OT-II or $CD8^+$OT-I T cells bearing an OVA specific MHC-class-II or -I restricted TCR respectively. As determined by ^{3}H-thymidine incorporation, proliferation of OT-II and OT-I T cells in response to BMDCs loaded with soluble OVA (30 μg/mL) was comparable in WT and hSIGN BMDC cultures, suggesting that expression of the human transgene does not affect the capacity of the DCs to process and present antigens to T cells (Fig.1A,B). Instead, OT-II and OT-I T-cell proliferation induced by hSIGN BMDCs targeted with αDC-SIGN:OVA (0.5 μg/mL) was significantly higher than proliferation induced by WT BMDCs targeted in the same way (Fig.1A,B). Targeting WT or hSIGN BMDCs with a similar amount of soluble OVA (0.5 μg/mL) or the same concentration of isotype:OVA induced only marginal proliferation in both OT-II and OT-I cultures (Fig.1A,B). To determine whether proliferation in response to αDC-SIGN:OVA was dose-dependent, we next evaluated the total number of proliferating OT-II and OT-I T cells using CFSE-based proliferation assays. As shown in Fig.1C, proliferation of OT-II T cells could be

induced by hSIGN BMDCs using αDC-SIGN:OVA concentrations as low as 0.05 µg/mL (Fig.1C, upper panel)., whereas induction of OT-I cell proliferation was first detected at concentrations 10-times higher (0.5 µg/mL; Fig.1C, lower panel). Conjugation of the αDC-SIGN antibody to OVA was a prerequisite to induce proliferation, since addition of comparable concentrations of αDC-SIGN and soluble OVA to BMDC cultures had no effect on T-cell proliferation (Supporting Information Fig.1C), excluding the possibility, that DC-SIGN ligation *per se* enhances the antigen presentation capacity of DCs. Furthermore, proliferation in response to αDC-SIGN:OVA-targeted hSIGN BMDCs was not due to DC activation, since treatment of WT or hSIGN BMDCs with αDC-SIGN:OVA alone did not induce expression of co-stimulatory markers on the BMDCs (data not shown). Moreover, addition of αCD40 to induce DC maturation, only marginally enhanced the proliferation capacity of OT-II T cells cultured with αDC-SIGN:OVA treated hSIGN BMDCs (Fig.1C, upper panel). In contrast, OT-I T-cell proliferation was markedly enhanced when αCD40 was added to the hSIGN BMDCs targeted with αDC-SIGN:OVA (Fig.1C, lower panel). BMDCs targeted with concentrations as high as 1µg/mL of isotype:OVA, even in the presence of αCD40, did not induce OT-II T-cell proliferation (Supporting Information Fig.2B). Proliferation of OT-I T cells in response to BMDCs targeted with isotype:OVA was only detected in the presence of αCD40 at a concentration of 1 µg/mL (Supporting Information Fig.2C), probably as a consequence of the increased cross-presentation capacity of activated DCs.

We next examined the production of IFN-γ by the $CD4^+$OT-II and $CD8^+$OT-I T cells expanded in vitro via αDC-SIGN:OVA in the presence or absence of αCD40. Upon re-stimulation with PMA/Iono similar frequencies of IFN-$\gamma^+CFSE^{low}CD4^+$OT-II T cells were detected in WT and hSIGN BMDCs targeted with αDC-SIGN:OVA alone (Fig.1D,E upper panels). Addition of αCD40 during αDC-SIGN:OVA targeting did not significantly enhance the frequency of IFN-γ-producing OT-II T cells (Fig.1D,E upper panels). In contrast, the

frequency of IFN-γ^+CFSE^{low}CD8$^+$OT-I T cells increased significantly in hSIGN cultures, but only if BMDCs were treated with αDC-SIGN:OVA plus αCD40 and not with αDC-SIGN:OVA alone (34.8±5.7% vs. 11.1±7.5%). Moreover, the percentage of IFN-γ-producing OT-I T cells in hSIGN cultures targeted with αDC-SIGN:OVA plus αCD40 was 10- to 15-fold higher than in WT cultures (34.8±5.7% vs. 2.0± 1.1%) (Fig.1D,E lower panels).

Taken together, these results suggest that, similar to human DCs, antigen delivery via αDC-SIGN antibodies to murine DCs, expressing the human DC-SIGN receptor, induces highly efficient antigen presentation through both MHC-class-II and MHC-class-I molecules.

DC-SIGN targeting drastically enhances antigen-specific T-cell responses in vivo

Having shown the ability of transgenic hSIGN DCs to induce CD4$^+$ and CD8$^+$ T-cell proliferation in vitro, we next used the hSIGN mouse model to determine whether targeting antigen to DCs via human DC-SIGN also leads to enhanced T-cell responses in vivo. In hSIGN mice, human DC-SIGN is specifically expressed on CD11c^{high}SiglecH$^-$ cDCs (Supporting Information Fig.3A,left), but not on B cells, NK1.1$^+$ cells, CD4$^+$ or CD8$^+$ T cells (data not shown and [4, 23]). Moreover, similar expression levels of human DC-SIGN were detected on CD8$^+$, CD4$^+$ and CD8$^-$CD4$^-$ splenic cDCs subsets (Supporting Information Fig.3B). In addition, similar to the expression pattern of DC-SIGN in humans and in correlation with the lower CD11c expression on pDCs [16, 18], DC-SIGN was only expressed on a small fraction of pDCs (Supporting Information Fig.3A, right).

In vivo targeting was investigated by adoptively transferring congenic CD45.1$^+$CD4$^+$OT-II and CD45.1$^+$CD8$^+$OT-I T cells into CD45.2$^+$ WT or hSIGN recipient mice. After injection of either αDC-SIGN:OVA or isotype:OVA with or without co-administration of αCD40, mice were bled on the indicated days and the expansion of transferred cells was determined by flow cytometry after gating on CD45.1$^+$ T cells (Fig.2 and Supporting Information Fig.4B,C).

