

Lung dendritic cells induce migration of protective T cells to the gastrointestinal tract

Darren Ruane,^{1,3,7} Lucas Brane,^{1,7} Bernardo Sgarbi Reis,² Cheolho Cheong,^{1,4} Jordan Poles,^{1,7} Yoonkyung Do,⁵ Hongfa Zhu,⁶ Klara Velinzon,¹ Jae-Hoon Choi,⁸ Natalie Studt,¹ Lloyd Mayer,⁷ Ed C. Lavelle,³ Ralph M. Steinman,¹ Daniel Mucida,² and Saurabh Mehandru^{1,7}

¹Laboratory of Cellular Immunology and Physiology and ²Laboratory of Mucosal Immunology, The Rockefeller University, New York, NY 10065

³Adjuvant Research Group, School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland

⁴Laboratory of Cellular Immunology and Physiology, Institut de Recherches Cliniques de Montréal and Department of Microbiology and Immunology, Université de Montréal, Montreal, Quebec H2W 1R7, Canada

⁵School of Nano-Bioscience and Chemical Engineering, Ulsan National Institute of Science and Technology, Ulsan 689-805, Republic of Korea

⁶Department of Pathology; and ⁷Division of Gastroenterology, Department of Medicine and the Immunology Institute, Mount Sinai School of Medicine, New York, NY 10029

⁸Department of Life Science, College of Natural Sciences, Hanyang University, Seoul 133-791, Republic of Korea

Developing efficacious vaccines against enteric diseases is a global challenge that requires a better understanding of cellular recruitment dynamics at the mucosal surfaces. The current paradigm of T cell homing to the gastrointestinal (GI) tract involves the induction of $\alpha 4\beta 7$ and CCR9 by Peyer's patch and mesenteric lymph node (MLN) dendritic cells (DCs) in a retinoic acid-dependent manner. This paradigm, however, cannot be reconciled with reports of GI T cell responses after intranasal (i.n.) delivery of antigens that do not directly target the GI lymphoid tissue. To explore alternative pathways of cellular migration, we have investigated the ability of DCs from mucosal and nonmucosal tissues to recruit lymphocytes to the GI tract. Unexpectedly, we found that lung DCs, like CD103⁺ MLN DCs, up-regulate the gut-homing integrin $\alpha 4\beta 7$ in vitro and in vivo, and induce T cell migration to the GI tract in vivo. Consistent with a role for this pathway in generating mucosal immune responses, lung DC targeting by i.n. immunization induced protective immunity against enteric challenge with a highly pathogenic strain of *Salmonella*. The present report demonstrates novel functional evidence of mucosal cross talk mediated by DCs, which has the potential to inform the design of novel vaccines against mucosal pathogens.

CORRESPONDENCE

Saurabh Mehandru:
Saurabh.mehandru@mssm.edu

Abbreviations used: ALDH, aldehyde dehydrogenase; CCR9, chemokine receptor 9; dnRAR, dominant negative RAR; DT, diphtheria toxin; DTR, DT receptor; GI, gastrointestinal; IEC, intraepithelial cells; IEL, intraepithelial lymphocytes; LPL, lamina propria lymphocytes; MDA, melanoma differentiation-associated protein; MLN, mesenteric LN; PP, Peyer's patch; RA, retinoic acid; RAG, recombination activating gene; RALDH, retinal dehydrogenase; RAR, retinoic acid receptor; SkDC, skin-draining LN DC; SpDC, spleen DC; TECK, thymus expressing chemokine.

Because efficient trafficking of immune cells to the gastrointestinal (GI) tract is critical for host defense against pathogenic challenge, studying cellular recruitment pathways to the GI tract is the key to developing novel vaccines against mucosally transmitted diseases, including HIV-1 infection.

Naive T cells acquire the capacity to migrate to extra-lymphoid tissues once activated by their cognate antigen (Butcher et al., 1999; von Andrian and Mackay, 2000). These antigen-experienced effector T cells migrate preferentially to the tissues where they first encountered the antigen (Kantele et al., 1999; Campbell and

Butcher, 2002). For example, early observations demonstrated that cells activated in the GI tract home back to the intestinal effector sites (Cahill et al., 1977; Hall et al., 1977). Integrin $\alpha 4\beta 7$ and chemokine receptor 9 (CCR9) are among the best studied gut-specific homing molecules (Berlin et al., 1995; Zabel et al., 1999). The $\alpha 4\beta 7$ ligand mucosal addressin cell adhesion molecule-1 (MAdCAM-1) mediates recruitment of T cells to the intestinal lamina propria (Berlin et al., 1995), and the CCR9 ligand TECK,

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expressed by small intestinal epithelial cells, recruits T cells to the small bowel (Zabel et al., 1999).

DCs are well recognized as the initiators of the adaptive immune response (Steinman and Cohn, 1973), as well as mediators of tolerance to self-antigens in steady-state conditions (Hawiger et al., 2001). Additionally, there is increasing evidence regarding the role played by DCs as conductors of immunological traffic to the skin and the GI tract (Johansson-Lindbom et al., 2003, 2005; Mora et al., 2003, 2005; Sigmundsdottir et al., 2007). DCs can imprint T cells to migrate to the tissue in which the T cells were originally activated. For example, gut-associated DCs induce the gut-homing receptors $\alpha 4\beta 7$ and CCR9 on T cells upon activation (Johansson-Lindbom et al., 2003; Mora et al., 2003; Stagg et al., 2002).

RA is necessary and sufficient to induce gut-homing receptors on T cells (Iwata et al., 2004). The main pathway of RA biosynthesis *in vivo* is dependent on the intracellular oxidative metabolism of retinol (Napoli, 1999; Duester, 2000), catalyzed by a family of alcohol dehydrogenases including retinal dehydrogenase (RALDH), a class I aldehyde dehydrogenase that mediates the irreversible oxidation of retinal to RA. RA in turn is thought to induce RALDH-2 in a positive feedback loop (Yokota et al., 2009; Hammerschmidt et al., 2011; Villablanca et al., 2011) and RA levels correlate with the ability of the intestinal DCs to induce gut-tropic T cells. Vitamin A is introduced via dietary or biliary sources (Jaensson-Gyllenbäck et al., 2011). Among the cellular sources of RA in the intestinal mucosa are DCs (Iwata et al., 2004), stromal cells (Hammerschmidt et al., 2008; Molenaar et al., 2009), intestinal epithelial cells (Bhat, 1998; Lampen et al., 2000), and intestinal macrophages (Denning et al., 2007), with the DCs likely playing a key role in the induction of gut-homing phenotype on T cells. Among the intestinal DCs, the CD103⁺ DC subsets express high levels of RALDH-2 and are capable of generating high levels of RA (Johansson-Lindbom et al., 2005; Coombes et al., 2007; Sun et al., 2007; Jaensson et al., 2008). In contrast, the CD103⁻CD11b⁺CX3CR1⁺ macrophage-like population in the intestinal lamina propria expresses RALDH-1 and not RALDH-2 and exhibits a lower RA-producing capacity (Schulz et al., 2009; Denning et al., 2011), and therefore a decreased capacity to induce gut-homing potential on T cells (Jaensson et al., 2008).

Collectively, a paradigm has emerged wherein only the intestinal CD103⁺ DCs, which are capable of metabolizing vitamin A, can induce GI-specific homing on T cells (Jaensson et al., 2008). This paradigm however, is difficult to reconcile with reports of GI T cell responses after *i.n.* delivery of antigens that do not directly target the GI lymphoid tissue. For example, mice infected *i.n.* with influenza virus show no activated virus-specific CD8⁺ T cells in the mesenteric LNs (MLNs) or Peyer's patches (PPs), yet flu-specific CD8⁺ cells within the lung-associated tissues express $\alpha 4\beta 7$ and memory CD8⁺ T cells are established within the small intestinal epithelium (Masopust et al., 2010). Similarly, a recent study demonstrates that *i.n.* challenge with H1N1 influenza results in the accumulation of T_H17 cells within the small intestinal

lamina propria (SILP; Esplugues et al., 2011). Furthermore, Ciabattini et al. (2011) have demonstrated that after *i.n.* immunization, antigen-specific T cells are generated in the mediastinal LN and migrate to the MLN in an $\alpha 4\beta 7$ - and CD62L-dependent manner. Additionally, it is known that lungs harbor prominent extrahepatic stores of vitamin A (Okabe et al., 1984; Dirami et al., 2004). Its metabolite, RA, plays an important role in pulmonary alveolar development (Dirami et al., 2004) and has a putative therapeutic role in emphysema (Massaro and Massaro, 1997). Finally, although RA production has been considered to be the forte of gut-resident DCs, other DC populations also express RALDH, particularly lung-resident DCs that express RALDH-2 (Heng and Painter, 2008; Williams et al., 2010). However, the ability of lung DCs to induce GI-specific T cell homing has not yet been reported.

All of the aforementioned factors led us to hypothesize that lung DCs would up-regulate the expression of gut-homing molecules integrin $\alpha 4\beta 7$ and CCR9 on T cells, which in turn would license the migration of T cells to the GI tract. Our hypothesis was made credible by the concept of a common mucosal immunological system proposed by Bienenstock et al. (1978) more than 30 years ago. Indeed, there is increasing appreciation of the mucosal immune system as an integrated network of tissues, cells, and effector molecules, although the cellular factors that link different mucosal compartments are not well understood (Gill et al., 2010).

In this study, we show that lung DCs can imprint expression of the gut-homing integrin $\alpha 4\beta 7$ and CCR9 on co-cultured T cells *in vitro* and on adoptively transferred cells *in vivo*, licensing T cells to migrate to the GI lamina propria and confer protective immunity against intestinal pathogens. We define a new pathway of DC-mediated mucosal cross talk and challenge the existing dogma that only GI-resident DCs can recruit antigen-specific T cells back to the gut.

RESULTS

Lung DCs induce expression of integrin $\alpha 4\beta 7$ and CCR9 on T cells *in vitro* and *in vivo*

We conducted a screen to examine the ability of mucosal and nonmucosal DCs to induce gut-homing integrin- $\alpha 4\beta 7$ and CCR9 expression. CD11c⁺ cells were isolated from murine lung, spleen (SpDC), MLNs, and skin-draining LNs (SkDC) and co-cultured with CFSE-labeled T cells from OVA-reactive CD4⁺ T cells from OT-II mice at a ratio of 1:2 DC/T cells for 5–7 d. Unexpectedly, we observed that the expression of integrin $\alpha 4\beta 7$ and CCR9 was significantly up-regulated by lung DCs, similar to MLN DCs, with $\alpha 4\beta 7$ and CCR9 up-regulation most prominent on proliferating (CFSE^{lo}) cells (Fig. 1, a–c).

