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CD301b$^+$ dermal dendritic cells drive T helper-2 cell mediated immunity

Yosuke Kumamoto[1], **Melissa Linehan**[1], **Jason S. Weinstein**[1], **Brian J. Laidlaw**[1], **Joseph E. Craft**[1,2], and **Akiko Iwasaki**[1,*]

[1]Department of Immunobiology, Yale University School of Medicine, New Haven, CT, 06520 USA

[2]Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, 06520 USA

Summary

Unlike other types of T helper (Th) responses, whether the development of Th2 cells requires instruction from particular subset of dendritic cells (DCs) remains unclear. By using an *in vivo* depletion approach, we have shown that DCs expressing CD301b were required for the generation of Th2 cells after subcutaneous immunization with ovalbumin (OVA) along with papain or alum. CD301b$^+$ DCs are distinct from epidermal or CD207$^+$ dermal DCs (DDCs) and were responsible for transporting antigen injected subcutaenoulsy with Th2-type adjuvants. Transient depletion of CD301b$^+$ DCs resulted in less effective accumulation and decreased expression of CD69 by polyclonal CD4$^+$ T cells in the lymph node. Moreover, despite intact cell division and interferon-γ production, CD301b$^+$ DC depletion led to blunted interleukin-4 production by OVA-specific OT-II transgenic CD4$^+$ T cells and significantly impaired Th2 cell development upon infection with *Nippostrongylus brasiliensis*. These results reveal CD301b$^+$ DDCs as the key mediatorsof Th2 immunity.

Keywords

Th2; dendritic cell subset; T cell priming

Introduction

Proper differentiation of T helper (Th) cells into distinct effector subsets – Th1, Th2, Th17 or regulatory T cells – dictates protective immunity against distinct classes of pathogens (Sacks and Noben-Trauth, 2002; Zhu et al., 2010). It is generally believed that the fate of effector Th differentiation is determined by the nature of antigen presenting cells (APCs) that prime naïve CD4$^+$ T cells (Moser and Murphy, 2000). In the case of differentiation into Th1, Th17 or regulatory T cells, migratory dendritic cells (DCs) that capture antigens in the peripheral organ and carry them to the draining lymph nodes (dLNs) play direct role in Th cell differentiation (Brewig et al., 2009; Guilliams et al., 2010; Igyarto et al., 2011; King et al., 2010). Previous studies have shown that a subset of DCs distinct from those induce Th1

*To whom correspondence should be addressed: Akiko Iwasaki, Ph.D., Department of Immunobiology, Yale University School of Medicine, 300 Cedar Street, New Haven, CT 06520, Phone: (203) 785-2919, FAX: (203) 785-4972, akiko.iwasaki@yale.edu.

cell differentiation, namely CD8α$^-$ DCs, preferentially generate Th2 cell responses both *in vivo* and *in vitro*, suggesting the presence of subset-specific Th2 cell-driving cue (Maldonado-Lopez et al., 1999; Pulendran et al., 1999; Tang et al., 2010). However, despite previous demonstration that innate cell types including DCs and basophils play an indispensable role in eliciting Th2 cell-mediated immune responses (Hammad et al., 2010; Leon et al., 2012; Ohnmacht et al., 2010; Otsuka et al., 2013; Perrigoue et al., 2009; Phythian-Adams et al., 2010; Smith et al., 2012; Sokol et al., 2009; Tang et al., 2010; Yoo et al., 2010; Yoshimoto et al., 2009), whether the differentiation of naïve CD4$^+$ T cells into Th2 cells *in vivo* requires a specialized population of APCs in the priming phase remains unclear (Kool et al., 2012; Pulendran and Artis, 2012).

Cysteine proteases, such as papain and house dust mite antigen DerP1, exemplify a class of noxious substance reflects activity of helminthes and allergens (Palm et al., 2012). Skin represents an essential protective barrier against infectious agents including helminthes. It is well documented that subcutaneous injection of certain cysteine protease alone can trigger robust Th2 cell responses *in vivo* (Kikuchi et al., 2006; Sokol et al., 2008; Tang et al., 2010), though the type of APCs or pattern recognition receptors that directly recognizes those proteases or their activity to induce Th2 immunity is unknown. In the skin, there are three distinct subsets of DCs that serve as APCs that potentially migrate to the dLN to activate naïve lymphocytes (Merad et al., 2008). Epidermal Langerhans cells (LCs) and CD103$^+$ dermal DCs (DDCs) are the two subsets of DCs that express CD207 (Langerin) and are important for differentiation of Th17 and Th1 cells, respectively (Kaplan et al., 2012). It has been previously reported that skin-resident CD207$^+$ DCs, including LCs and CD103$^+$ DDCs, do not efficiently transport protein antigens co-injected with papain to the dLN and are therefore not responsible for the papain-induced Th2 cell responses (Tang et al., 2010). Although these findings leave open the possibility that other subset of skin-migratory DCs, namely CD207$^-$ DDCs, in mediating Th2 cell differentiation, *in vivo* role of this dominant DC subset in the dermis is largely unknown.

Based on the selective expression of a CD301b, also known as macrophage galactose-type C-type lectin 2 (*Mgl2),* in CD207$^-$ DDCs (Kumamoto et al., 2009), we generated mice in which the DDC population could be specifically and inducibly depleted. Here, we have examined the requirement for CD301b$^+$ DDCs in the generation of Th2 cell mediated immunity following immunization with antigen plus papain. We have extended our analysis to another widely-used Th2-type adjuvant, alum, as well as to a natural rodent pathogen, *Nippostrongylus brasiliensis*, that induces robust Th2 cell responses. Our results reveal a requirement for CD301b$^+$ DDCs in the generation of Th2 cell-mediated immunity, and describe the key defects in CD4$^+$T cells primed in the absence of these DDCs.

Results

CD301b is expressed by dermal and submucosal DC subsets that are distinct from CD207$^+$ DCs

To address the function of DDCs *in vivo*, we utilized CD301b, a specific marker of CD207$^-$ DDCs in the skin (Kumamoto et al., 2009). We developed a monoclonal antibody (mAb) against CD301b that is not cross-reactive to a closely related homologue, CD301a (Supplementary Figure 1). CD301a is expressed more broadly by DCs and plasmacytoid DCs, whereas CD301b is expressed by DDCs in the dermis, cutaneous LNs but not in the spleen (Kumamoto et al., 2009). Staining naïve skin-draining LN (dLN) cells with the anti-CD301b mAb confirmed that CD301b$^+$ DDCs expressed neither CD103 nor CD326, markers for CD207$^+$ DDCs and LCs, respectively (Nagao et al., 2009) (Figure 1A). Moreover, CD301b$^+$ CD11c$^+$ cells were present only in the dermis and submucosa underneath squamous stratified epithelia, and were excluded from the epithelial layer

inhabited by the LCs (Figure 1B). These results indicate that expression of CD301b marks a unique subset of DCs within the subepithelial regions in multiple mucosal organs and skin.

CD301b$^+$ DDCs efficiently transport protein antigen injected in the footpad

To test the ability of DC subsets to take up antigens *in vivo*, we immunized mice in the footpad with fluorescently-labeled ovalbumin (OVA) together with the Th2-type adjuvant papain or the Th1-type adjuvant CpG and analyzed cells that had taken up OVA in the dLN 24 hours after injection. Although CD301b$^+$ DDCs were capable of taking up OVA regardless of the adjuvant used, papain induced selective uptake of OVA by CD301b$^+$ DDCs (Figure 2 and Supplementary Figure 2). In addition, the percentage of CD301b$^+$ DC in the dLNs increased dramatically in papain-injected mice compared to PBS-injected mice or naïve control, suggesting that OVA uptake occurred in CD301b$^+$ DDCs that had migrated from the dermis to the dLN. Together with a previous report showing that CD301b$^+$ DDCs are the earliest migrants following hapten painting on the skin (Kumamoto et al., 2009), our data demonstrates the CD301b$^+$DDCs as a key transporter for cutaneous antigens to the dLN *in vivo*.