Treatment of hSIGN mice with αDC-SIGN:OVA alone led to a significant expansion of OT-II, but not OT-I T cells, in blood (Fig.2A,B), spleen (Fig.2C) and LN (data not shown). Moreover, a massive expansion of both OT-II and OT-I T cells was observed in the blood, spleen and LN of hSIGN mice treated with αDC-SIGN:OVA plus αCD40 (Fig.2A-C, and data not shown). Under these conditions, the expansion of OT-II T cells in hSIGN mice reached a maximum between day 5 to 7 post-immunisation, whereas the peak of OT-I T cells was already observed on day 5. At this time point almost 80% of all $CD8^+$ T cells in the blood of hSIGN mice were $CD45.1^+$OT-I T cells (Fig.2B). In contrast, in WT mice treated with αDC-SIGN:OVA with or without αCD40, no expansion of OT-II or OT-I T cells was observed (Fig.2A-C). Similarly, no expansion of OT-II or OT-I T cells was observed after treatment of WT or hSIGN mice with isotype:OVA with or without αCD40 (Supporting Information Fig.4B,C).

Thus, in vivo targeting of DCs via αDC-SIGN antibodies leads to efficient antigen presentation and proliferation of $CD4^+$ and $CD8^+$ T cells.

DC-SIGN targeting promotes strong cytotoxic responses in vivo

CTL responses are a prerequisite for inducing effective immunity against intracellular pathogens. Therefore, we next analysed the IFN-γ production capacity of the expanded OT-I T cells using ex vivo recall assays. SIINFEKL re-stimulation of spleen cells from mice vaccinated with αDC-SIGN:OVA alone induced IFN-γ production by transferred $CD45.1^+CD8^+$OT-I T cells in both WT and hSIGN mice. Furthermore, administration of αCD40 together with αDC-SIGN:OVA, led to a slight but significant increase in the percentage of IFN-γ-producing $CD45.1^+CD8^+$OT-I T cells in hSIGN mice compared with that in WT control mice (Supporting Information Fig.5A). Re-stimulation with an irrelevant peptide (myelin oligodendrocyte glycoprotein, MOG_{35-55}) did not induce IFN-γ production (Supporting Information Fig.5B). Most importantly, the total numbers of IFN-γ-producing

CD45.1$^+$CD8$^+$OT-I T cells found in the spleen of hSIGN mice vaccinated with αDC-SIGN:OVA plus αCD40, were approximately 15-fold higher than in WT mice vaccinated in the same way or mice treated with αDC-SIGN:OVA alone (Fig.3A), suggesting that this immunisation strategy is highly efficient in expanding CD8$^+$ T cells with the capacity to produce IFN-γ.

We next asked whether these expanded cells also display cytotoxic activity. To address this issue, we used a modified protocol to analyse specific killing [25]. The capacity of the adoptively transferred CD8$^+$OT-I T cells to kill OVA-pulsed splenocytes in vivo was analysed 6 days after immunisation. A strong CTL response could be observed in hSIGN, but not in WT mice, that were immunised with αDC-SIGN:OVA plus αCD40 as evidenced by a dramatic decrease in the number of the CFSE^{low} OVA-pulsed target cells (Fig.3B,C). Again, specific killing of target cells could neither be observed in WT mice treated with αDC-SIGN:OVA and αCD40 nor in hSIGN mice treated with isotype:OVA (Fig.3B,C and data not shown).

Thus, targeting OVA to DCs via αDC-SIGN antibodies induces a massive expansion of antigen-specific CD8$^+$ T cells that produce high amounts of IFN-γ and show strong cytotoxic activity.

Vaccination with αDC-SIGN:OVA plus αCD40 protects against *Listeria monocytogenes* infection

Next, we analysed the efficacy of the DC-SIGN-mediated vaccination in a more physiological setting without transferring OT-II or OT-I T cells. To this end, WT and hSIGN mice were treated with a single dose of αDC-SIGN:OVA plus αCD40 and infected 7 days later intravenously with 5x10^4 LM-OVA (Fig.4A). To determine the effect of αDC-SIGN:OVA/αCD40 vaccination on the endogenous CD8$^+$ T cell pool, mice were bled before infection, and OVA-specific CD8$^+$ T cells were determined by SIINFEKL-pentamer staining.

At this time point, increased levels of antigen-specific $CD62L^{low}$ SIINFEKL-specific $CD8^+$ T cells were detected in the blood of vaccinated hSIGN mice, but not in vaccinated WT or non-vaccinated mice (Fig.4B,C). Consequently, the bacterial burden in the liver and spleen of vaccinated hSIGN mice, was significantly lower than in vaccinated WT or non-vaccinated mice 3 days after infection (Fig.4D,E). The lower bacterial burden was accompanied by higher frequencies of $CD8^+$ IFN-γ-producing T cells present in the spleen of infected hSIGN mice vaccinated with αDC-SIGN:OVA/ αCD40 compared with that in control mice (Fig.4F). Taken together, our results demonstrate that DC-SIGN vaccination, leads to the expansion of the (non-transgenic) endogenous pool of $CD8^+$ OVA-pecific T cells that can efficiently control *L. monocytogenes* infection.

Vaccination with αDC-SIGN plus αCD40 induces long-lasting protective immunity

Another important aspect in the design of new vaccination strategies is their capacity to provide long-lasting immunity. Therefore, we next studied the efficacy of DC-SIGN targeting following a standardised long-term protocol [26]. Briefly, a single dose of αDC-SIGN:OVA plus αCD40 was given intraperitoneally to WT and hSIGN mice and 21 days later the animals were boosted with OVA/IFA subcutaneously (Fig.5A). On day 35 after the initial vaccination, mice were bled to determine the levels of OVA-specific antibodies and subsequently infected with $2x10^5$ CFU LM-OVA intravenously. While both WT and hSIGN mice showed augmented levels of OVA-specific IgG1 when compared to non-vaccinated mice (Fig.5B), a significant increase in the level of OVA-specific Th1-associated IgG2c was only observed in vaccinated hSIGN mice, but not in vaccinated WT mice or in mice that were not vaccinated (Fig.5C and Supporting Information Fig.7A). After infection, bacterial burden in the spleen and liver of vaccinated hSIGN mice was significantly lower than in vaccinated WT or non-vaccinated mice (Fig.5D and Supporting Information Fig.7B). This was accompanied by higher frequencies of $CD62L^{low}$ SIINFEKL-specific $CD8^+$ T cells in the spleen and blood of

hSIGN vaccinated mice but not in vaccinated WT or non-vaccinated mice (Fig.5E and data not shown). Although the OVA/IFA boost alone also caused a reduction in the CFU mainly in the liver, hSIGN mice that received the combination of of αDC-SIGN:OVA and OVA/IFA boost showed slightly lower bacterial load than the OVA/IFA boost alone. Interestingly, the reduction in CFU induced by the boost alone was not accompanied by an increase in the frequencies of $CD62L^{low}$ SIINFEKL-specific $CD8^{+}$ T cells (Fig.5E). More importantly, long-lasting immunity was also achieved when only one administration of αDC-SIGN:OVA plus αCD40 was performed without an additional OVA/IFA boost (Fig.5D).