To test whether lung DCs can up-regulate $\alpha 4\beta 7$ *in vivo*, we adoptively transferred CFSE-labeled CD45.1⁺ OT-II cells to naive CD45.2 mice and immunized the mice after 2 h with OVA protein and polyICLC delivered *i.n.*, *s.c.*, *i.t.*, or orally (*per os*; *p.o.*). On day 4 after immunization, the percentage of CD45.1⁺ $\alpha 2^+$ CD4⁺CFSE^{lo} cells expressing $\alpha 4\beta 7$

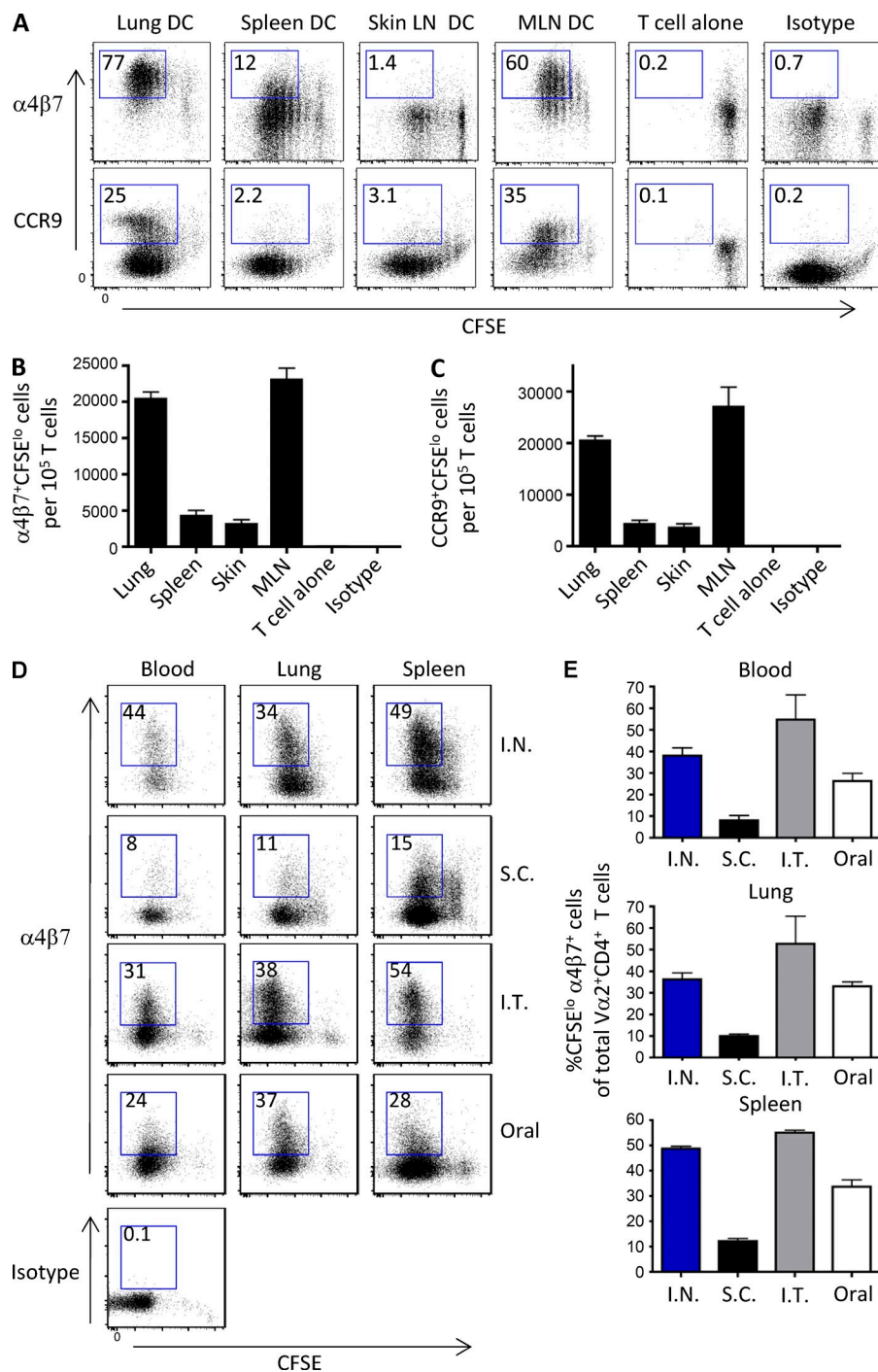


Figure 1. Lung DCs induce integrin $\alpha 4\beta 7$ and CCR9. (A–C) CD11c⁺ DCs isolated from the lung, spleen, skin-draining LNs, or MLNs were pulsed with OVA and cultured with CFSE-labeled OT-II T cells at a ratio of 1:2 for 5–7 d. (A) T cell expression of $\alpha 4\beta 7$ (top) and CCR9 (bottom) was measured by flow cytometry and plotted against CFSE dilution. (B and C) Quantification of the number of proliferated (CFSE^{lo}) $\alpha 4\beta 7^{\text{+}}$ (B) or CCR9⁺ (C) cells showing cumulative data from three independent experiments. (D and E) CFSE-labeled CD45.1⁺ $\text{V}\alpha 2^{\text{+}}\text{CD}4^{\text{+}}$ OT-II cells were transferred into naive CD45.2⁺ recipients. 2 h after transfer, mice were immunized with OVA protein + polyI:LC or cholera toxin via the indicated route, and the percentage of CFSE^{lo}CD45.1⁺ $\text{V}\alpha 2^{\text{+}}\text{CD}4^{\text{+}}$ cells expressing $\alpha 4\beta 7$ was determined in blood, lung, and spleen 4 d after immunization. (D) Representative flow cytometry data showing CFSE dilution versus $\alpha 4\beta 7$ expression. (E) Cumulative data from three independent experiments showing frequency of $\alpha 4\beta 7^{\text{+}}$ CFSE^{lo} cells among total $\text{V}\alpha 2^{\text{+}}\text{CD}4^{\text{+}}$ cells.

was determined in the blood, lung, and spleen. As shown in Figs. 1 (d and e), a significantly higher percentage of CFSE^{lo} $\alpha 4\beta 7^{\text{+}}$ cells were induced in the blood, lung, and spleen after i.n., i.t., and p.o. immunization than in the blood or spleen after s.c. immunization. Similar findings were observed in the case of CD8⁺OT-I cells (unpublished data).

We next examined the ability of lung DC subsets to induce $\alpha 4\beta 7$. Lung MHC-II⁺CD11c^{hi} DCs (gating strategy shown in Fig. S1) were FACS-sorted into CD103⁺CD11b⁻

and CD11b⁺CD103⁻ DC populations and co-cultured with CFSE-labeled OT-II cells. Unexpectedly, both CD103⁺CD11b⁻ and CD11b⁺CD103⁻ lung DC subsets induced $\alpha 4\beta 7$ integrin, with expression of $\alpha 4\beta 7$ being even higher in the CD11b⁺CD103⁻ populations. As previously described (Jaensson et al., 2008), among the MLN DCs, CD103⁺CD11b⁻ DCs induced high levels of $\alpha 4\beta 7$, whereas CD11b⁺CD103⁻ DCs did not (Fig. 2, a and b). Additionally, the lung CD11b⁺CD103⁻ population was sorted into CD24⁺CD64⁻ and CD24⁻CD64⁺

populations. We used these markers to distinguish between macrophages and DCs contained within the heterogeneous CD11b⁺ population (Langlet et al., 2012; Tamoutounour et al., 2012). Additionally, although not the focus of this study, a combination of MAR-1 and CD64 can also be used to distinguish between cDCs and MoDCs contained within the CD11b population (Plantinga et al., 2013). We observed striking differences between the CD24⁺ and CD64⁺ subpopulations, with the CD11b⁺CD24⁺ cells inducing both integrin α 4 β 7 and CCR9. In contrast, the CD11b⁺CD64⁺ population was poor in inducing both T cell proliferation and gut-homing molecule induction (Fig. 2, c–e).

Lung DCs license T cells to migrate to the intestinal lamina propria via α 4 β 7 induction

Targeting of lung DCs by i.n. delivery (Vermaelen et al., 2001) was confirmed in a time course experiment (unpublished data) where fluorescently labeled antibodies directed against CD11c were administered to C57BL/6 mice i.n. Both CD103⁺CD11b⁻ and CD11b⁺CD103⁻ DC subsets were

targeted in the mediastinal LNs and lungs, with the peak labeling occurring 24 h after antibody administration. However, we could not detect antibody-labeled cells in the spleen, MLN, or the GI tissues even after 72 h. To rule out a possible role of gastric acid or GI proteases, cleaving the injected antibodies, orally delivered CD11c was administered to C57BL/6 mice. Peak labeling was detected in the small intestines after 16 h, targeting the CD11b⁺ population (unpublished data). Therefore, transport of labeled antibodies through the GI tract does not affect its ability to be used to stain gut DCs in vivo. To confirm this, we compared i.n. and intratracheal (i.t.) delivery of fluorescent antibodies and found that i.t. immunization targets DCs in the lung similar to those targeted by i.n. immunization (unpublished data).

We next conducted a time course experiment to study the kinetics of induction of integrin α 4 β 7 and the migration of adoptively transferred cells into the GI tissues: SILP, colon, and MLNs. CFSE-labeled CD45.1⁺ OT-II cells were adoptively transferred to naive CD45.2 recipients that were immunized after 2 h with OVA protein and polyICLC-delivered i.n. We