CD301b$^+$DDCs are depleted upon injection of diphtheria toxin in Mgl2DTR mice

To selectively and transiently deplete CD301b$^+$ DDC *in vivo*, we generated mice in which a diphtheria toxin receptor (DTR)-GFP cassette was genetically targeted into the first coding exon of CD301b, or the *Mgl2* gene (Supplementary Figure 3A). Both homozygous and heterozygous mice were healthy and born at the expected Mendelian ratio. The GFP fluorescence in the homozygotes confirmed selective expression of the DTR-GFP fusion protein in CD301b$^+$ DDCs (Supplementary Figure 3B,C). From hereafter we use only *Mgl2*$^{+/DTR-GFP}$ heterozygotes for experiments and refer to them as Mgl2DTR mice. In Mgl2DTR mice, a single intraperitoneal injection of DT selectively depleted CD301b$^+$ DDCs in the skin-draining LNs (Figure 3A,B). The depletion lasted for at least six days following a single DT injection (Figure 3C). When mice were painted with a fluorescent dye TRITC on the skin, the dLNs of the CD301b$^+$ DDC-depleted mice were almost completely devoid of TRITC-bearing cells at 24 hours after the painting (Figure 3D). These data indicate that, unlike the CD207$^+$ DCs (Kissenpfennig et al., 2005), CD301b$^+$ DDCs account for the majority of the migratory skin-derived DCs early after sensitization in the skin.

Experiments with congenic bone marrow chimeric mice indicated that CD301b$^+$ DDCs are derived from circulating radio-sensitive precursors (Supplementary Figure 4A,B), consistent with the published reports (Bogunovic et al., 2006; Ginhoux et al., 2007). Unexpectedly, however, despite undetectable expression of either DTR-GFP or endogenous CD301b protein by LCs, DT injection also reduced LC frequency within the epidermis (Supplementary Figure 4C), but not in the skin-dLNs (Figure 3B and Supplementary Figure 4D). Depletion of epidermal LCs was not observed in bone marrow chimeric mice in which Mgl2DTR bone marrow cells were transplanted into lethally-irradiated wild-type (WT) mice (Mgl2DTR->WT chimera), while conversely, DT treatment of WT->Mgl2DTR chimera resulted in ablation of LCs (Supplementary Figure 4D). These data indicate that LC depletion requires Mgl2DTR expression in radioresistant cells, and is not a secondary effect of CD301b$^+$DDC removal.

CD301b$^+$DDCs are required for recruitment and activation of CD4$^+$T cells induced by Th2-type adjuvants

DCs support primary immune responses by inducing LN expansion and stimulating antigen (Ag)-specific T cells (Kumamoto et al., 2011; Webster et al., 2006). To determine the role of CD301b$^+$ DDCs in the initiation of adaptive immune responses, we assessed T cell priming following depletion of these cells. Footpad injection with papain induced hypertrophy in the

draining popliteal LN in WT mice (Figure 4A). In contrast, in the DT-treated Mgl2DTR mice, dLN hypertrophy was significantly impaired, which was largely explained by the reduction in the number of $CD4^+$ T, $CD8^+$ T and B cells, with a significant reduction in the proportion of $CD4^+$ T cells (Figure 4B–G). In addition, upregulation of CD69, but not CD25, in $CD4^+$T cells in the dLN was abrogated in DT-treated Mgl2DTR mice (Figure 4H,I). The defect in $CD4^+$ T cell accumulation and CD69 upregulation was due to the depletion of $CD301b^+$ DDCs but not to the depletion of epidermal LCs, as Mgl2DTR->WT chimera injected with DT replete with LCs (Supplementary Figure 4) also showed the same phenotype (Supplementary Figure 5). Furthermore, selective depletion of epidermal LCs in huLangerin-DTR mice (Bobr et al., 2010) had no effect on $CD4^+$ T cell accumulation or CD69 expression following papain immunization (Supplementary Figure 5). These data indicate that both LN recruitment and activation of $CD4^+$T cells require $CD301b^+$ DDCs, but not LCs, upon immunization with papain.

To extend the role of $CD301b^+$ DDCs in Th2 cell-mediated immunity, we tested another Th2-type adjuvant, alum. Similar to papain, immunization with alum in WT mice induced dLN hypertrophy as well as CD69 upregulation in $CD4^+$ T cells, both of which were abrogated in $CD301b^+$DDC-depleted Mgl2DTR mice (Figure 5A–C). In contrast, immunization with a Th1-type adjuvant CpG induced CD69 upregulation independently of $CD301b^+$ DDCs, while the accumulation of $CD4^+$ T cells in the dLN was still partially impaired in $CD301b^+$ DDC-depleted mice (Figure 5D–F). These results identify $CD301b^+$ DDCs as the key DCs required for the optimal accumulation of $CD4^+$ T cells to the dLNs, whereas the requirement of these cells for CD69 upregulation in $CD4^+$ T cells is specific to Th2-type adjuvants.

$CD301b^+$ DDCs are required for optimal screening for antigen-specific naïve $CD4^+$T cells in the dLN

To test whether $CD301b^+$ DDCs mediate antigen-specific Th cell priming, we adoptively transferred carboxyfluorescein succinimidyl ester (CFSE)-labeled naïve OVA-specific transgenic $CD4^+$T cells (OT-II cells) into Mgl2DTR mice. Immunization with OVA and papain in the footpad led to CFSE dilution in OT-II cells in the dLN of DT-treated mice that was identical to that of PBS-treated mice, indicating that antigen-induced division of OT-II cells did not require $CD301b^+$ DDCs (Figure 6A). However, reflecting the defect in accumulation of polyclonal $CD4^+$ T cells to the reactive dLN (Figure 4 and 5B), the absolute number of OT-II cells in the dLN was reduced in the DT-treated mice compared to PBS-treated mice (Figure 6C). Consistently, in the non-draining LN where the circulating pool is the only source of divided OT-II cells, the relative abundance of undivided OT-II cells was larger in $CD301b^+$ DDC-depleted mice (Figure 6A,D), suggesting that systemically, more OT-II cells are left unscreened and thus remained unprimed in the absence of $CD301b^+$ DDCs. Together, these results support the idea that $CD301b^+$ DDCs are required for the optimal screening of antigen-specific naïve $CD4^+$ T cells within the dLN.

$CD301b^+$ DDCs are required for development of antigen-specific Th2 cells

Thus far, our data indicated that $CD301b^+$DDCs are required for the maximal recruitment of naïve antigen-specific $CD4^+$ T cells into the reaction induced by Th2-type adjuvants. To test whether the OT-II cells primed in the absence of $CD301b^+$ DDCs were qualitatively affected in addition to their migration defect, we analyzed expression of activation markers in those OT-II cells. At two days after immunization with OVA and papain, OT-II cells primed in the absence of $CD301b^+$ DDCs had lower CD69 expression compared to intact mice (Figure 7A), as expected from the CD69 defect in polyclonal CD4 T cells (Figure 4). Nevertheless, those OT-II cells that underwent division cycles upregulated Th1 cell-associated activation marker CD44 (Baaten et al., 2010; Guan et al., 2009) and transcription factor T-bet (Szabo

et al., 2000) normally (Figure 7A). By four days of immunization, CD44 and T-bet expression in OT-II cells were downregulated in cells that have undergone several divisions. However, expression of these molecules remained high upon cell division in OT-II cells primed in CD301b$^+$ DDC-depleted hosts (Figure 7A). In contrast, upregulation of Th2 cell-regulator transcription factor GATA3 (Zhang et al., 1997; Zheng and Flavell, 1997) in divided OT-II cells was impaired in DT-treated Mgl2DTR mice (Figure 7A), suggesting that OT-II cells primed in the absence of CD301b$^+$ DDC are impaired in Th2 and skewed toward Th1 phenotype.