Therefore, these results indicate that a single injection of αDC-SIGN:OVA plus αCD40 followed by a boost, can induce a long-lasting and antigen-specific cellular and humoral immunity which provides protection against *L. monocytogenes* infections.

Discussion

Vaccinations offer one of the most powerful tools in medicine to control infectious diseases. However, attempts to use common vaccine strategies against many important intracellular infectious agents remain unsuccessful [27]. A prerequisite for effective vaccination against intracellular pathogens is the induction of a long-lasting $CD8^+$ T-cell response that is able to induce sterilising immunity. Here, we made use of a recently described method to deliver antigens to DCs via conjugated or recombinant antibodies carrying a specific antigenic protein [1]. We focused on DC-SIGN, a C-type lectin receptor, which acts via capturing and internalising pathogens for antigen presentation to T cells [17, 28]. The high expression of DC-SIGN in lymphoid tissue DCs, mucosal and dermal DCs [15, 29], together with the strong endocytic capacity of this receptor, makes DC-SIGN an ideal candidate for delivering antigens to DCs.

Although in the past several studies have demonstrated the potential of human DC-SIGN as a therapeutic target in vitro [3, 4, 19, 20, 24], until recently no mouse model was available to study DC-SIGN targeting in vivo [23]. As for many other C-type lectins, not only the expression pattern, but also the function of DC-SIGN differs among mice and humans. While the internalisation motif of the DC-SIGN receptor is highly conserved in humans and primates, murine DC-SIGN homologues lack this motif and therefore do not functionally mirror their human counterpart [30, 31]. Using hSIGN transgenic mice [23], we show here that in the presence of an additional co-stimulatory stimulus such as αCD40 in vitro targeting of OVA to BMDCs via anti-human DC-SIGN antibody AZN-D1 leads to a potent $CD4^+$ and $CD8^+$ T-cell proliferative response. Previous studies using human monocyte-derived DCs and peripheral blood lymphocytes showed that the AZN-D1 antibody or a chimeric antibody carrying the same recognition site as AZN-D1 can be rapidly internalised upon binding to the DC-SIGN receptor and induce efficient antigen presentation to T cells [19, 32]. Similarly, mannosylated antigens that bind the carbohydrate recognition domain of DC-SIGN can

induce strong cross-presentation by both human DCs and hSIGN murine BMDCs [3, 4, 20]. Thus, consistent with and in extension to our previous observations, our results indicate that transgenic murine DCs expressing the human DC-SIGN behave similar to their human counterparts and can efficiently present antigen in the context of MHC-class-I or MHC-class-II when targeted via the DC-SIGN receptor [4, 24].

The potential of DC-SIGN targeting has been previously evaluated in a humanised mouse model using immunodeficient mice reconstituted with human cord blood cells [21]. In this model, the authors show that vaccination with a humanised antibody derived from the AZN-D1 antibody conjugated to keyhole limpet hemocyanin (KLH) can efficiently reduce tumour growth [21]. Although this study represents an important proof of principle for DC-SIGN targeting in vivo, the human cord blood engrafted mouse model has many limitations. On the one hand, the low cell frequency after engraftment does not allow for the characterization of the effector cell populations (e.g. $CD4^+$ *vs*. $CD8^+$ T cells) and therefore little is known about the mechanism behind tumour protection. On the other hand, the lack of immune cell renewal limits the study of long-term effects after DC-SIGN vaccination [21]. With our system, we could demonstrate for the first time, that delivering specific antigens to DCs via DC-SIGN in vivo can not only propagate adoptively transferred $CD4^+$OT-II and $CD8^+$OT-I T cells, but also induce a potent endogenous cytotoxic $CD8^+$ T-cell response that promotes efficient eradication and long-term protection from the intracellular pathogen *L. monocytogenes*. Contrary to Kretz-Rommel et al. [21], who did not find any advantage of using a co-stimulatory stimulus such as αCD40, in our study the protective effect of αDC-SIGN vaccination was only observed when αDC-SIGN was used in combination with αCD40 as an adjuvant. Our results are consistent with studies performed using other immune targets on DCs, illustrating that targeting DCs in vivo can lead to immunity or tolerance according to the presence or absence of an adjuvant [33-37]. Moreover, the ability of DCs to cross-present

antigens to $CD8^+$ T cells is enhanced in the presence of DC maturation signals [5]. In our experimental system, addition of the αDC-SIGN:OVA antibody alone did not activate DCs, similarly to what was described by Tacken et al. [19], using the same fusion protein as Kretz-Rommel et al. [21]on human DCs. In fact, in the absence of adjuvant we did not observe any cross-presentation in vivo, but the expansion of $CD4^+$ T cells with a Treg-cell phenotype (data not shown). Thus, we believe that immunity mediated by DC-SIGN targeting requires the presence of an adjuvant. In the study by Kretz-Rommel et al [21] this adjuvant activity might be, at least in part, given by the KHL´s intrinsic adjuvant capacity. Alternatively, the human monocyte derived DCs that were transferred into immunodeficient mice represent inflammatory DCs and might not need additional activation. A third possibility would be that different antigens would bias the route in which the antigen is presented towards class I or class II molecules [38, 39], suggesting that immune responses might be evaluated individually for each conjugated antigen.