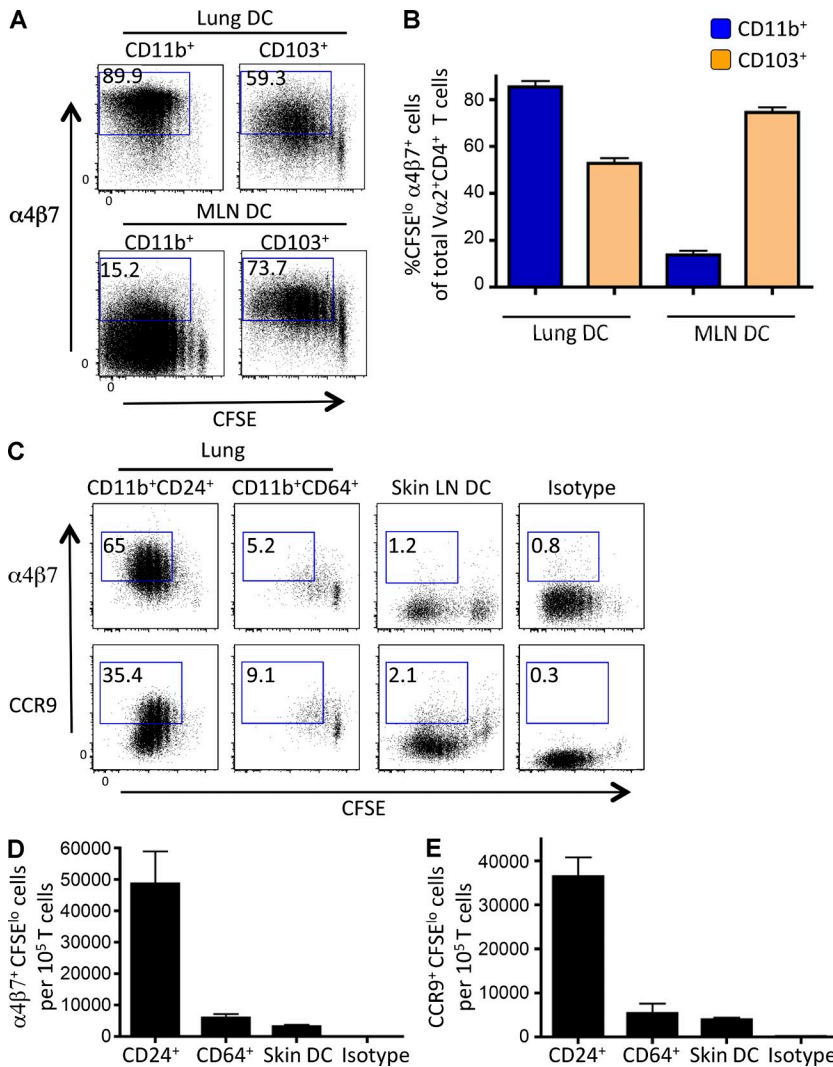


Figure 2. Lung CD103⁺ and CD24⁺11b⁺ DCs induce integrin α 4 β 7 and CCR9 in vitro. (A and B) MHCII⁺CD11c^{hi}CD11b⁺CD103⁻ and MHCII⁺CD11c^{hi}CD11b⁻CD103⁺ DCs were flow-sorted from the lung or MLN, pulsed with OVA, and cultured with CFSE-labeled OT-II T cells at a ratio of 1:2 for 5–7 d. (A) T cell expression of α 4 β 7 induced by lung DCs (top) and MLN DCs (bottom) was measured by flow cytometry and plotted against CFSE dilution. (B) Quantification of the number of proliferated (CFSE^{lo}) α 4 β 7⁺ cells showing cumulative data from three independent experiments. (C–E) Lung-derived MHCII⁺CD11c^{hi}CD103⁻CD11b⁺CD24⁺ and MHCII⁺CD11c^{hi}CD103⁻CD11b⁺CD64⁺ cells were flow-sorted. Additionally, MHCII⁺CD11c^{hi} DCs were isolated from skin-draining LNs. These DCs were pulsed with OVA and cultured with CFSE-labeled OT-II T cells at a ratio of 1:2 for 5–7 d (C) T cell expression of α 4 β 7 (top) and CCR9 (bottom) was measured by flow cytometry and plotted against CFSE dilution. (D and E) Quantification of the number of proliferated (CFSE^{lo}), α 4 β 7⁺ (D) or CCR9⁺ (E) cells showing cumulative data from three independent experiments.

tested for the induction of integrin $\alpha 4\beta 7$ on the transferred cells on days 1, 2, 3, 4, and 7 after transfer and quantified the frequency of transferred cells in GI tissues at the same time points (Fig. 3, a and b). Proliferation of the transferred cells was first detected in the mediastinal LNs on day 2 after transfer. On day 3, a sharp rise in the expression of integrin $\alpha 4\beta 7$ was noted on the transferred cells in the mediastinal LNs and the lungs, peaking at day 4 and regressing by day 7. The frequency of transferred $CD45.1^+V\alpha 2^+CD4^+$ T cells showed a striking rise on day 4 in the GI tissues (SILP, colon, and MLNs) corresponding

to the peak expression of integrin $\alpha 4\beta 7$ on lung and mediastinal LN-resident transferred cells. Additionally, we did not detect proliferation of the CFSE-labeled $CD45.1^+$ OT-II cells in the MLN on days 1, 2, and 3. The earliest site, where T cell proliferation was observed after i.n. immunization, was the mediastinal LNs (Fig. 3 c). In contrast, proliferating cells were detected in the MLNs by day 4, suggesting that T cell activation initially occurred in the mediastinal LNs and not the MLNs.

Next, we wanted to confirm that the transferred $CD45.1^+V\alpha 2^+CD4^+$ T cells detected in the GI tissues after

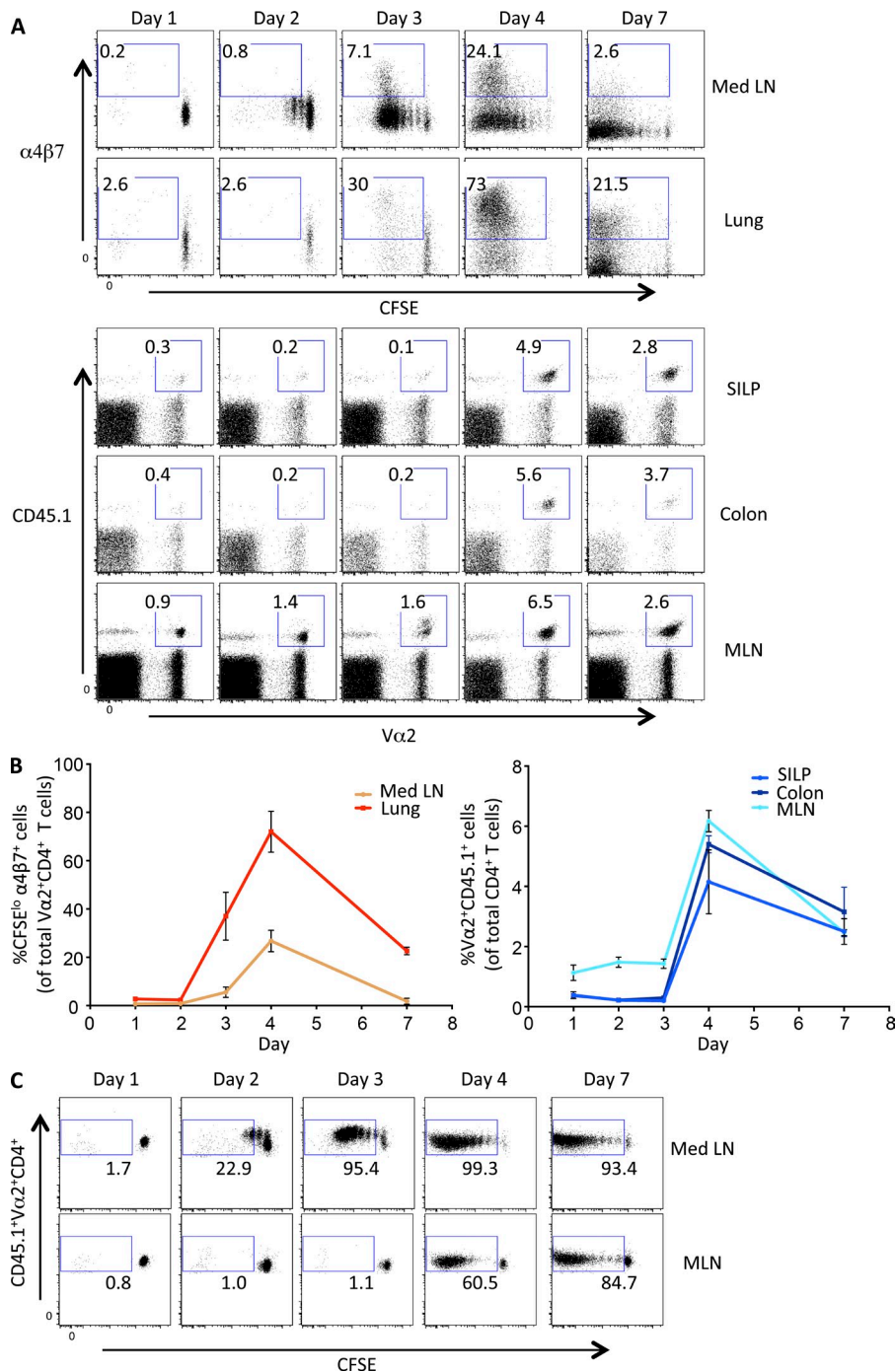


Figure 3. After i.n. immunization, integrin $\alpha 4\beta 7$ is induced in the mediastinal LN- and lung-resident cells, followed by the appearance of migratory cells in the GI tissues. $CD45.1^+V\alpha 2^+CD4^+$ OT-II cells were transferred into naive $CD45.2^+$ recipients. 2 h after transfer, mice were immunized with OVA protein + polyI:CLC via the i.n. route, and the transferred $CD45.1^+V\alpha 2^+CD4^+$ cells in mediastinal LN (med LN), lung, SILP, colon, and MLN were examined on days 1, 2, 3, 4, and 7 after immunization. (A) Representative flow cytometry data showing CFSE dilution versus $\alpha 4\beta 7$ expression in the med LN and lung at the respective time points (top two rows) and the frequency of adoptively transferred $V\alpha 2^+CD45.1^+CD4^+$ T cells in the SILP, colon and MLN (bottom three rows). (B) Cumulative data showing frequency of $\alpha 4\beta 7^+CFSE^0$ cells among total $V\alpha 2^+CD4^+$ cells (left) and the frequency of $V\alpha 2^+CD45.1^+$ cells among total $CD4^+$ T cells (right). (C) Proliferation (CFSE dilution) of the adoptively transferred $CD45.1^+CD4^+V\alpha 2^+$ T cells is compared between the med LN (top) and MLN (bottom) of the recipient mice on days 1, 2, 3, 4, and 7.

i.n. immunization were being generated in the lung-draining LNs and not gut-draining LNs as a result of inadvertent swallowing of i.n.-delivered antigen. We used FTY-720, an inhibitor of lymphocyte egress from LNs (Fig. 4) whereby CFSE-labeled CD45.1⁺ OT-II cells were adoptively transferred to naive CD45.2 recipients that were immunized after 2 h with OVA protein and polyICLC-delivered i.n. On days 0–3, recipient mice were administered FTY-720 (1 μg/g mouse). The mice were sacrificed on day 4, and the frequency of transferred CD45.1⁺Vα2⁺CD4⁺ T cells was quantified in the mediastinal LNs, MLNs, SILP, and colon. An increase in the frequency of transferred cells was noted in the mediastinal LNs. In contrast, we noted a decrease in the frequency of transferred cells in the MLNs, suggesting that antigen exposure and expansion of the transferred OT-II cells was occurring in the mediastinal LNs and not MLNs. As expected, a significant decrease in the frequency of transferred cells was noted in SILP and colon due to their arrest within the mediastinal LN (Fig. 4, a and b). Furthermore, as shown in Fig. 4 c, in the absence of gut exposure, the frequency of CFSE^{lo} antigen-specific T cells was dramatically lowered in the gut

compartments in the FTY-treated group. Thus, based on the in vivo labeling, time course, and FTY experiments, we were able to conclude that after i.n. delivery of antigen, integrin α4β7 was induced on transferred T cells within the mediastinal LNs and lungs and that inadvertent swallowing of i.n.-delivered antigen was not a factor in our experimental system.