Notably, OT-II cells primed in CD301b$^+$ DC-depleted hosts were severely impaired in interleukin (IL)-4 production (Figure 7B,C). The defect in IL-4 production was observed as early as day 4 post-immunization (Figure 7B), suggesting that imprinting toward Th2 cell differentiation required CD301b$^+$ DDCs at the very beginning of Th2 cell differentiation *in vivo*. In addition, the defect in IL-4 production as well as the increased relative abundance of undivided fraction of OT-II cells in the non-dLN and spleen were also observed in mice depleted of CD301b$^+$ DDC and immunized with OVA and alum (Supplementary Figure 7A,B), suggesting that the requirement of CD301b$^+$ DDCs for Th2 cell differentiation is not specific to papain. IFN-γ production upon immunization with papain and OVA (Supplementary Figure 7B) or infection with herpes simplex virus (Supplementary Figure 7C) was generally intact or even slightly exaggerated in CD301b$^+$ DDC-depleted animals, indicating the selective role of CD301b$^+$ DDC in Th2 cell responses. Similarly, IFN-γ production following immunization with CpG was also not affected by depletion of CD301b$^+$ DDCs (Supplementary Figure 7D). In addition, consistent with a previous report (Tang et al., 2010), the defect in Th2 cell differentiation in Mgl2DTR mice was not due to depletion of epidermal LCs, as specific depletion of LCs in huLangerin-DTR mice did not result in impaired IL-4 production (Supplementary Figure 7E). Among multiple layers of cellular and molecular mechanisms identified for Th2 cell-mediated immunity to date (Paul and Zhu, 2010; Pulendran and Artis, 2012), these results highlight the critical requirement for CD301b$^+$DDCs in early stages in the development of Th2 cells.

CD301b$^+$DCs alone are insufficient forthe generation of Th2 cell responses

To gain mechanistic insight into the properties of CD301b$^+$ DC that impart upon it Th2-cell differentiation function, we next examined surface expression of Th2 cell-associated molecules in CD301b$^+$DCs and other skin-derived DC subsets in the dLNs of papain-immunized WT mice. In addition to their modest expression of known Th2 cell-driving molecules such as ICOSL, OX40L and Jagged-1, CD301b$^+$ DCs showed highest expression of receptor for IL-33, which has recently been shown to be a potent inducer for Th2 cell differentiation and allergy (Besnard et al., 2011; Rank et al., 2009) (Supplementary Figure 8A). Of note, CD301b$^+$DCs also expressed the highest amount of PD-L2 among other DC subsets, which is a potential regulator for Th2 cell differentiation (Ishiwata et al., 2010; Matsumoto et al., 2008; Oflazoglu et al., 2004).

Next, we assessed the sufficiency of CD301b$^+$ DCs in priming Th2 cell responses in vivo. To this end naïve mice that were given OT-II T cells were injected with CD301b$^+$ DCs that were sorted from papain-immunized dLNs and pulsed *in vitro* with major histocompatibility complex class II (MHCII)-restricted OVA$_{323–339}$ peptide. Despite robust division of OT-II cells, we found no evidence for Th2 cell differentiation in the recipients of DC transfer (Supplementary Figure 8B), suggesting that additional requirement exists to induce Th2 cell differentiation upon this mode of immunization.

CD301b$^+$DCs are required for Th2 but not Tfh or germinal center responses induced by *N. brasiliensis*

To determine the role of CD301b$^+$ DDCs in a more physiological setting, we infected Mgl2DTR mice with *N. brasiliensis*, a natural rodent hookworm pathogen that induces strong Th2 cell responses. In this model, once injected subcutaneously, the parasites quickly migrate to the lung through the bloodstream, where they expand and trigger robust type 2 cytokine production (Camberis et al., 2003). Notably, depletion of CD301b$^+$ DDCs impaired IL-4 production from endogenous polyclonal CD4$^+$ T cells in the dLNs of the initial injection site (Figure 7D). In addition, production of IL-4 by CD4$^+$ T cells in the lung-draining mediastinal LN was also reduced, suggesting the presence of corresponding CD301b$^+$ DC subset in the lung that promote Th2 cell differentiation (Figure 7D). However, CD301b$^+$ DC-depleted animals produced normal amount of parasite-specific class-switched IgG1 antibodies as well as follicular helper CD4$^+$ T (Tfh) cells and germinal center B cells (Figure 7E–G). In addition, total IgE was induced comparably in the absence of CD301b$^+$ DC (Figure 7G). These data indicate that upon infection with a parasite, CD301b$^+$DCs are required for the development of Th2 but not Tfh or B cell responses involving germinal center B cells, IgG1 or IgE responses.

Discussion

In this study, we demonstrated that CD301b$^+$ DDCs are the major migratory DC subset that transport protein antigen upon immunization in the footpad with papain. CD301b$^+$DDCs were also required for accumulation of naïve CD4$^+$T cells to the dLNs, thereby facilitating the rate of screening for antigen-specific T cells. Furthermore, *in vivo* depletion of CD301b$^+$ DDCs resulted in selective loss of IL-4 production from antigen-specific CD4$^+$ T cells after immunization with papain or alum, or after infection with a Th2 cell-inducing parasite. Interestingly, while the impaired Th1, Th2 or Th17 cell differentitation by the depletion of DC subsets has been usually associated with a severe defect in the division of antigen-specific CD4$^+$ T cells in the dLN (Brewig et al., 2009; Fahlen-Yrlid et al., 2009; Hervouet et al., 2010; Igyarto et al., 2011; Kim et al., 2010; Magalhaes et al., 2011), the depletion of CD301b$^+$ DDCs abrogated the differentiation of Th2 cells without affecting the division of cognate CD4$^+$ T cells. These data suggest that the instruction for the Th2 cell differentiation may not be coupled to the signal for cell division. Consistent with our study, genetic mutation that leads to a loss of the CD301b$^+$ DDC subset results in a similar deficiency in Th2 cell-mediated immunity (see accompanying study by Gao et al.). Further, our results are consistent with the previous finding that antigen co-injected with papain is preferentially presented *ex vivo* by CD207$^-$ CD8α$^-$ DCs (Tang et al., 2010), which includes the CD301b$^+$ DDC population. While we cannot formally rule out CD301b expression by cell types other than DDCs, together, these studies indicate the role of CD301b$^+$ DDC in controlling Th2 cell responses.

Compared to the notable advances over the years in understanding the development of other types of Th cell responses, our understanding in the development and maintenance of Th2 cell responses is still far from complete. While accumulating evidence indicates involvement of DCs in Th2 cell-mediated immunity, either in priming or maintenance (Hammad et al., 2010; Leon et al., 2012; Phythian-Adams et al., 2010; Smith et al., 2011; Tang et al., 2010), the key DC subset required for Th2 cell-mediated immunity *in vivo*, particularly in the skin, has not been well characterized. In the lung, a recent work by Lambrecht and colleagues demonstrates that CD11b$^+$ DCs and FcεRI$^+$ monocyte-derived DCs are required for infiltration of eosinophils and other cell types in the lung, a hallmark of type 2 inflammation induced by inhalation of house-dust mite extracts (Hammad et al., 2010; Plantinga et al., 2013). However, given that eosinophilic infiltration and type 2 cytokine production do not necessarily require CD4$^+$ T cells (Halim et al., 2012), it remains to be determined whether a

specific subset of DCs is required for Th2 cell differentiation. Our current study shows that in the skin, the CD103^{-}DDC subset plays a key role in the generation of Th2 immunity after allergen and helminth challenge.