One of the most controversial aspects to translate DC targeting as a vaccination strategy into humans is the difficulty to find the "optimal" receptor for targeting DCs in humans. First, different receptors have different capacities to induce immune T-cell responses. Recent work by Castro et al. [36] compared a panel of conjugated antibodies directed against various DC receptors (e.g. CD11c, CD205 , MHC-II, CD40, TLR2 and FcRII/III) for their ability to induce T-cell proliferation and activation. From this study the authors proposed CD11c as the best candidate for vaccine development based on its capacity to induce the most potent $CD4^+$ and $CD8^+$ T-cell responses when compared to other candidates. The stronger potency of the αCD11c conjugates to induce T-cell responses was explained by the high efficiency of this receptor to capture antigen [36]. However, in mice the CD11c receptor is expressed on all DCs, while DEC-205 is only expressed on a subset (approximately 20%) of the $CD11c^+$ DCs [40], influencing thus the frequency of DCs able to present the antigen. We did not directly

compare DC-SIGN and CD11c targeting, however, while Castro et al. [36] reported that targeting CD11c leads only to an OT-I T-cell expansion of 30%, using similar experimental conditions, we here show that DC-SIGN targeting resulted in an expansion of OT-I T cells that reached 80% of all $CD8^+$ T cells in the blood of vaccinated mice. Hence, considering that in hSIGN mice the human DC-SIGN is expressed under the control of the CD11c promoter, and therefore the expression pattern of both receptors is comparable DC-SIGN vaccination might represent a better choice for inducing strong immune responses.

Another factor to consider when using DC targeting in a therapeutic setting is that the outcome of an immune response can be influenced not only by the receptor targeted, but also by the type of DCs carrying the receptor. For example, it has been postulated that delivering antigens via receptors mostly expressed on $CD8^-$ DCs (e.g. DCIR2, CIRE, FIRE) [41, 42] mainly induce Th2 responses, while targeting DEC-205, which is predominantly expressed on $CD8^+$ DCs, promotes preferentially a $CD8^+$ T-cell response [10]. Indeed, conjugated antibodies directed against DEC-205 proved to be promising at inducing potent CTL responses in different models [2, 6, 34, 43]. However, vaccination approaches using DEC-205 might be accompanied by additional side effects due to the ability of this antibody to target cell populations other than DCs. Restricted expression of DC-SIGN in immature DCs and macrophages might therefore represent an advantage over DEC-205 [15, 29].

In this study we provide for the first time evidence that targeting a human DC receptor in vivo can induce a protective CTL immune response against the intracellular pathogen *L. monocytogenes*. Thus, although further studies using anti-DC-SIGN antibodies conjugated to clinically relevant antigens might be necessary, we propose DC-SIGN as a novel candidate for DC-based vaccine strategies against intracellular pathogens.

Materials and methods

Mice

hSIGN mice were described previously [23] and CD45.1^{+}, OT-I and OT-II mice were obtained from Jackson Laboratories. All animals were bred and maintained under specific pathogen-free conditions at the animal facility of the Helmholtz Centre for Infection Research (HZI, Braunschweig, Germany) or the TWINCORE, Centre for Experimental and Clinical Infection Research (Hannover, Germany). Sex- and age-matched mice between 8- and 16-weeks of age were used. All animal experiments were performed in accordance with institutional, state and federal guidelines (Permit number: 09/1677 and 12/0732).

Flow cytometry

The following antibodies and reagents were purchased from eBioscience: anti-CD11c (N418), anti-CD8α (53-6.7), anti-CD4 (GK1.5), anti-CD4 (RM4-5), anti-CD45.1 (A20), anti-IFN-γ (XMG1.2), anti-CD62L (MEL-14), anti-CD19 (eBio1D3), anti-CD3e (145-2C11), anti-NK1.1 (PK136), Streptavidin and Brefeldin A. Anti-CD209 (120507) was purchased from R&D Systems and anti-SiglecH (551) from Biolegend. For ex vivo DC analysis, spleens were digested with 0.5mg/mL Collagenase D (Roche) and 50µg/mL DNase I (Roche) prior to staining. Fluorescence minus one controls were used to set the gates in the respective populations. Data acquisition was performed using a LSRII (BD, Biosciences) or CyAn™ ADP (Beckman Coulter). Data analysis was performed with FlowJo software (Tree Star). Dead cells were excluded by PI, DAPI, ethidium bromide monoazide (EMA), all from Sigma, or Aqua fluorescent reactive dye (Invitrogen) staining. Cellular aggregates were excluded by gating singlets as described in the Supporting Information. SIINFEKL-specific CD8^{+} T cells were stained using H2k^{b}-SIINFEKL-specific pentamer (ProImmune) for 1h in the dark on ice.

In vitro proliferation assay

GM-CSF derived BMDCs were generated from BM cells using standard protocols [44]. On day 7, BMDCs were incubated with αDC-SIGN:OVA, isotype:OVA or soluble OVA, with or without anti-CD40, at the indicated concentrations for 6-16h, washed and incubated in a 1:2 ratio with $CD4^+$ or $CD8^+$ T cells obtained from OT-II or OT-I mice, respectively. OT-II and OT-I T cells were enriched by negative magnetic sorting using the Dynal Mouse CD4 or CD8 negative T-cell isolation kit following the manufacturer's protocol (Invitrogen). Co-cultures were performed for 4 days at 37°C in 96-well round bottom plates (Cellstar). Cell proliferation was determined by adding 1 μCi/well ^{3}H-thymidine (Hartmann Analytic) to the cultures after 3 days of co-culture and assessment of thymidine incorporation 16h later. Alternatively, proliferation was evaluated by staining the cells with CFSE or CellTrace Violet Cell Proliferation Kits (Invitrogen) followed by flow cytometry analysis. For simplification, we refer to proliferating cells as $CFSE^{low}$ cells and non-proliferating cells as $CFSE^{high}$, independently from the dye used.

Vaccination with anti-DC-SIGN:OVA antibodies

The monoclonal anti-DC-SIGN antibody (clone: AZN-D1, IgG1) used in this study was previously described [15]. Briefly, the AZN-D1 or an isotype control antibody (10E2, anti-human Langerin, mouse IgG1,κ) were conjugated to OVA using the cross-linking agent sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate according to the manufacturer's protocol (sulfo-SMCC; Pierce). WT or hSIGN mice were injected with 3-10 μg of αDC-SIGN:OVA or isotype:OVA with or without αCD40 (5-25 μg/mouse of clone 1C10 or 50 μg/mouse of clone FGK45.5). For long-term experiments, mice received 50 μg OVA mixed at a 1:1 ratio with incomplete Freund's adjuvant (IFA, Sigma) subcutaneously into the tail base and the neck, 21 days after primary immunisation [26].