Next, we sought to compare the efficiency of lung DC-primed and skin DC-primed T cells to migrate to the GI tract. Mice were immunized i.n. or s.c. (2 h after adoptive transfer of CFSE-labeled CD45.1⁺ OT-II cells to naive CD45.2⁺ mice) with OVA protein and polyICLC. On day 7, after immunization, the frequency of CD45.1⁺Vα2⁺ cells in the small intestines and colon was 3–10 fold higher after i.n. immunization than after s.c. immunization, demonstrating that lung DC-primed congenic OT-II cells migrated to the lamina propria with a significantly greater efficiency than skin DC-primed OT-II cells (Fig. 5, a and b).

We next sought to examine the effect of blocking integrin α4β7 on lung DC-primed, α4β7-expressing cells to the GI tract. After adoptive transfer of CFSE-labeled CD45.1⁺OT-II cells and i.n. immunization with OVA/polyICLC, half of the

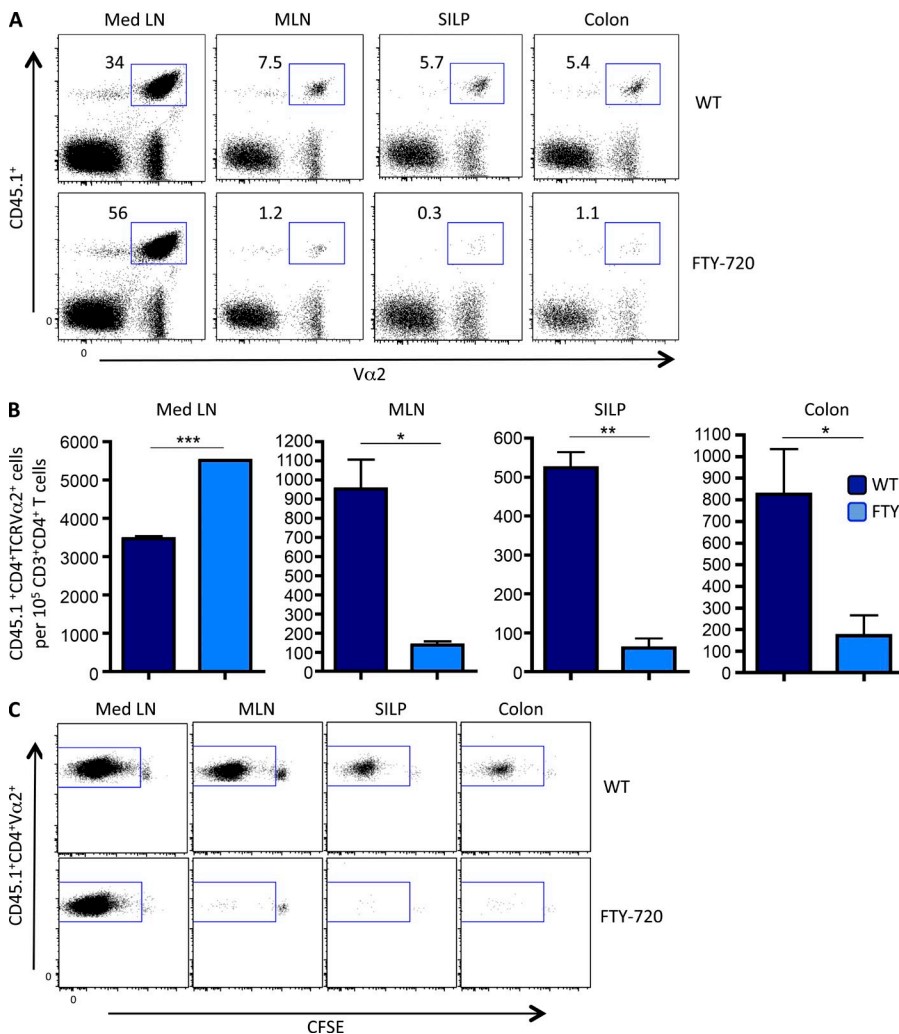


Figure 4. Administration of FTY-720 leads to accumulation of transferred CD45.2⁺Vα2⁺CD4⁺ OT-II cells in the mediastinal LNs, but not in the MLNs, after i.n. vaccination. CFSE-labeled CD45.1⁺ Vα2⁺CD4⁺OT-II cells were adoptively transferred to naive CD45.2⁺ recipients that were immunized after 2 h with OVA protein and polyICLC, delivered i.n. On days 0–3, the recipient mice were administered FTY-720 (1 μg/g mouse). The mice were sacrificed on day 4, and the frequency of transferred CD45.1⁺Vα2⁺CD4⁺ T cells was quantified in the med LN, MLN, SILP, and colon. (A) Representative flow cytometry plot comparing the frequency of adoptively transferred CD45.1⁺ CD4⁺ Vα2⁺ OT-II cells between WT mice (top) and FTY-720 administered mice (bottom). (B) Quantification of the number of transferred CD45.1⁺CD4⁺Vα2⁺ OT-II cells in the med LN, MLN, SILP, and colon showing cumulative data from three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (C) Proliferation of the adoptively transferred CD45.1⁺CD4⁺Vα2⁺ T cells is compared between WT and FTY-720 administered mice in the indicated tissues.

mice received 100 μ g of anti- $\alpha 4\beta 7$ antibody delivered i.p. on days 0 and 3, with the other half receiving PBS as a control. As shown in Fig. 5 (c and d), anti- $\alpha 4\beta 7$ antibody administration led to a significant reduction in the migration of CD4⁺ T cells to the small intestinal and colonic lamina propria, as well as the intraepithelial compartments. Conversely, an increase in the percentage of CD45.1⁺V $\alpha 2$ ⁺CD4⁺ T cells was noted in systemic sites such as the blood, spleen, and lung where $\alpha 4\beta 7$ is not required for cellular migration. This demonstrated that i.n. immunization-induced OT-II cell migration to the GI tract was mediated by $\alpha 4\beta 7$.

Lung DC-mediated induction of integrin $\alpha 4\beta 7$ is RA- and TGF- β -mediated

RA has an unequivocal role in inducing $\alpha 4\beta 7$ (Iwata et al., 2004) and CD103⁺ DCs from PPs, and MLNs are capable of metabolizing vitamin A into RA (Johansson-Lindbom et al., 2005). To investigate the mechanisms mediating integrin $\alpha 4\beta 7$ induction by lung DCs, we started by determining the presence of retinal to RA-oxidizing enzymes, aldehyde dehydrogenases (ALDHs) in lung DCs, using a fluorescent ALDH substrate called ALDEFLOUR (Yokota et al., 2009). Both CD103⁺CD11b⁻ and CD103⁻CD11b⁺ lung DC subsets

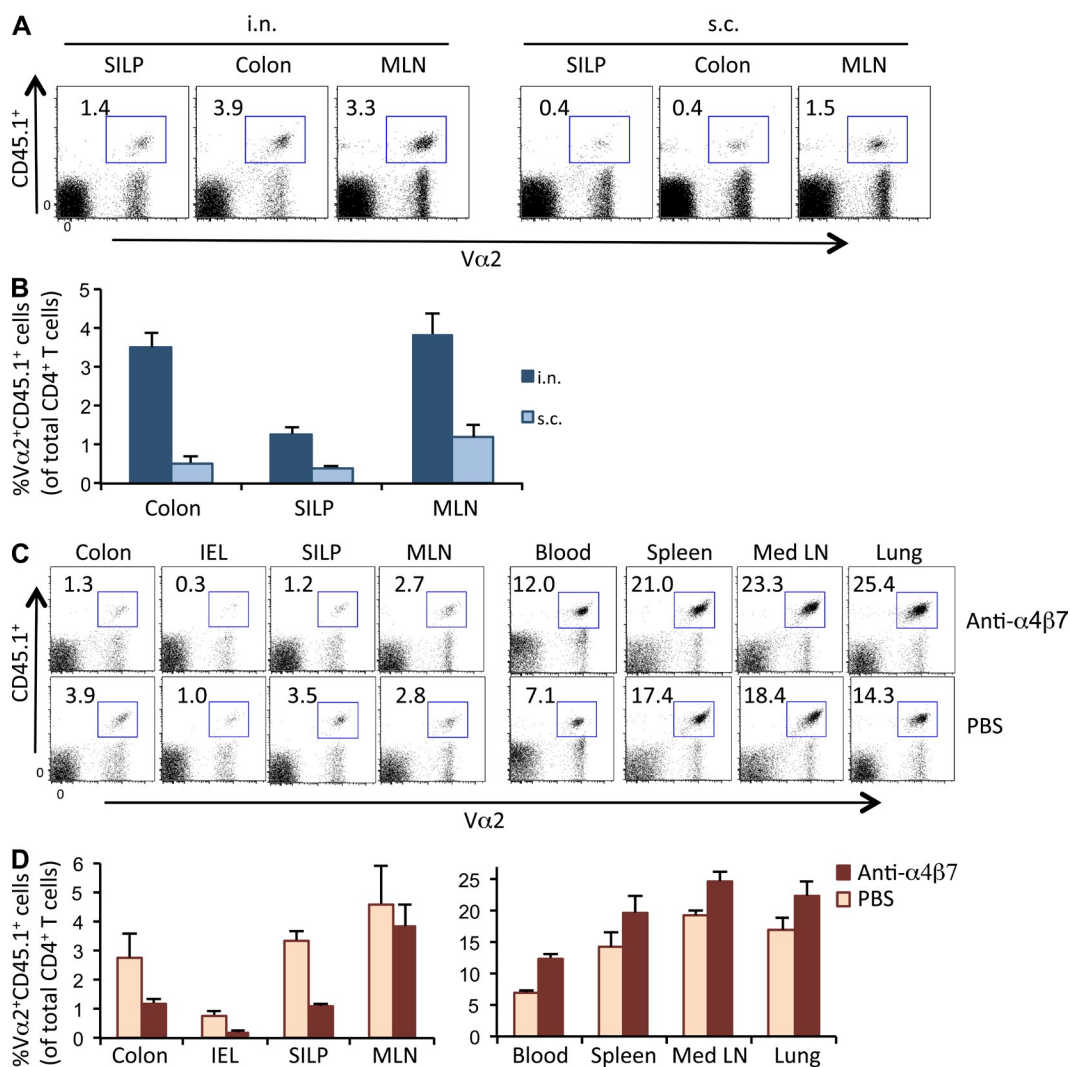


Figure 5. i.n. immunization-induced migration of T cells to the GI tract is integrin $\alpha 4\beta 7$ -dependent and is more efficient than s.c. immunization-induced T cell migration to the GI tract. (A and B) 2 h after adoptive transfer of CFSE-labeled CD45.1⁺V $\alpha 2$ ⁺CD4⁺ OT-II cells to naive CD45.2⁺ mice, the recipient mice were immunized i.n. or s.c. with OVA protein and polyI:CLC. On day 7 after immunization, the frequency of CD45.1⁺V $\alpha 2$ ⁺CD4⁺ T cells was determined in the SILP, colon, and MLN. Representative flow cytometry plots (A) and cumulative data from three independent experiments (B) are shown. (C and D) After 2 h of adoptive transfer of CFSE-labeled CD45.1⁺V $\alpha 2$ ⁺CD4⁺ OT-II cells to naive CD45.2⁺ mice, the recipient mice were immunized i.n. with OVA protein and polyI:CLC. On days 0 and 3, mice received 100 μ g of anti- $\alpha 4\beta 7$ antibody delivered i.p. with the control mice receiving 100 μ l of PBS i.p. On day 7 after immunization, the frequency of CD45.1⁺V $\alpha 2$ ⁺CD4⁺ T cells was determined in the colon, IEL, SILP, MLN, blood, spleen, med LN, and lung. Representative flow cytometry plots (C) and cumulative data from three independent experiments comparing anti- $\alpha 4\beta 7$ and PBS administered mice (D) are shown.