It was reported previously that a subset of DCs in certain conditions is sufficient to promote Th2 cell differentiation when loaded with antigen and injected into naïve recipients (Hammad et al., 2010; Maldonado-Lopez et al., 1999; Pulendran et al., 1999; Smith et al., 2012). Similarly, sensitization with CD301b^{+}DCs alone is sufficient for developing contact hypersensitivity against FITC, a typical Th2 cell-type hapten (Kumamoto et al., 2009). In contrast, other studies have shown additional requirement of non-DC accessory cells such as basophils in Th2 cell differentiation in some models including papain-induced skin immunity (Otsuka et al., 2013; Perrigoue et al., 2009; Sokol et al., 2009; Tang et al., 2010). Our results are consistent with the latter, in that adoptive transfer of CD301b^{+} DCs isolated from LN draining papain injection site is not sufficient to prime Th2 cell-mediated immune responses in the recipient host. Thus, the other cell types involved in orchestrating Th2 priming in vivo remains to be elucidated.

With regards to antibody responses, our results with *N. brasiliensis* infection indicated no impairment in the germinal center B cells, IgG1 or IgE responses in mice depleted of CD301b^{+}DCs. Further, our results suggest that development of follicular T helper (Tfh) cells can be uncoupled and may require a distinct DC subset from development of Th2 cell responses. Although function of Th2 and Tfh cells has often been considered interchangeable for type 2 inflammation, the fact that Tfh cell-defective Bcl6-deficient animals show an enhanced Th2 cell (Dent et al., 1997) response and that an instructive signal from APC is required for Bcl6 expression in Tfh cell development (Choi et al., 2011) strongly support the idea that those two types of CD4^{+} T cells require different APCs for their differentiation.

Previous studies have shown that depletion of either basophils or total CD11c^{+} DC population abrogates Th2 cell responses (Hammad et al., 2010; Otsuka et al., 2013; Phythian-Adams et al., 2010; Smith et al., 2012; Sokol et al., 2008; Tang et al., 2010). The relatively narrow window of the basophil presence in the dLN restricts their interaction with only a limited subset of migratory DCs, a time course consistent with CD301b^{+} DDC migration. In addition, the accumulation of CD4^{+} T cells mediated by CD301b^{+} DDC may facilitate interaction of CD4^{+} T cells with late-arising Th2 cell drivers such as late-activator APCs (Yoo et al., 2010) or chemokine receptor CXCR5-expressing conventional DCs (Leon et al., 2012). The unique ability of CD301b^{+} DDC to promote Th2 cell responses likely involves mechanisms at different levels. First, as shown in this study, CD301b^{+} DDCs express several Th2 cell-associated molecules including IL-33 receptor (this study) as well as very high amount of CD40 even at the steady state (Kumamoto et al., 2009), a molecule required for Th2 cell-mediated immunity (MacDonald et al., 2002). Second, CD301b^{+} DDCs are the major antigen-bearing DCs in the dLN early after immunization with papain. Third, CD301b^{+} DDCs provide signals in the LN needed to both increase the size of naïve precursor pool and to retain antigen-specific CD4^{+}T cells for optimal Th2 cell differentiation. CD69 acts as a LN retention signal for antigen-specific lymphocytes by inhibiting sphingosine-1-phosphate receptor 1 downstream of type I IFN receptor (Shiow et al., 2006). While CpG is a well-known inducer of type I IFN, the absolute requirement of CD301b^{+} DDC for CD69 upregulation by CD4^{+} T cells upon immunization with papain or alum suggests an intriguing possibility that DDCs replace the role of type I IFNs during helminth infection for retention of pathogen-specific CD4^{+} T cells. In addition to demonstrating the requirement for DDCs, the results of our studies have implications for treatment of Th2 cell-mediated diseases. Modulating the function of CD301b^{+}DDCs may be

a potential therapeutic target for atopic dermatitis and other type of allergic diseases in which Th2 cells are implicated (Cookson, 2004).

Experimental Procedures

Mice

Age- and sex-matched 2–5 month old mice were used in each experimental group. C57BL/6 (B6) and B6. SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ (congenic CD45.1 mice on B6 background) were obtained from National Cancer Institute. For generating Mgl2DTR mice, a DTR-GFP cassette (gift from Steffen Jung) linked to a floxed neomycin resistant cassette (NeoR) was targeted into *Mgl2* exon 2 in bacterial artificial chromosome (BAC) R23-67I17 by recombination-mediated genetic engineering (gift from Neal Copeland). The recombined allele was then retrieved onto a targeting vector pEZ-Frt-lox-DT (Schenten et al., 2002) (gift from Dominik Schenten) and linearized by digesting with SacII. The linearized vector was electroporated into B6 embryonic stem (ES) cells. Successfully-recombined ES clone was identified by Southern blotting and injected into WT B6 mice. The probes used for Southern blotting was amplified from BAC R23-67I17 by using specific primers (probe 1 forward primer: agaggaatatgggacggcacact, probe 1 reverse primer: aggccacaacccaagctagagtgc, probe 2 forward primer: atcttgccaggctggtcattgttaagttca, probe 2 reverse primer: gtatcatggatgtagcctccctgtcatttatag) and labeled with Rediprime II (GE Healthcare). The offspring were tested for the presence of DTR-GFP cassette by PCR (forward primer: agcaaagaccccaacgagaa, reverse primer: cgtccatgccgagagtgat) in the genomic DNA and backcrossed once with B6. FVB-Tg(EIIa-Cre) mice (gift from Richard Flavell) to remove the floxed NeoR. The NeoR-removed Mgl2DTR mice were maintained on WT B6 background. The removal of NeoR and EIIa-cre transgene from the genomic DNA was confirmed by PCR (NeoR forward primer: tgatcgacaagaccggcttcca, NeoR reverse primer: tgcatacgcttgatccggctac, Cre forward primer: gcggtctggcagtaaaaactatc, Cre reverse primer: gtgaaacagcattgctgtcactt). In the case we needed to distinguish *Mgl2*$^{DTR/DTR}$ homozygotes from the heterozygotic Mgl2DTR mice, the WT *Mgl2* exon 2 was detected by PCR (forward primer: tgcgtttgtcaaaacatgacaatgagatat, reverse primer: atacttagagcaaactttggagactccat). CD45.1 OT-II TCR transgenic mice (gift from Richard Flavell) were maintained on *Rag1*$^{-/-}$ background. HuLang-DTR transgenic mice were a gift from Daniel Kaplan. For bone marrow chimera experiments, mice were irradiated with two doses of 475 rads and transplanted with ~10^6 bone marrow cells. All bone marrow chimeric mice were treated with Sulfatrim in drinking water for 4 weeks and then maintained on regular water for another 4 or more weeks before being used in experiments. All procedures used in this study complied with federal guidelines and institutional policies of the Yale Animal Care and Use Committee.