In vivo T-cell priming

One day before immunisation, 2-3x10^6 OVA-specific congenic CD45.1$^+$CD8$^+$OT-I or CD45.1$^+$CD4$^+$OT-II T cells were adoptively transferred intravenously into WT or hSIGN mice. Blood and lymphoid organs (spleen, inguinal LN) were analysed at indicated time points. LN (1x10^6) and spleen cells (1x10^7) were re-stimulated with 1 µg/mL $OVA_{257–264}$ (SIINFEKL) or MOG_{35-55} peptide (Department of Chemical Biology, HZI). After 2h Brefeldin A (eBioscience) was added for additional 3h before staining.

In vivo cytotoxicity assay

WT or hSIGN mice were adoptively transferred with 3.5x10^6 CD8$^+$ enriched OT-I T cells and vaccinated 1 day later. After 6 days, splenocytes from naïve WT mice were pulsed with SIINFEKL peptide (1x10^{-6} M) for 30 min at 37°C, washed, and labelled with a low concentration of CFSE (1µM), or left unpulsed and labelled with a high concentration of CFSE (10 µM). Pulsed (CFSE^{low}) and non-pulsed (CFSE^{high}) cells were mixed at a 1:1 ratio and 3.5x10^7 cells were injected intravenously into vaccinated and non-vaccinated recipient animals (protocol modified from [25]). After 5 h, splenocytes from recipient mice were analysed by flow cytometry to determine the percentage of CFSE^{low} and CFSE^{high} cells. Specific lysis was quantified using the following equation: % specific lysis= 1-($r_{non-vaccinated}/r_{vaccinated}$)]×100, with the ratio (r) being determined as the % of CFSE^{high} divided by the % of CFSE^{low} cells.

***Listeria monocytogenes* infection**

Vaccinated and non-vaccinated control mice were infected intravenously with a low dose LM-OVA (5x10^4 CFU, short-term vaccination protocol) to analyse the primary immune response, or a high dose (2x10^5 CFU, long-term vaccination protocol) to investigate a secondary immune response. The OVA-expressing *L. monocytogenes* 140403s strain (DMX,

Philadelphia) was kindly provided by H. Shen [45]. LM-OVA was grown in Tryptic Soy Broth (TSB, BD, Biosciences) until early log phase. Bacterial density was adjusted according to a standard growth curve after measuring OD at 600 nm. Bacterial burden was determined in liver and spleen of infected mice. Livers were collected in 1mL sterile PBS (Gibco) and mechanically disrupted. Spleens were lysed using 0.1% v/v Triton-X in dH_2O. Suspensions were plated in serial dilutions on TSB agar containing 50µg/mL erythromycin (Sigma). CFUs were calculated after incubating agar plates for 2 days at 37°C.

ELISA

OVA-specific IgG1 and IgG2c were assessed in serum samples by ELISA as previously described [46]. To evaluate antibody concentrations IgG1 (clone OVA-14, Sigma) was used as a standard. IgG2c was assessed by measuring serum dilutions from 1:500 to 1:8000. Biotinylated anti-mouse IgG1 (A85-1, Pharmingen) or alkaline phosphatase conjugated anti-mouse IgG2c (Southern Biotech) antibodies were used.

Statistical analysis

Data analysis was performed using GraphPad Prism Software 5.0. Statistics were calculated using One-Way ANOVA or Mann-Whitney t-test as indicated in the figure legends. Means are given as ±SD with *p*-values considered significant as follows: $*p<0.05$; $**p<0.01$; and $***p<0.001$.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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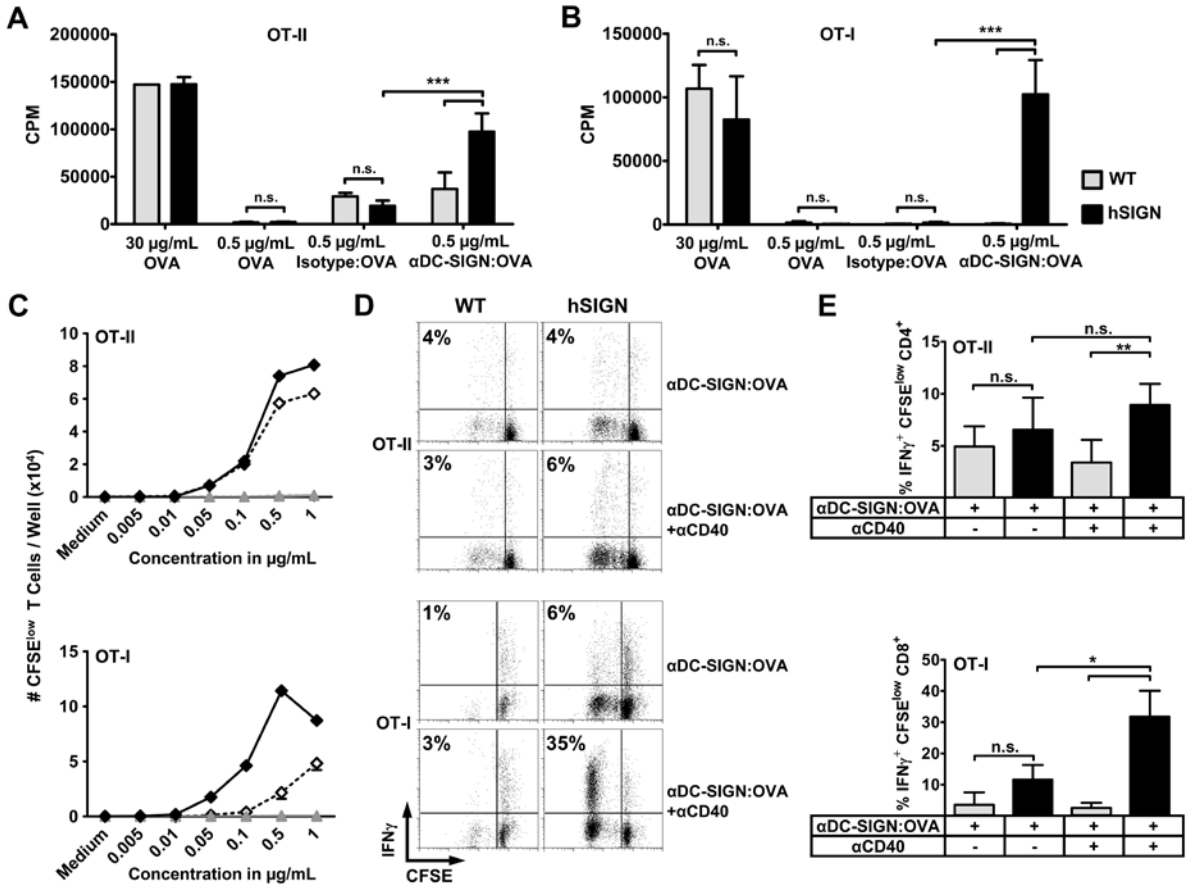