demonstrated the presence of ALDH at levels comparable to MLN CD103⁺CD11b⁻ DCs (Fig. 6 a). This is in concurrence with data generated by the Immgen Consortium (Heng and Painter, 2008) and a similar study by Guilliams et al. (2010), although the ability of lung DCs to induce integrin $\alpha 4\beta 7$ was not examined in either of those studies. Next, we investigated the impact of inhibiting retinoic acid receptor (RAR) signaling

on $\alpha 4\beta 7$ induction. Adding 1 μ M RAR- β inhibitor LE540 to DC/OT-II cultures resulted in a significant reduction of $\alpha 4\beta 7$ expression (Figs. 6, b and c). To test for RAR and TGF- β signaling in vivo, we used a dominant-negative RAR (dnRAR) mouse crossed to OT-II on a RAG background (Rajaii et al., 2008; Pino-Lagos et al., 2011), as well as a TGF- β R^{def} OT-II mouse, respectively (see Materials and methods). WT OT-II

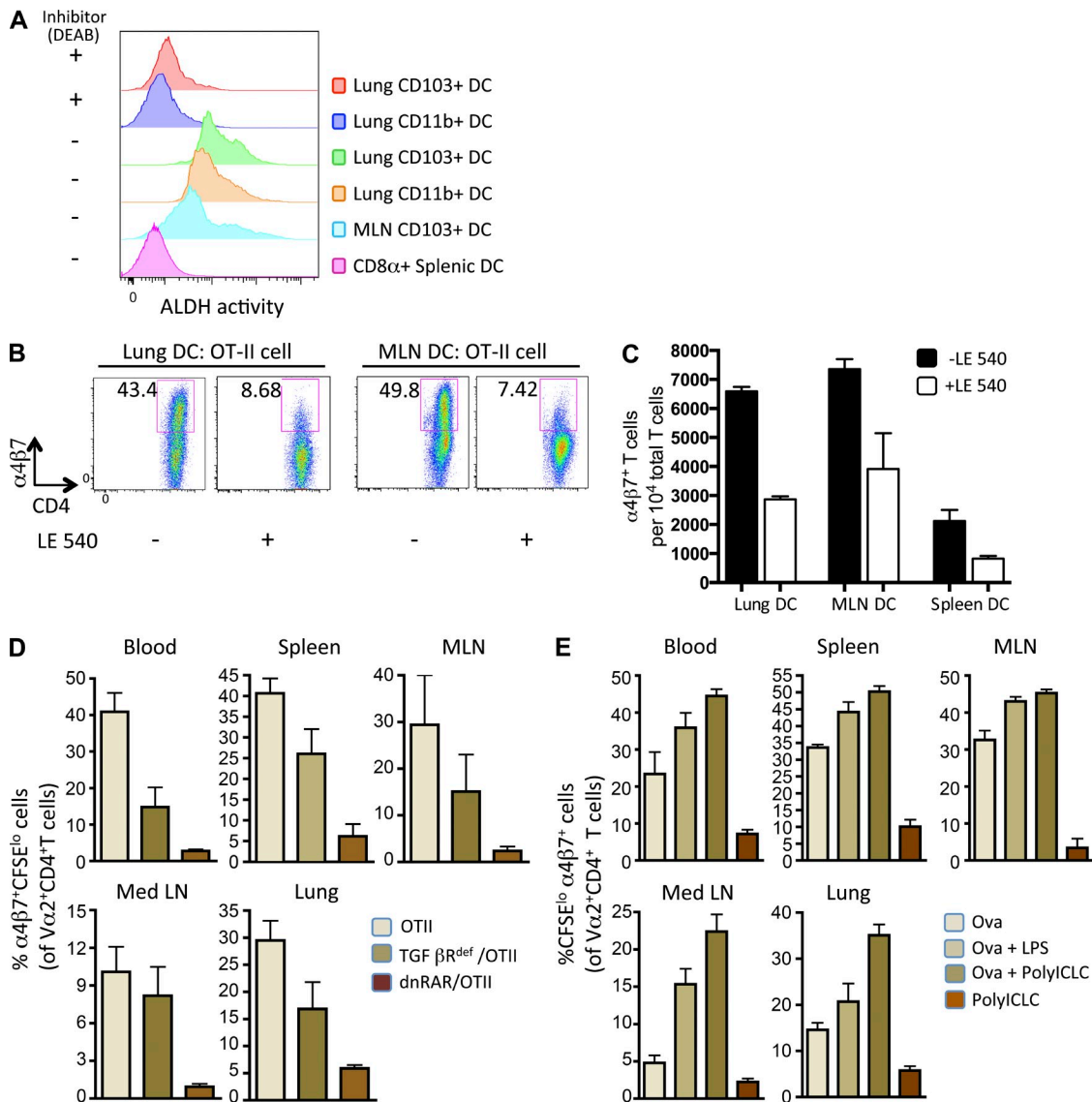


Figure 6. Induction of integrin $\alpha 4\beta 7$ by lung DCs requires RA and TGF- β signaling. (A) Demonstration of ALDH activity in lung-derived DCs using flow cytometry. The MFI of a fluorescent ALDH substrate was quantified for lung DCs in the presence (top two curves) or absence (middle two curves) of the ALDH inhibitor-DEAB. MLN CD103⁺ DCs and splenic CD8 α ⁺ DCs served as the positive and negative controls, respectively (bottom two curves). (B and C) CD11c⁺ DCs isolated from the lung and MLN were pulsed with OVA and cultured with CFSE-labeled OT-II T cells at a ratio of 1:2 for 5–7 d. RAR- β inhibitor, LE540 (1 μ M), or DMSO control is added to the DC/OT-II cultures. (B) Representative flow cytometry plots comparing the expression of $\alpha 4\beta 7$ on CD4⁺OT-II cells with and without RAR- β antagonist LE-540. (C) Quantification of the number of $\alpha 4\beta 7$ ⁺V α 2⁺CD4⁺ T cells induced by the respective DCs, in the absence (black bars) or presence (white bars) of RAR- β antagonist LE-540. (D) WT CD45.1⁺V α 2⁺CD4⁺OT-II cells, dnRAR-OT-II cells, and TGF- β R^{def} OT-II cells (described in the Materials and methods) were transferred into CD45.2⁺ recipient mice and OVA/polyI/CLC was administered i.n. Cumulative data from three experiments comparing the percentage of $\alpha 4\beta 7$ ⁺CFSE^{lo}V α 2⁺CD4⁺ T cells between the WT OT-II, dnRAR OT-II, and TGF- β R^{def} OT-II recipients is shown. (E) After the transfer of CD45.1⁺V α 2⁺CD4⁺OT-II cells, mice were immunized with OVA alone, with OVA + lipopolysaccharide (LPS), with OVA + polyI/CLC, or with polyI/CLC alone. Cumulative data from three experiments comparing the percentage of $\alpha 4\beta 7$ ⁺CFSE^{lo}V α 2⁺CD4⁺ T cells between these mouse groups.

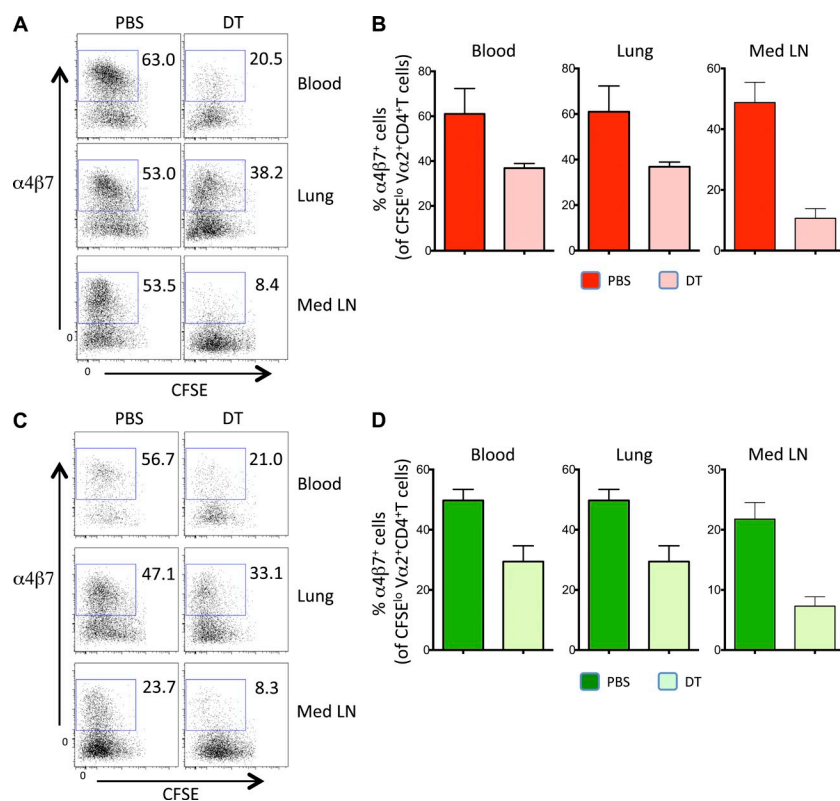


Figure 7. After i.n. immunization, induction of integrin $\alpha4\beta7$ is mediated by DCs. DT was administered to CD11c-DTR chimeras (CD11c-DTR bone marrow into WT mice), or zDC-DTR chimeras (zDC-DTR bone marrow into WT mice) (described in the Materials and methods). 24 h later, we transferred CFSE-labeled CD45.1⁺V α 2⁺CD4⁺ OT-II cells to CD11c-DTR chimeras (A and B) or to zDC-DTR chimeras (C and D). Mice administered PBS served as the respective controls. Representative flow cytometry plots (A and C) and cumulative data from three experiments each (B and D), showing the in vivo induction of integrin $\alpha4\beta7$ on CFSE^{lo}CD45.1⁺V α 2⁺CD4⁺ OT-II cells in CD11c-DTR and zDC-DTR mice, respectively.

cells, dnRAR-OT-II cells, and TGF- β R^{def} OT-II cells were transferred into congenic mice, and OVA/polyICLC was administered i.n. We found that $\alpha4\beta7$ induction was attenuated on TGF- β R^{def}OT-II cells and virtually absent on dnRAR-OT-II cells, suggesting important roles for TGF- β and RAR signaling in lung DC-mediated $\alpha4\beta7$ induction (Fig. 6 d).