Immunization

All immunization procedures were conducted in the rear footpad with 20 μl injection volume per footpad. In experiments involving DT injection, 0.5 μg DT (List Biological Laboratories) was injected intraperitoneally. For *in vivo* antigen uptake experiments, WT B6 mice were injected in both hind legs with 2 μg Alexa Fluor 488-conjugated OVA together with 50 μg papain (Sigma) or CpG2216 (Invivogen). The draining popliteal LNs were harvested for analysis 24 hours after immunization. For LN T cell accumulation and activation, WT B6, Mgl2DTR or huLang-DTR mice were treated with DT on days −1 and +1. On day 0, they were immunized with 50 μg papain (without OVA), 10 μg CpG2216 with 5 μg purified OVA (Worthington Biochemical Corporation), or 5 μg OVA precipitated in 50% alum in PBS (Imject Alum, Thermo Scientific) in the right hind leg. On day 3, the left non-draining and the right draining popliteal LNs were harvested for analysis. For OT-II transfer experiments, CD4^{+}T cells were isolated from naïve CD45.1*Rag1*$^{-/-}$OT-II animals

by MACS cell sorter with mouse $CD4^+$T cell isolation kit (Miltenyi Biotec) and labeled with CFSE (Invitrogen) according to the manufacturer's protocol. One million OT-II cells were adoptively transferred retro-orbitally into Mgl2DTR recipients on day −1 that were subsequently treated with DT or PBS 2 hours after the adoptive transfer. The depleted or undepleted recipients were then immunized on day 0 with 5 μg purified OVA with 50 μg papain in the both hind legs and treated with DT or PBS every 2–3 days. The draining popliteal LNs (on both sides), non-draining brachial LNs and the spleen were harvested on days 4 and 8. For adoptive immunization with sorted DC subsets, WT donor mice were subcutaneously immunized with papain in the footpads and in the tail base, then DCs were isolated with MACS cell sorter using anti-CD11c Microbeads (Miltenyi Biotec) from axillary, brachial, inguinal and popliteal dLNs 24 hours after immunization. Isolated DCs were then stained for CD11c, MHCII and CD301b and $CD301b^+$ or $CD301b^-$ $MHCII^{hi}$ DCs were sorted by FACSAria II cell sorter (>95% purity). The sorted DCs were pulsed with 10 μg/ml $OVA_{323–339}$ peptide for 60 min, washed extensively with PBS, and injected into the right footpad of naïve WT mice that had been given naïve OT-II cells one day earlier. Seven days after adoptive immunization, the draining popliteal LNs were harvested and stimulated with PMA and ionomycin for further analysis.

N. brasiliensis infection and detection of serum antibodies

Mice were treated with DT every third day starting on day −1. On day 0, mice were injected subcutaneously at the tail base with 500 worms per mouse third-stage larvae (L3) of *N. brasiliensis* in 0.2 mL PBS with gentamycin (Gibco). For serum antibody ELISA against L3 antigens, the L3 larvae were sonicated and homogenized and used as coating antigens for ELISA. For detecting anti-L5 serum antibodies, stage 5 (L5) were isolated from intestines of mice infected with L3 larvae 7 days earlier and homogenated. Five micrograms of lysates were used to coat microtiter plates, which were then incubated with the sera, then with anti-mouse IgG1 and IgE secondary antibodies (Southern Biotech).

Flow-cytometry

For staining surface antigens, LNs and spleens were minced and digested with 2 mg/ml collagenase D (Roche) for 30 minutes. In the case of splenocytes, erythrocytes were lysed by briefly suspending cells in ACK lysis buffer. Cells were re-suspended in 2 mM EDTA in PBS and incubated with fluorochrome-conjugated mAb cocktail for 20min on ice. For intranuclear transcriptional factor staining, cells were fixed and permeabilized with Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience). For intracellular cytokine staining, cells were stimulated in a 96-well round-bottom plate with 20 ng/ml PMA and 1 μg/ml ionomycin (Sigma) for 1.5 hours at 37°C, then incubated another 4.5 hours at 37°C with additional Protein Transport Inhibitor Cocktail (eBioscience). Cells were then fixed and permeabilized with BD Cytofix/Cytoperm Kit (BD Biosciences), then incubated with anti-cytokine mAbs for 60 minitues on ice. Anti-CD4 (GK1.5), CD8a (53-6.7), CD25 (PC61), CD11b (M1/70), CD11c (N418), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD326 (G8.8), OX40L (RM134L), IFN-γ (XMG1.2) and IL-4 (11B11) mAbs were purchased from BioLegend. Anti-CD44 (IM-7), CD103 (2E7), T-bet (4B10), Jagged-1 (HMJ1-29), PD-L2 (TY25), ICOSL (HK5.3) and GATA-3 (TWAJ) mAbs were purchased from eBioscience. Anti-B220 (RA3-6B2), CXCR5 (2G8), CD95 (Jo2) and GL7 mAbs were purchased from BD Biosciences. Anti-pan CD301 mAb (ER-MP23) was purchased from Santa Cruz Biotechnology. Anti-IL-33 receptor (245707) was purchased from R&D systems. Anti-MHCII (M5/114.15.2) mAb was purified in house from the culture supernatant of the hybridoma and conjugated with Alexa Fluor 680 (Invitrogen). All flow-cytometry samples were scanned on BD FACS LSRII (BD Biosciences) and analyzed by FlowJo software (Version 9.3.2, TreeStar).

Anti-CD301b hybridoma

Anti-CD301b hybridoma 11A10-B7-2 was established by standard limiting dilution of YB2/0 rat myeloma cells fused with dLN cells of rats immunized recombinant CD301b. cDNA clones for *Mgl1* and *Mgl2* were purchased from OpenBiosystems (clone ID 4456238 and 40044847, respectively) and the extracellular domain of each protein was amplified by PCR (common forward primer: catgccatgggatcccaaaattcccagttaa, *Mgl1* reverse primer: ccgctcgagtaaattacttggcgagagct, *Mgl2* reverse primer: ccgctcgagttatggaactgaggctataa) and cloned into NcoI and XhoI sites of pET21d vector (gift from Ruslan Medzhitov). The recombinant proteins were expressed in Rosetta (DE3) E. coli (Novagen) and purified with a lactose-agarose column (Sigma) as previously described(Kumamoto et al., 2004). To immunize, two female WKY rats were injected in the footpad on both hind legs with 0.1 mg recombinant CD301b together with TiterMax Gold (CytRx) on days 0, 5 and 13. On day 14, the draining popliteal and inguinal LNs were excised and pooled, then the cells were fused with YB2/0 non-secreting rat myeloma cells and selected in a standard HAT media. One CD301b-specific clone, 11A10-B7-2 (IgG2a, λ), that does not cross-react with CD301a was selected by ELISA of the supernatant against recombinant CD301a and CD301b proteins and subcloned by limiting dilution. The hybridoma cells were cultured in serum-free media (CD hybridoma, Invitrogen) supplemented with 0.5x cholesterol (Invitrogen) and insulin-transferrin-selenium supplement (Sigma) and the mAb was purified from the culture supernatant with protein G-agarose (Millipore). For the use in flow-cytometry, purified 11A10-B7-2 mAb was labeled with Lightning-Link APC Antibody Labeling Kit (Novus Biologicals).

Immunohistochemistry

Axillary and brachial LNs were removed from DT- or PBS-treated WT B6 or Mgl2DTR mice and frozen in OCT compound (Sakura Finetek). Frozen sections (8 μm thickness) were fixed in ice-cold acetone and non-specific binding was blocked by pre-incubating the sections with 5% bovine serum albumin, 1% normal goat serum in PBS. The sections were incubated with 2.5 μg/ml purified anti-CD301b 11A10-B7 mAb or rabbit anti-GFP polyclonal Ab (Invitrogen). Biotinylated donkey anti-rat IgG (H+L) and donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch) were used to detect mAb CD301b and anti-GFP polyclonal Ab, respectively. After incubating with the secondary Abs, the bound Abs were detected by streptavidin-horseradish peroxidase (Zymed) and Tyramide-Cy3 (PerkinElmer). For double staining, fixed sections were first incubated with anti-CD207 (929F3) or anti-CD11c (N418) mAbs, followed by secondary antibodies biotinylated donkey anti-rat IgG (H+L) and donkey anti-hamster IgG (H+L) (Jackson ImmunoResearch), respectively. The secondary Abs were detected by SA-HRP and Tyramide-Cy3. The sections were then incubated with biotin-conjugated anti-mouse MGL2 (R&D) that was subsequently detected by SA-HRP and Tyramide-FITC. The nuclei were stained with DAPI. Images were captured by BX51 fluorescence microscope (Olympus) and processed with PictureFrame software (Optronics).