Fig. 1. In vitro targeting of OVA to DCs via αDC-SIGN induces OT-II and OT-I T-cell proliferation. (A) $CD4^+$OT-II and (B) $CD8^+$OT-I T cells were co-cultured with targeted WT or hSIGN BMDCs and proliferation was determined by ^{3}H-thymidine incorporation. Mean ± SD of triplicate wells is depicted from one out of three experiments. ***p<0.001, one-way ANOVA. (C) Dose-dependent proliferation of CFSE-labelled $CD4^+$OT-II (top) and $CD8^+$OT-I (bottom) T cells co-cultured with WT (grey lines) or hSIGN (black lines) BMDCs targeted with increasing doses of αDC-SIGN:OVA with (solid lines) or without (dotted lines) αCD40. Mean of duplicate wells from one out of three experiments is depicted. (D, E) Percentage of IFN-γ-producing $CD8^+$OT-I or $CD4^+$OT-II T cells (re-stimulated with PMA/Iono) after 4-5 days of co-culture with WT or hSIGN BMDCs targeted with 0.5 µg/mL αDC-SIGN-OVA with or without αCD40. (D) Representative plots for T-cell proliferation and IFN-γ staining after 4 days of co-culture are shown. (E) Data are shown as mean + SD of 5 samples (OT-I) or

6 samples (OT-II) pooled from three independent experiments. *$p<0.05$; **$p<0.01$; Mann-Whitney t-test.

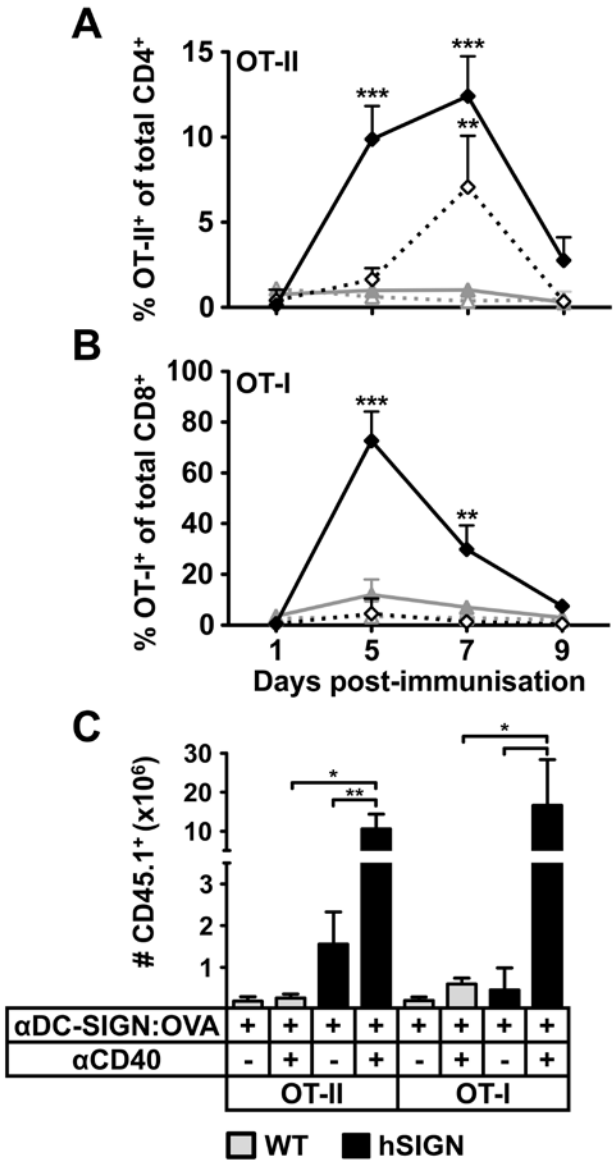

Fig. 2. In vivo DC-SIGN targeting induces OT-II and OT-I T-cell proliferation. Expansion of adoptively transferred (A) CD45.1$^+$CD4$^+$OT-II or (B) CD45.1$^+$CD8$^+$OT-I T cells in the blood of WT (grey lines) or hSIGN (black lines) mice on day 1 to 9 post-immunisation. Dotted lines represent groups receiving only αDC-SIGN-OVA, solid lines represent groups receiving αCD40 in addition to αDC-SIGN-OVA. (C) Total numbers of CD45.1$^+$CD4$^+$OT-II and CD45.1$^+$CD8$^+$OT-I T cells in the spleen of WT and hSIGN mice at day 5 after immunisation, calculated from the total cell count after flow cytometric analysis. Data are shown as mean ± SD, n=3-5 mice/group, from one experiment representative of

three (A, B), **p<0.01; and ***p<0.001; one-way-ANOVA, or two (OT-II) and three (OT-I) experiments (C), *p<0.05 and **p<0.01; Mann-Whitney t-test.