Because low levels of integrin $\alpha4\beta7$ can be induced by cellular activation, we examined the role of adjuvant in $\alpha4\beta7$ induction. After CD45.1⁺OT-II cell transfer into naive CD45.2⁺ hosts, the recipient mice were immunized i.n. with OVA alone, OVA/LPS, OVA/polyICLC, and polyICLC alone. PolyICLC alone did not induce $\alpha4\beta7$, whereas OVA alone did, demonstrating the need for antigen presentation. Additionally, OVA/LPS also induced $\alpha4\beta7$, suggesting that $\alpha4\beta7$ induction was not adjuvant specific (Fig. 6 e).

Finally, to test for involvement of polyICLC-induced RIG-I- and TLR3-mediated DC maturation on $\alpha4\beta7$ induction, we transferred CD45.1⁺OT-II cells into WT and MDA-5^{-/-}TLR3^{-/-} hosts (McCartney et al., 2009). No significant difference was noted between the MDA-5^{-/-}TLR3^{-/-} and WT mice (unpublished data). Thus, we show that expression of $\alpha4\beta7$ on T cells after i.n. immunization is not induced in a nonspecific fashion by polyICLC alone.

After i.n. immunization, induction of integrin $\alpha4\beta7$ is mediated by DCs

To test for the role of DCs in the induction of $\alpha4\beta7$ after i.n. immunization, we used two different experimental systems. First, we used CD11c-DTR mice (Jung et al., 2002). CD11c-DTR

chimeras (CD11c-DTR bone marrow into WT mice) were generated (see Materials and methods) to avoid the lethality of DT treatment in CD11c-DTR mice (Zammit et al., 2005). 24 h after diphtheria toxin (DT) administration, we transferred CD45.1⁺ OT-II cells and immunized with OVA/polyICLC. CD11c-DTR mice administered PBS served as controls. Significantly lower levels of $\alpha4\beta7$ were induced on the transferred V α 2⁺CD45.1⁺CD4⁺CFSE^{lo} cells after DT-mediated ablation of DCs (Fig. 7, a and b). Because CD11c is also expressed on various cells, including activated monocytes, macrophages, and plasmacytoid DCs (pDCs), the CD11c-DTR model cannot definitively distinguish the role of classical DCs (cDCs) from activated monocytes and macrophages (Probst et al., 2005; Zammit et al., 2005; Bennett and Clausen, 2007) in $\alpha4\beta7$ induction. To discern the role of lung cDCs in $\alpha4\beta7$ induction, we used the recently described zDC-DTR mice (Meredith et al., 2012a,b). In these mice, a zinc finger transcription factor, *Zbtb46*, which is specific to cDCs, is conditionally ablated, thus distinguishing the role of cDCs from other cell types expressing CD11c. Again, zDC-DTR chimeras (zDC-DTR bone marrow into WT mice) were generated (see Materials and methods) to avoid the lethality of DT treatment in zDC-DTR mice (Meredith et al., 2012a). CD45.1⁺OT-II cells were transferred into zDC-DTR chimeras 24 h after DT ablation, and the mice were immunized with OVA/polyICLC delivered i.n. zDC-DTR mice administered PBS served as controls. Lung DC depletion after DT administration was confirmed (unpublished data). Significantly lower levels of $\alpha4\beta7$ were induced on the transferred

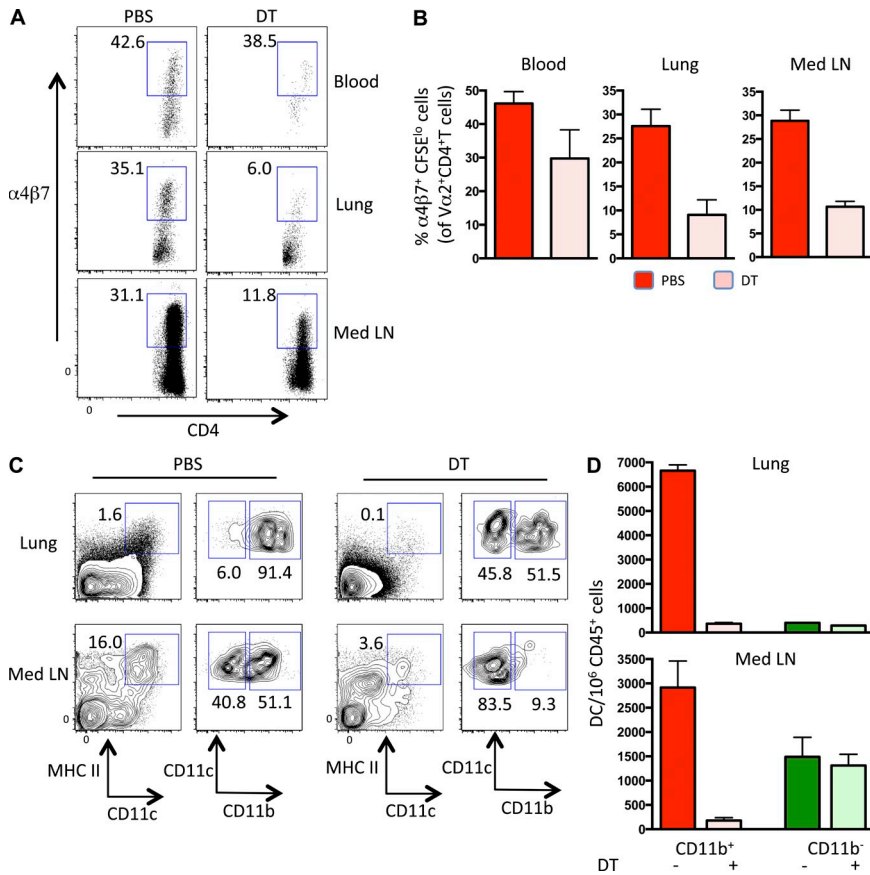


Figure 8. Ablation of CD11b⁺ cells attenuates the induction of $\alpha 4\beta 7$ on transferred OT-II cells after i.n. immunization. Two doses of DT were administered to CD11b-DTR chimeras (CD11b-DTR bone marrow into WT mice; described in the Materials and methods) on days 0 and 1. On day 3 after DT, CFSE-labeled CD45.1⁺V α 2⁺CD4⁺ OT-II cells were transferred and the mice were immunized i.n. with OVA/polyICLC. PBS-administered CD11b-DTR mice served as controls. Representative flow cytometry plots (A) and cumulative data from three independent experiments showing the induction of integrin $\alpha 4\beta 7$ on adoptively transferred CD45.1⁺V α 2⁺CD4⁺ OT-II cells in PBS or DT-administered animals (B). (C) Representative flow cytometry plots comparing the frequency of MHCII⁺CD11c⁺-gated CD11c⁺CD11b⁺ cells in the lung (top) and med LN (bottom) of CD11b-DTR bone marrow chimeric mice administered PBS or DT. (D) Quantification of CD11b⁻ and CD11b⁺ DC in the lung and med LN of CD11b-DTR bone marrow chimeric administered PBS or DT, showing cumulative data from three independent experiments.

V α 2⁺CD45.1⁺CD4⁺CFSE^{lo} cells after DT-mediated ablation of cDCs (Fig. 7, c and d). Thus, using two different methods of DC depletion, we confirmed that lung DCs mediated the induction of integrin $\alpha 4\beta 7$ in vivo.

Ablation of lung CD11b⁺ cells attenuates the induction of $\alpha 4\beta 7$, whereas depletion of langerin⁺ and Batf-dependent DCs does not

Contrary to the MLN, where only CD103⁺ DCs (and not CD11b⁺ DCs) up-regulate gut-homing phenotype (Johansson-Lindbom et al., 2005), we have found that both CD103⁺ and CD11b⁺ lung DC subsets express ALDH (Fig. 6) and that both lung DC subsets up-regulated $\alpha 4\beta 7$ and CCR9 in vitro (Fig. 1). Here, we wanted to test the effect of ablating specific lung DC populations on the induction of $\alpha 4\beta 7$ in vivo. To deplete CD11b⁺ lung DCs, we used CD11b-DTR mice (Duffield et al., 2005). CD11b-DTR chimeras were created (CD11b-DTR bone marrow into WT mice). Two doses of DT (25 ng/g) were administered on days 0 and 1. On day 3, CD45.1⁺ OT-II cells were adoptively transferred, and the mice were immunized with OVA and polyICLC. 4 d later, we examined the transferred cells for $\alpha 4\beta 7$ induction. CD11b-DTR chimera that received PBS instead of DT served as controls. As shown in Fig. 8 (a and b), the $\alpha 4\beta 7$ level

on transferred V α 2⁺CD45.1⁺CD4⁺CFSE^{lo} cells in the blood, lung and mediastinal LN were significantly lower in the DT injected mice compared with mice that received PBS. Additionally, we examined the transferred CD45.1⁺ T cells in the spleen and MLN of recipient mice and observed similar attenuation of $\alpha 4\beta 7$ induction (unpublished data). We tested multiple doses of DT and found that two doses of 25 ng/g mouse, 1 d apart, were optimal in effecting depletion of CD11b⁺ lung DCs and mediastinal LN DCs (Fig. 8, c and d). One dose of DT resulted in monocyte depletion in the blood, but not in lung tissue, and three doses of DT were lethal after i.n. administration of PolyICLC (unpublished data).