Statistical analysis

All experiments were performed at least twice and the data were pooled where indicated. All p values were calculated by two-tailed Student's t-test by Prism software (version 4.0b, GraphPad). Data are presented as mean ± SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

APC	antigen-presenting cell
DC	dendritic cell
DDC	dermal dendritic cell
dLN	draining lymph node
LC	Langerhans cell
LN	lymph node
ndLN	non-draining lymph node
OVA	ovalbumin
Th	T helper

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Highlights

- CD301b is expressed by majority of DCs in dermis and submucosa.
- CD301^{+}DCs facilitate CD4 T cell accumulation in the reactive lymph nodes.
- Transient depletion of CD301b^{+}DCs results in impaired Th2 responses in vivo.

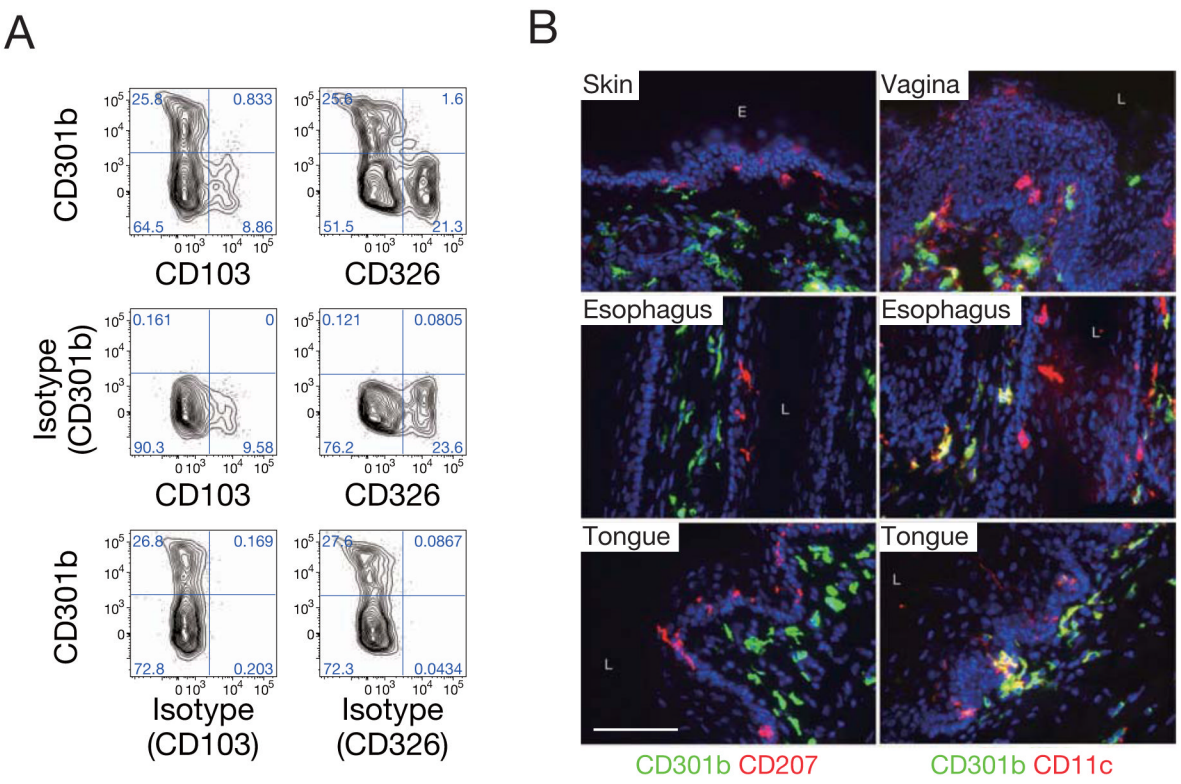

Figure 1. Expression of CD301b by the major DC population in the dermis and submucosae

(**A**) Expression of CD301b, CD326 (LC marker) and CD103 (CD207^{+}DDC marker) in naïve cutaneous LN. Cells within the CD11c^{+}MHCII^{+}B220^{-} gate are shown. (**B**) Frozen sections of skin, vagina, esophagus and tongue from naïve C57BL/6 mice were stained with Ab against CD301b (green), CD207 (red, left) and CD11c (red, right). Scale bar represents 100 μm. E: Epidermis, L: Lumen. These data are representative of three or more similar experiments. See also Figure S1.

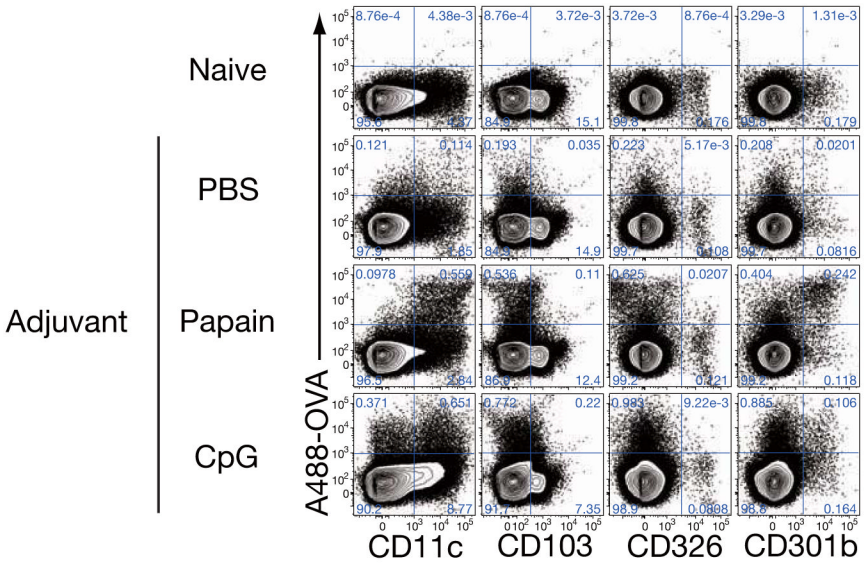

Figure 2. Uptake of soluble protein antigen by CD301b^{+}DDCs in the skin-draining LNs

Mice were injected with Alexa Fluor 488 (A488)-labeled OVA protein with the indicated adjuvant in the footpad. Twenty-four hors later, the draining popliteal LNs were harvested and stained for DC markers. The total live LN cells are shown. These data are representative of three independent experiments. See also Figure S2.