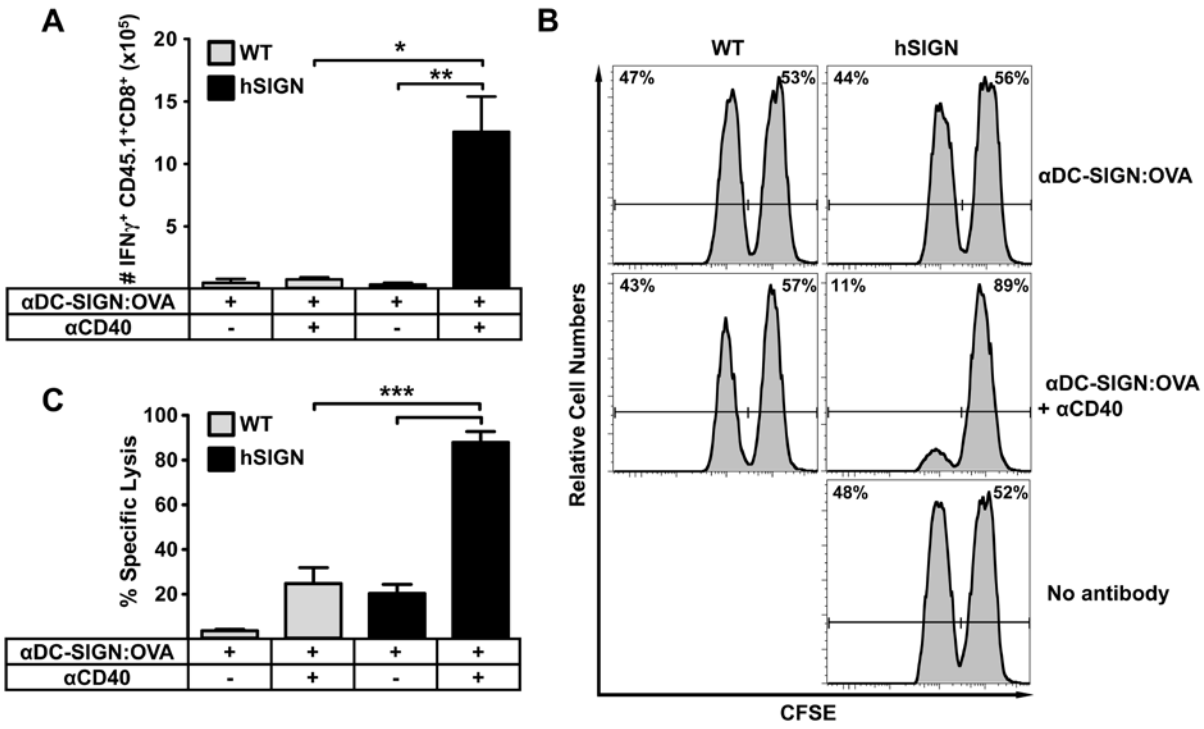

Fig. 3. In vivo DC-SIGN targeting leads to the expansion of IFN-γ-producing cytotoxic T lymphocytes. After transfer of CD45.1$^+$CD8$^+$OT-I T cells, WT and hSIGN mice were immunised with αDC-SIGN-OVA with or without αCD40 and IFN-γ production was analysed by flow cytometry. (A) Total numbers of IFN-γ-producing CD45.1$^+$CD8$^+$OT-I T cells in immunised WT and hSIGN mice after ex vivo re-stimulation with SIINFEKL peptide, calculated from the total cell count after flow cytometric analysis. Data are shown as mean ± SD, n=3-5 mice/group, One representative experiment of three is shown. *p<0.05 and **p<0.01; Mann-Whitney t-test. (B, C) Specific lysis induced by in vivo DC-SIGN targeting after transferring CD8$^+$OT-I T cells into WT and hSIGN mice on day 6 after immunisation. (B) Representative flow cytometry plots show the percentage of OVA-pulsed (CFSE^{low}) and unpulsed (CFSE^{high}) splenocytes present in the spleen of WT and hSIGN mice 5 h after transfer. (C) Frequencies from (B) were used to calculate the specific lysis as described in the *Materials and Methods*. Data are shown as mean ± SD of n=3-4 mice/group and are from one out of three experiments performed. ***p<0.001; one-way-ANOVA.