To examine the effect of depleting CD103⁺ lung DCs, we used two systems: temporal (*Langerin DTR*; Kissenpfennig et al., 2005) and constitutive (*Batf3*^{-/-}; Hildner et al., 2008). In the *Langerin DTR* mice, 1 d after administration of DT, CD45.1⁺OT-II cells were adoptively transferred and the mice were immunized with OVA and polyICLC. 4 d later, we examined the transferred cells for $\alpha 4\beta 7$ induction. *Langerin DTR* mice administered PBS served as controls. There was no difference in the levels of $\alpha 4\beta 7$ between PBS- and DT-treated mice (Fig. 9, a and b). Next, we compared *Batf3*^{-/-} and WT mice. Again, after adoptive transfer of congenic OT-II cells and immunization with OVA/PolyICLC, no difference was observed between the levels of $\alpha 4\beta 7$ induced on the adoptively

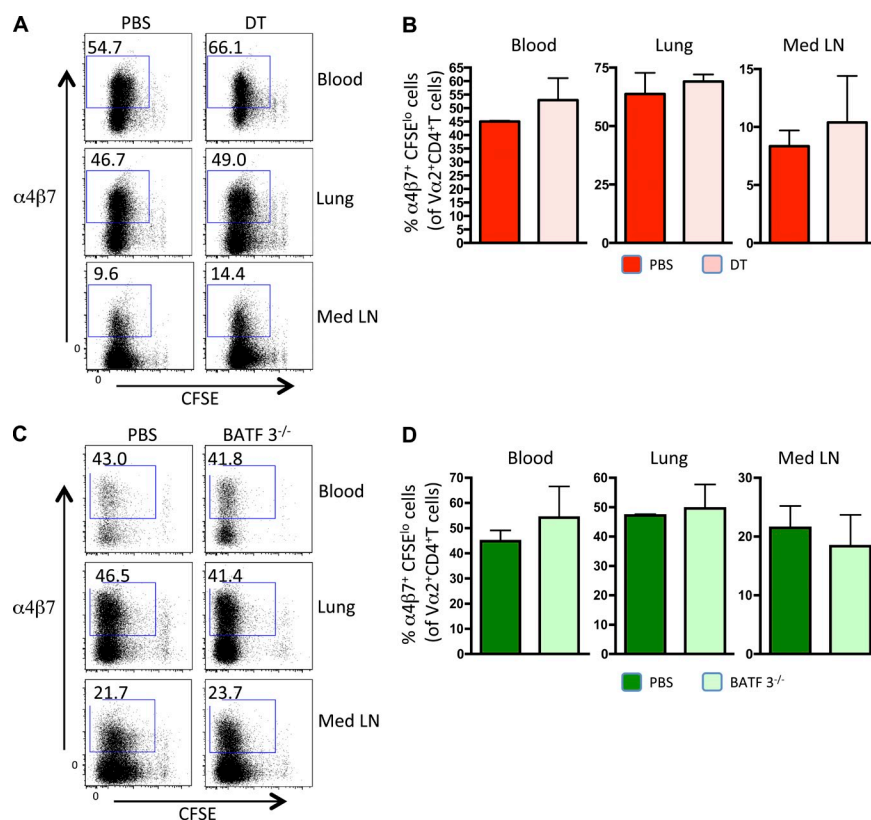


Figure 9. Ablation of CD103⁺ DCs does not impact the induction of $\alpha 4\beta 7$ on transferred OT-II cells after i.n. immunization. (A and B) DT or PBS was administration to *langerin*-DTR mice (described in the Materials and methods). 24 h later CFSE-labeled CD45.1⁺V α 2⁺CD4⁺ OT-II cells were transferred and the mice were immunized with OVA/polyI/CLC. Representative flow cytometry plots (A) and cumulative data from three experiments (B) are shown, comparing the expression of $\alpha 4\beta 7$ on CFSE^{lo} CD45.1⁺V α 2⁺CD4⁺ OT-II cells. (C and D) CD45.1⁺V α 2⁺CD4⁺ OT-II cells were transferred to WT or *batf3*^{-/-} mice, followed by immunized with OVA/polyI/CLC, delivered i.n. Representative flow cytometry plots (C) and cumulative data from three experiments (D) are shown, comparing the expression of $\alpha 4\beta 7$ on CFSE^{lo} CD45.1⁺V α 2⁺CD4⁺ OT-II cells.

transferred cells (Fig. 9, c and d). Together, these data suggest that in vivo, lung CD11b⁺ DC subsets are more proficient in inducing $\alpha 4\beta 7$ than the CD103⁺ DCs.

Lung DCs induce protective immunity in the GI tract

Having observed that lung DCs can license T cells to migrate to the GI tract, we wanted to examine whether protective gut immunity can be induced after targeting of lung DCs by i.n. immunization. We first transferred CD45.1⁺ OT-II cells into naive mice and immunized i.n. or s.c., with unimmunized mice serving as controls. After gavage of pathogenic *Salmonella typhimurium* expressing OVA, all the unimmunized or s.c.-immunized mice died between days 6–9. In contrast, survival in the i.n. immunized mice was prolonged by 2–3 d (unpublished data). Transferred V α 2⁺CD45.1⁺CD4⁺ T cells were enriched in the small and large intestinal lamina propria and intraepithelial compartments in the i.n.-immunized mice compared with the s.c.-immunized animals (unpublished data). Having observed that i.n. immunization extended the survival of *Salmonella*-challenged animals in the transgenic OVA TCR model, we asked whether a protective immune response could be generated from the endogenous immune repertoire. WT mice were immunized with OVA/polyI/CLC (100 μ g and 50 μ g, respectively) delivered i.n. or s.c. in a prime-boost fashion or p.o. with OVA/Cholera Toxin (50 mg/ 2 μ g; a booster dose of the vaccine was given 4 wk after priming),

and challenged orally with *Salmonella*-OVA. Survival was significantly greater in the i.n.-immunized mice compared with unimmunized mice, with 36% of the mice demonstrating long term survival (>40 d, $P < 0.05$; Fig. 10 a). Pathological examination of the liver, spleen, and MLN demonstrated significant disruption of hepatic, splenic, and LN architecture, vasculitis, coagulative necrosis, and intravascular thrombi in the challenged, unimmunized mice compared with WT mice, similar to previous studies (Jones and Falkow, 1996; Griffin and McSorley, 2011). In contrast, the liver, spleen, and MLN of i.n.-immunized mice were indistinguishable from WT mice (Fig. 10 b). This is also reflected in the inflammation score (Wu et al., 2005) for each of the vaccinated groups (Fig. 10 c). Stool cultures showed a significantly higher level of *Salmonella* in the stool of i.n. immunized mice on days 2 and 4 after challenge (unpublished data), perhaps because of luminal sequestration of the bacteria. Interestingly, the i.n.-immunized mice showing long term survival continued to demonstrate detectable levels of *Salmonella* in the stool even at day 40 (unpublished data). Finally, we also immunized naive C57BL/6 mice with heat and paraformaldehyde inactivated *Salmonella typhimurium*, delivered i.n. or s.c. Significantly lower levels of *Salmonella* CFUs were observed in the spleen of i.n.-immunized mice compared with unimmunized mice (Fig. 10 d). Thus, using three different models of protection against pathogenic *Salmonella*, we found that i.n. immunization extends survival,