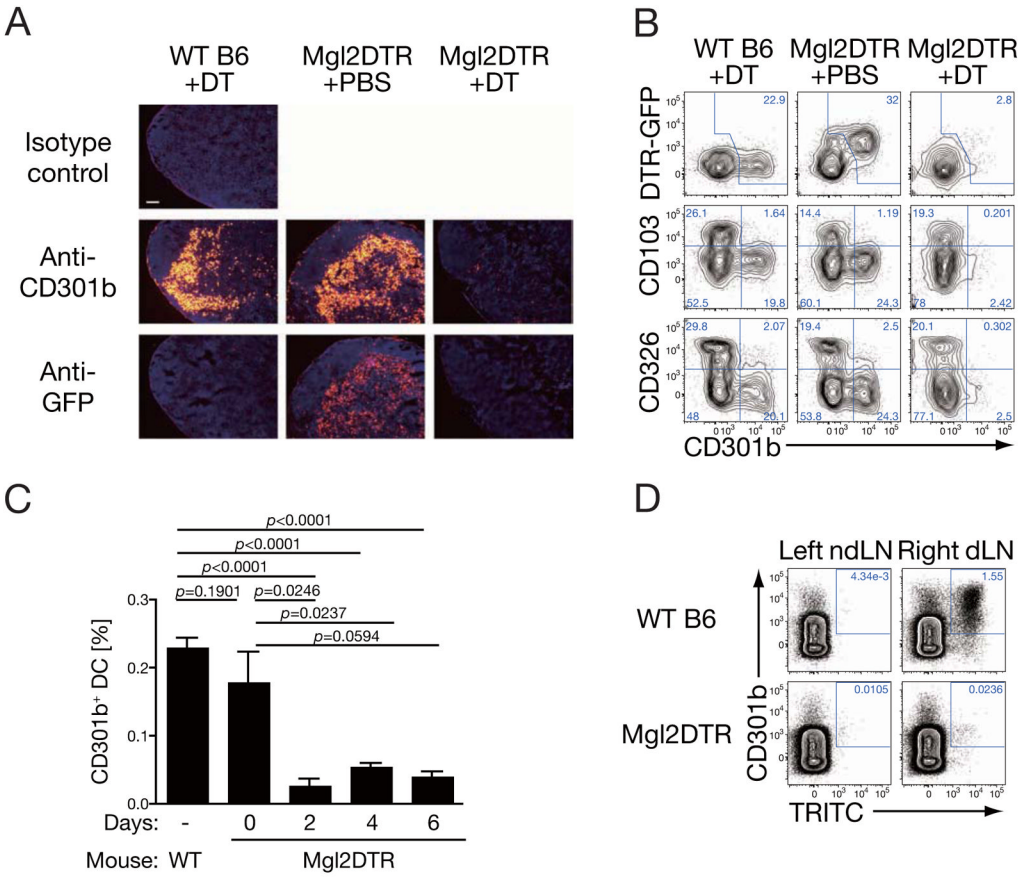

Figure 3. Depletion of CD301b$^+$ DDCs in Mgl2DTR mice

(**A**) Mice of indicated genotype were intraperitoneally injected with PBS or DT. Two days later, skin-draining LNs were harvested and stained for CD301b or DTR-GFP. Scale bar = 100μm. (**B**) Skin-draining LN DCs one day after DT injection were stained for CD301b and CD207$^+$DC markers. (**C**) Kinetics of CD301b$^+$DDC depletion in the skin-draining LNs in Mgl2DTR mice. The percentage of CD301b$^+$DCs in the total LN cells is shown as mean ± SEM. n = 4 – 6 per time point. (**D**) Depletion of CD301b$^+$DDCs abolishes the influx of hapten-bearing DCs in the dLN. DT-treated WT C57BL/6 (WT B6) or Mgl2DTR mice were painted with TRITC dissolved in 1:1 mixture of acetone:di-*n*-butylphthalate on the shaved abdomen. Twenty-four hours later, the draining (right) and non-draining contralateral (left) inguinal LNs were harvested. The total live LN cells are shown. These data are representative of two similar experiments. See also Figures S3 and S4.

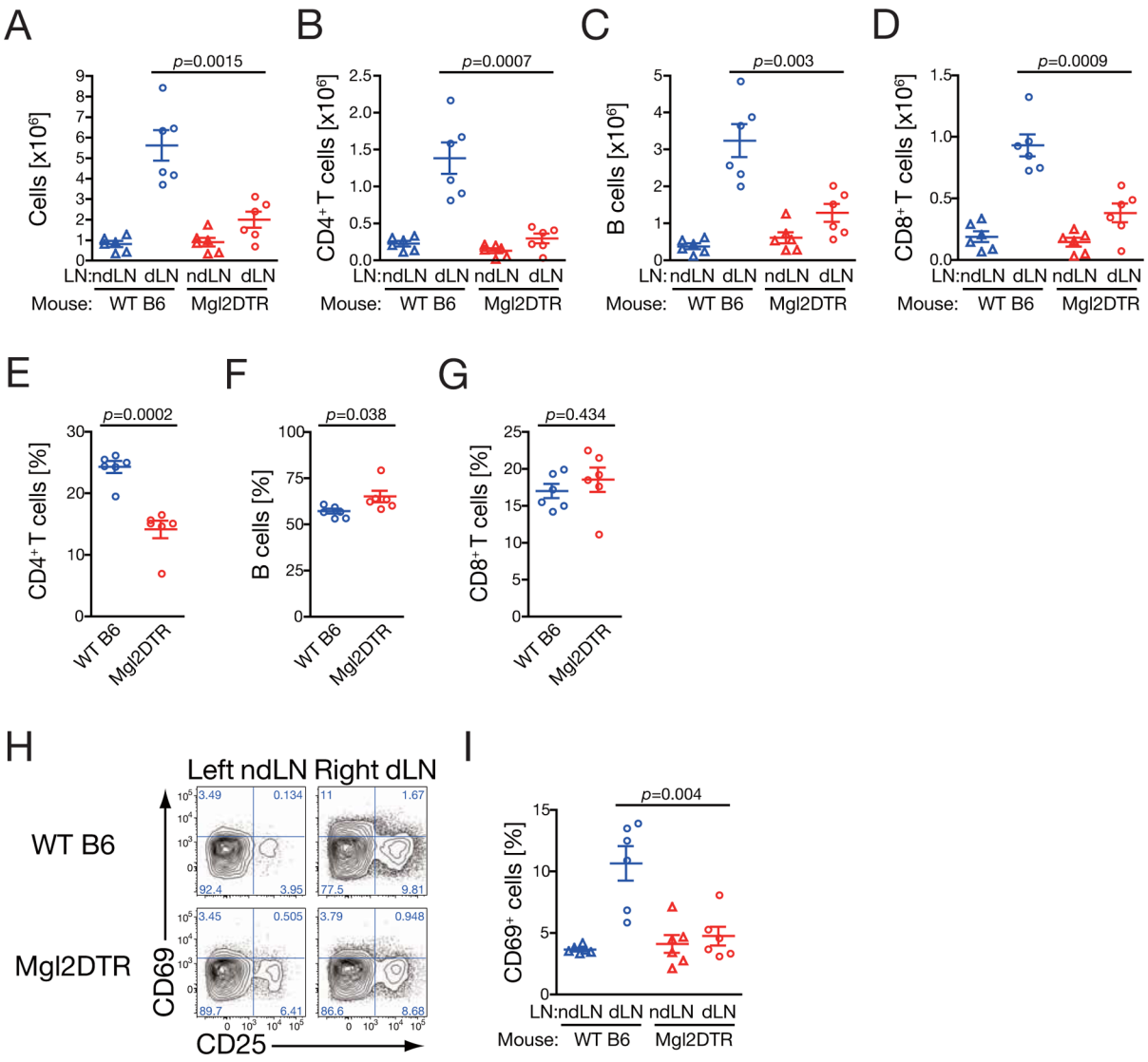

Figure 4. Requirement of CD301b$^+$ DDCs for accumulation and activation of polyclonal CD4$^+$T cells in the dLN upon immunization with papain

Wild-type or Mgl2DTR mice were intraperitoneally injected with DT. One day later, the mice were immunized with papain in the right hind footpad. (**A–G**) The draining right or non-draining left popliteal LNs were harvested three days after immunization and the total LN cellularity (**a**) and the number (**B–D**) and the percentage in the total dLN cells (**E–G**) of CD4$^+$T cells (**B,E**), B cells (**C,F**) and CD8$^+$T cells (**D,G**) are depicted. (**H,I**) Expression of CD69 and CD25 was analyzed in CD4$^+$T cells in (**B**) and the percentage of CD69$^+$CD25$^-$ cells among total CD4$^+$T cells were calculated. Data were pooled from two independent experiments with similar results and represented as mean ± SEM. Each pointrepresents one mouse. See also Figure S5.