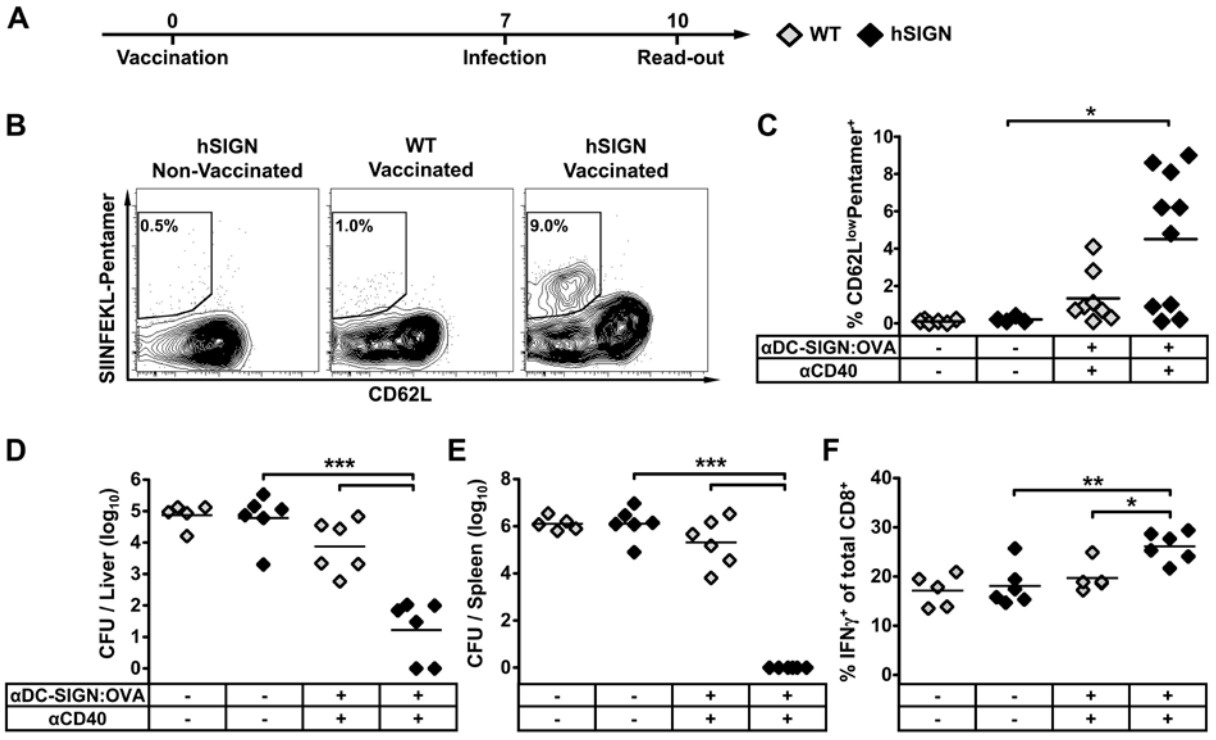

Fig. 4. Vaccination with αDC-SIGN:OVA and αCD40 reduces bacterial burden in a *Listeria monocytogenes* infection model. (A) Experimental scheme. (B) Representative plots showing expansion of endogenous antigen-specific $CD8^+$ T cells after DC-SIGN mediated vaccination, determined by CD62L and SIINFEKL-pentamer staining shortly before infection. (C) Quantification of $CD62L^{low}pentamer^+$ T cells before infection. Each symbol represents an individual mouse and data shown are from one experiment representative of three performed. *p<0.05; Mann-Whitney t-test. (D, E) On day 7 after initial vaccination mice were infected with LM-OVA ($5x10^4$ CFU) and 3 days later, the bacterial burden was assessed in (D) liver and (E) spleen. Each symbol represents an individual mouse and data are from one representative of two performed. (F) IFN-γ production was measured in spleen after ex vivo re-stimulation of spleen cells with PMA/Iono 3 days after infection. Each symbol represents an individual mouse and data are from one experiment performed. *p<0.05; **p<0.01 and ***p<0.001; one-way-ANOVA.

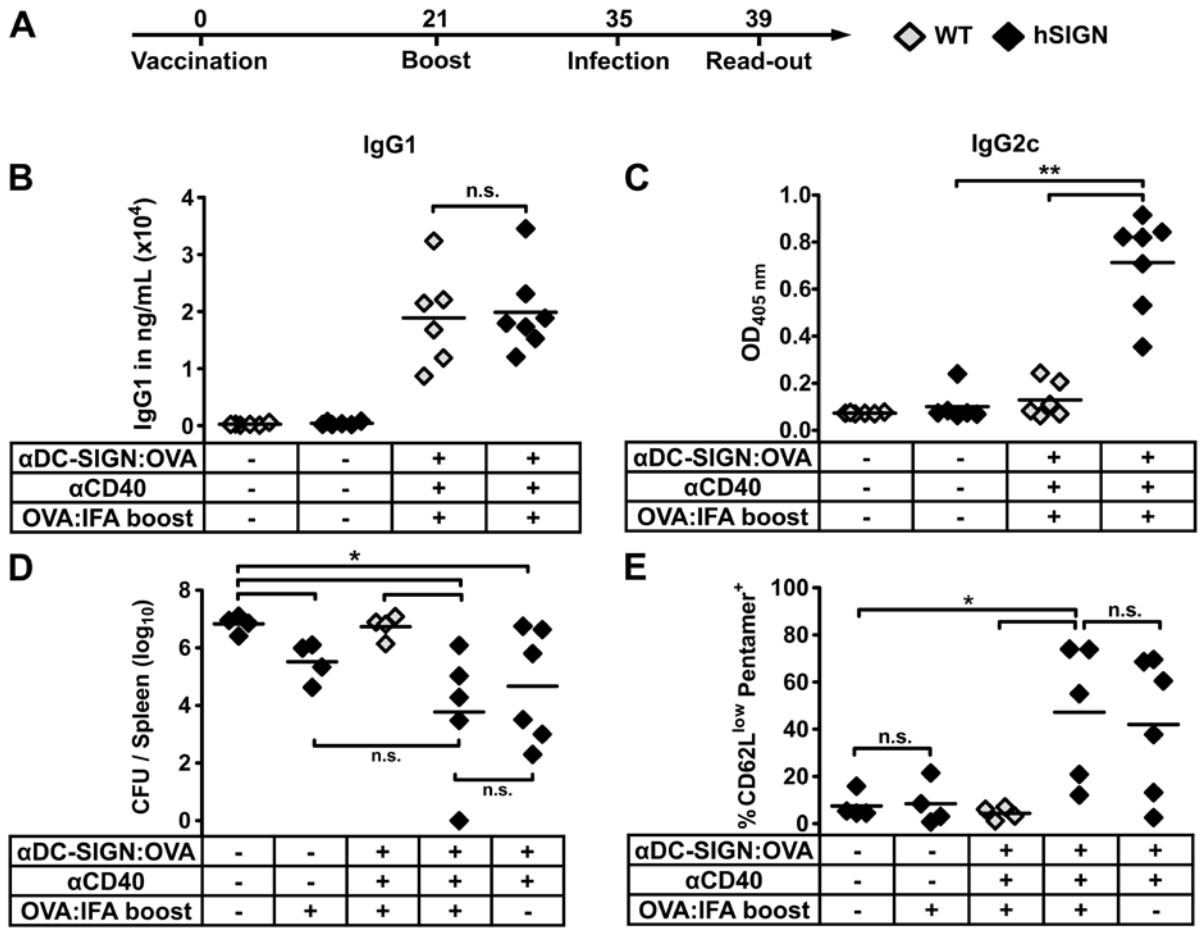

Fig. 5. Long-term protection after vaccination with αDC-SIGN:OVA. (A) Experimental scheme. (B, C) IgG1 and IgG2c serum levels of vaccinated or non-vaccinated WT and hSIGN mice were assessed at day 35 before infection with a high dose of LM-OVA ($2x10^5$ CFU). (D) Bacterial burden in the spleen 4 days after infection with LM-OVA ($2x10^5$ CFU). (E) Frequency of $CD62L^{low}pentamer^+$ T cells in the spleen of infected WT or hSIGN mice 4 days post-infection. (B-E) Each symbol represents an individual mouse and data shown are from one experiment representative of two (D, E) or three (A, B) experiments performed. *p<0.05; **p<0.01; Mann-Whitney t-test.