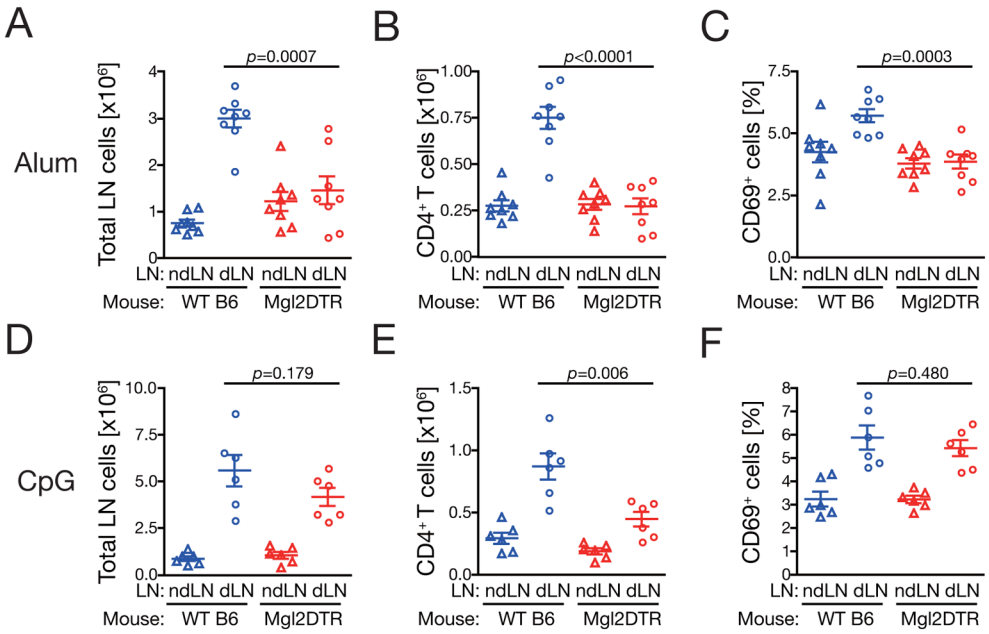

Figure 5. CD301b$^+$DDC requirement for activation of CD4$^+$T cells in response to Th1- and Th2-type adjuvants

As in Figure 4, DT-treated mice were immunized in the right footpad with OVA and alum (**A–C**) or OVA and CpG 2216 (**D–F**). Popliteal LNs were harvested on day 3. The total LN cellularity (**A,D**), the number of CD4$^+$T cells (**B,E**) and the percentage of CD69$^+$CD25$^-$ cells in total CD4$^+$T cells (**C,F**) are shown. Data were pooled from two independent experiments with similar results and represented as mean ± SEM. Each point represents one mouse.

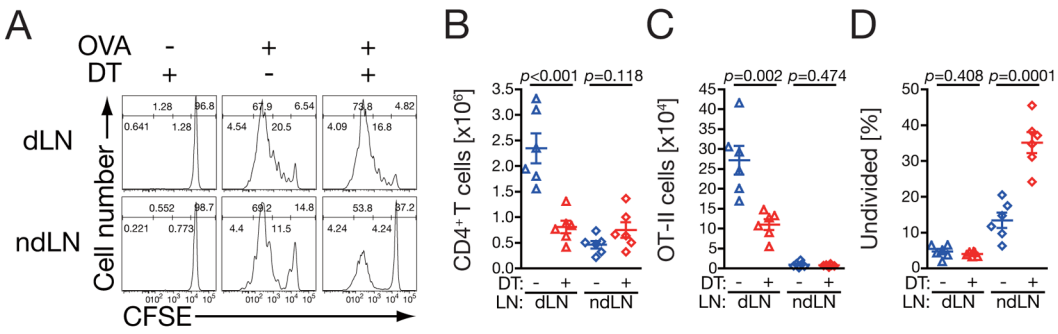

Figure 6. Requirement of CD301b^{+}DDCs for the optimal screening for antigen-specific CD4^{+} T cells upon immunization with papain

Mgl2DTR mice were adoptively transferred with CFSE-labeled OVA-specific naïve OT-II CD4^{+} T cells, and treated with DT or PBS. One day later, the mice were immunized with OVA and papain in the footpad. The draining and non-draining LNs were harvested four days after immunization. (**A**) Dilution of CFSE in OT-II cells. (**B**) The number of endogenous polyclonal CD4^{+}T cells per LN. (**C**) The number of OT-II cells per LN. (**D**) The percentage of undivided CFSE^{hi} cells among OT-II cells. Data were pooled from two independent experiments with similar results and represented as mean ± SEM. Each point represents one mouse.

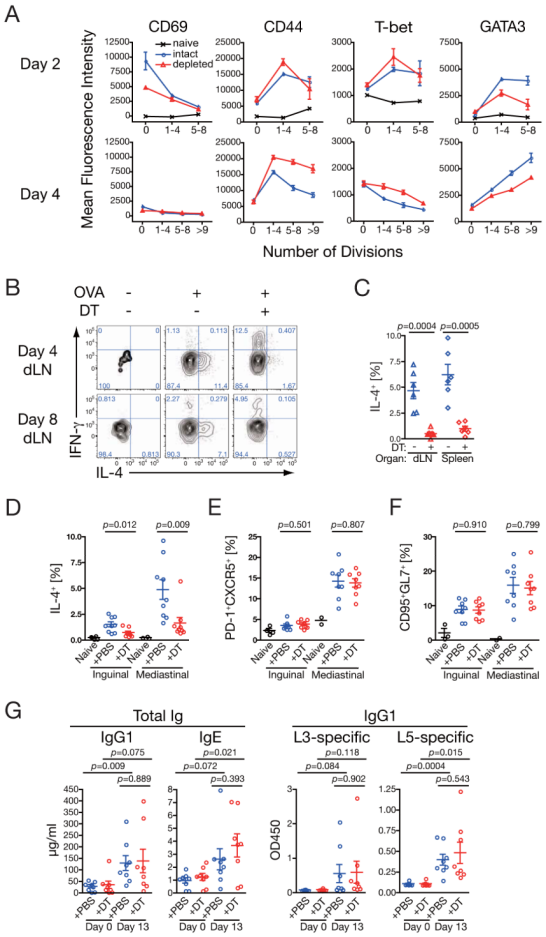

Figure 7. Requirement of CD301b$^+$DDCs for the papain- and hookworm-induced Th2 cell differentiation

(**A–C**) Mgl2DTR mice were adoptively transferred with CFSE-labeled OT-II cells and treated with DT or PBS as in Figure 6. (**A**) The OT-II cells were subdivided based on the number of divisions as shown in Figure 6A. Mean fluorescent intensity of indicated markers in OT-II cells are depicted against the number of cell divisions. The data are representative of two independent experiments and shown as mean ± SEM. n = 3 for each time-point. (**B,C**) The draining popliteal LNs and spleen were harvested on day 4 (**B**) or day 8 (**B,C**) post-immunization and re-stimulated *in vitro* with PMA and ionomycin for intracellular cytokine staining in gated OT-II cells. (**D-G**) Mgl2DTR mice were treated with either PBS or DT every 3 days starting at day −1. On day 0, mice were subcutaneously infected with *N. brasiliensis* at the tail base. At day 5 (**D**) and day 13 (**E** and **F**), indicated LNs were collected and stained for IL-4 following restimualtion with PMA and ionomycin (**D**) or for Tfh cell markers PD-1 and CXCR5 and germinal center B cell markers CD95 and GL7 without restimulation (**E** and **F**). Total CD4$^+$ T (**D** and **E**) and B (**F**) cells are shown. Sera were harvested before infection (day 0) or on day 13 and parasite-specific antibody was detected by ELISA (**G**). Data were pooled from two independent experiments with similar results and represented as mean ± SEM. See also Figures S6 and S7.