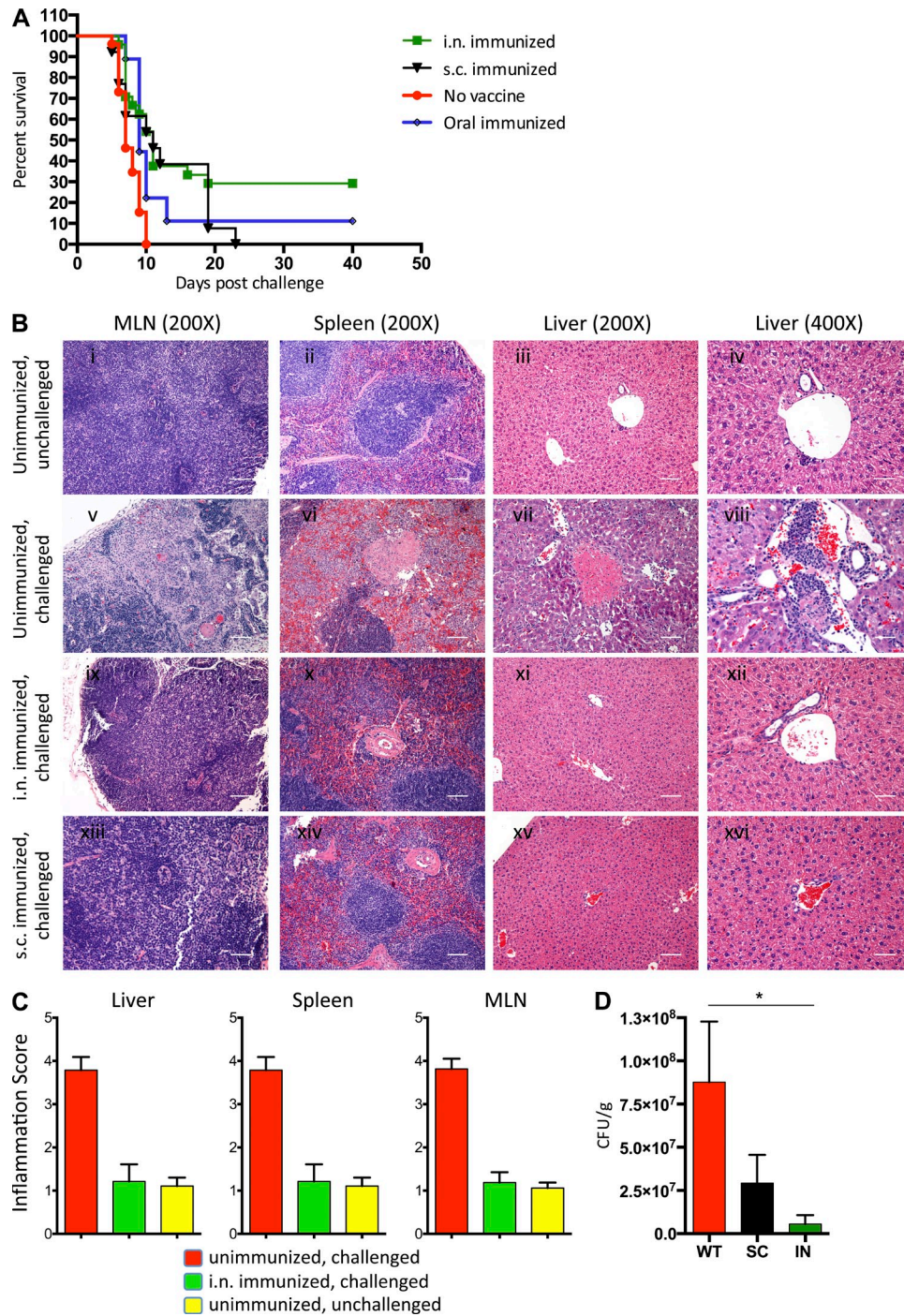


Figure 10. i.n. immunization protects against enteric challenge with highly pathogenic *Salmonella*. (A–C) C56Bl/6 mice were immunized with OVA/polyI/CLC delivered i.n. or s.c. in a prime-boost fashion or p.o. with OVA/CT, and challenged orally 7 d after the boost with *Salmonella*-OVA. Unimmunized mice challenged with *Salmonella*-OVA served as controls. Data from three independent experiments is presented here. (A) Kaplan-Meier curves comparing the survival of unimmunized (red) mice or mice immunized i.n. (green), s.c. (black), or p.o. (blue) against oral challenge with *Salmonella*-OVA (10⁶PFU). (B) Hematoxylin and eosin sections examining the MLN (i, v, ix, and xiii), spleen (ii, vi, x, and xiv), and liver (iii, vii, xi, and xv) at 200× magnification (bar, 100 μm), and liver (iv, viii, xii, and xvi) at 400× magnification (bar, 50 μm) from a WT (unimmunized, unchallenged) mouse (i–iv), an unimmunized, *Salmonella*-challenged mouse (v–viii), a representative i.n.-immunized, *Salmonella*-challenged mouse #1 (ix–xii), and a representative i.n.-immunized, *Salmonella*-challenged mouse #2 (xiii–xvi). (C) Cumulative data from three experiments showing the inflammation score from the WT (unimmunized, unchallenged), unimmunized, and *Salmonella*-challenged groups. (D) C56Bl/6 mice were immunized with heat and paraformaldehyde-inactivated *S. typhimurium* delivered i.n. or s.c. in a prime-boost fashion, and challenged orally 7 d after the boost with *Salmonella*-OVA. Unimmunized mice challenged with *Salmonella*-OVA served as controls. CFU of *S. typhimurium* per gram of splenic tissue is compared between the WT, s.c.-immunized, and i.n.-immunized mice.

and is associated with a striking absence of pathology in the liver and spleen in the immunized compared with unimmunized mice.

DISCUSSION

In this study, we propose a novel pathway of lung DC-mediated T cell migration to the GI lamina propria via induction of gut-homing integrin $\alpha 4\beta 7$. Several lines of evidence pointed us in this direction. First, lungs harbor extrahepatic stores of vitamin A (Okabe et al., 1984; Dirami et al., 2004). Second, lungs are anatomically juxtaposed to the liver (the largest store of vitamin A in the body), directly receiving hepatic blood. Third, lung DCs express the enzyme RALDH (Guilliams et al., 2010) and are capable of oxidizing Vitamin A into RA. Thus, lung DCs have access to the necessary substrate (Vitamin A) and are equipped with the enzymatic machinery required to metabolize it and generate RA. These facts led us to hypothesize that lung DCs may up-regulate integrin $\alpha 4\beta 7$ and induce a gut-homing phenotype on T cells.

We have used multiple experimental approaches to test our hypothesis. First, lung DCs co-cultured with OVA-specific OT-II cells in the presence of OVA express integrin $\alpha 4\beta 7$ at levels comparable to those induced by MLN CD103⁺ DCs. Second, integrin $\alpha 4\beta 7$ is induced on adoptively transferred OT-II cells after i.n. immunization. Third, T cells can be tracked to the GI lamina propria after i.n. immunization and fourth, T cell migration can be blocked by anti- $\alpha 4\beta 7$ antibody, causing the transferred T cells to accumulate in systemic compartments.

It may be argued that i.n. immunization may lead to inadvertent swallowing of antigen and targeting of GI-resident DCs that are known to induce gut migration of T and B cells. To exclude this possibility, we first used a time course experiment to identify the populations targeted by i.n. delivery of fluorescent, DC-specific antibodies. We could readily detect targeting of mediastinal LN- and lung-resident DCs, but not GI-resident DCs. Because this does not exclude migration of antigen to the GI tract, we performed experiments to block the egress of T cells from LNs using FTY-720. In doing so, we observed an accumulation of transferred cells in the mediastinal LNs and, in contrast, a drop in transferred cell frequency in MLNs in mice given FTY-720, thereby confirming that i.n. immunization-mediated expansion of transferred cells was occurring in the former and not the latter lymphoid sites. Finally, we observed early (day 2) proliferation of transferred cells within the mediastinal LNs and the appearance of integrin $\alpha 4\beta 7$ on mediastinal LN- and lung-resident cells (day 3), followed by the migration of transferred cells to GI compartments (day 4), which could be blocked with an anti- $\alpha 4\beta 7$ antibody. These data extend the work of Ciabattini et al. (2011), who recently demonstrated that after i.n. immunization, antigen-specific T cells trafficked to distal skin-draining LNs in a CD62L-dependent fashion and to the MLNs in an $\alpha 4\beta 7$ -dependent fashion. However, unlike in the present study, mechanisms of induction of $\alpha 4\beta 7$, i.e., the role of lung

DCs or the physiological relevance of these pathways was not explored by Ciabattini et al. (2011).

It is interesting to note that whereas migration to GI sites was blocked using an anti- $\alpha 4\beta 7$ antibody, migration to the systemic sites was not. In fact, there was a nonsignificant increase in the frequency of transferred cells in other sites examined, including the lungs. This lends specificity to our experimental approach, as migration of T cells to the lungs has been shown to be independent of $\alpha 4\beta 7$ /MAdCAM but dependent on L-Selectin/PNAd, $\alpha 4\beta 1$ /VCAM-1 and LFA-1 (Xu et al., 2003).

To examine the role of lung DCs in the *in vivo* induction of integrin $\alpha 4\beta 7$, we used two different conditional knock-out models. Use of the CD11c DTR leads to the depletion of 85–90% of CD11c⁺ DCs (Bennett and Clausen, 2007). However, because this model cannot distinguish between classical DCs, activated monocytes, and alveolar macrophages, all of which express CD11c and are therefore depleted (Probst et al., 2005; Zammit et al., 2005), we also used the newly described zDC-DTR model (Meredith et al., 2012a,b). Significantly attenuated numbers of transferred congenic cells expressing $\alpha 4\beta 7$ were seen in both models, demonstrating that lung DCs induced integrin $\alpha 4\beta 7$ after i.n. vaccination. These results need to be interpreted with the caveat that both DT-induced depletion of DCs and nasal administration of polyICLC are likely associated with lung inflammation (Tittel et al., 2012). We speculate that local inflammation is responsible for the variability observed in the levels of $\alpha 4\beta 7$ expressed on transferred cells isolated from various compartments, the expression being high on cells isolated from the lungs compared with the cells isolated from the mediastinal LN and blood, or from the spleen and MLN (unpublished data), where a more profound attenuation of $\alpha 4\beta 7$ was seen.

Thus, based on the data generated by different experimental approaches, we propose that after i.n. immunization, lung and mediastinal LN-resident DCs are targeted which in turn induce the expression of integrin $\alpha 4\beta 7$ on T cells, resulting in their localization to the GI tract. Herein, we provide novel functional evidence of mucosal cross talk mediated by DCs. This provides a mechanistic insight to the observation of T cell responses in the GI lamina propria after i.n. challenge (Esplugues et al., 2011; Masopust et al., 2010) or after the appearance of antigen-specific T cells in the MLN after i.n. immunization (Ciabattini et al., 2011).

Although the mechanisms underlying $\alpha 4\beta 7$ induction appear to be conserved between lung- and GI-resident DCs, the DC subsets involved show striking differences. For example, consistent with studies of $\alpha 4\beta 7$ induction by gut DCs (Iwata et al., 2004; Kang et al., 2011), our results show that RA and TGF- β are required in the lung DC-mediated induction of $\alpha 4\beta 7$. Notably, in addition to an *in vitro* culture system, we have used novel, dominant-negative RAR-OTII Rag^{-/-} mice and TGF- β receptor^{fl/fl}-CD4^{CRE} OT-II Rag^{-/-} mice to address these questions. Splenic OT-II cells from the dominant-negative RAR-OTII Rag^{-/-} mice and TGF- β receptor^{fl/fl}-CD4^{CRE} OT-II Rag^{-/-} mice were used as donor cells, so local

lung DC subsets in these mice are not relevant here. However, these mice will be described in detail in future studies.

In contrast to the existing literature showing that CD103⁺ MLN DCs induce integrin $\alpha 4\beta 7$, whereas the CD11b⁺ MLN DCs do not, we have found that both CD103⁺ and CD11b⁺ lung DC populations induced integrin $\alpha 4\beta 7$, the expression being higher in the lung CD11b⁺ DC induced co-cultures in vitro. Because these differences were novel and unexpected, we examined them by using in vivo DC depletion models. To deplete the CD103⁺ DC populations, we used *Langerin DTR* (Bennett et al., 2005) mice and could not detect any significant differences in the level of $\alpha 4\beta 7$ induction between the langerin depleted and replete mice. To further confirm the effect of CD103⁺ DC depletion, we used the *Batf3*^{-/-} mice, in which the deletion of the transcription factor *Batf3* results in ablation of cross presenting, CD103⁺ tissue DCs (Hildner et al., 2008). Again, no significant differences in the induction of $\alpha 4\beta 7$ were observed between WT and *Batf3*^{-/-} animals. Finally, to deplete the CD11b⁺ lung DCs, we used the CD11b DTR mice. Both CD11b-expressing alveolar macrophages and DCs are depleted in this model; however, the use of zDC-DTR demonstrates the role of classical DCs. Attenuated levels of $\alpha 4\beta 7$ were observed in the mice in which CD11b-expressing cells were depleted. It may be noted that although the dose of DT administered to the Langerin DTR and CD11b DTR mice was different, we were guided by previous studies on the dose required to deplete the respective DC subsets (Duffield et al., 2005; Kissenpfennig et al., 2005). Additionally, in this study, we confirmed the depletion of respective lung DC populations. Therefore, in contrast with MLN DCs, both CD103⁺ and CD11b⁺ lung DC populations induced integrin $\alpha 4\beta 7$ in vitro, although in vivo, the CD11b⁺ populations appeared to have a more dominant effect on the induction of $\alpha 4\beta 7$. Furthermore, within the heterogeneous CD11b⁺ population, we observed a striking difference between the CD24⁺ and CD64⁺ subsets, where the CD24⁺CD11b⁺ DCs induced robust levels of $\alpha 4\beta 7$ and CCR9, whereas CD64⁺CD11b⁺ cells did not. We speculate that whereas the migratory gut CD103⁺ DCs use dietary and biliary sources of vitamin A to generate RA (Jaensson-Gyllenbäck et al., 2011), the lung DCs either use hematogenous vitamin A, released from hepatic stores, or local stores of vitamin A (Dirami et al., 2004; Okabe et al., 1984). Thus, the predominance of CD103⁺ DC in mediating gut-homing, as in the case of MLN, is not observed in case of lung DCs. This further allows us to speculate that specific DC populations, i.e., CD103⁺ DCs, may not be preconditioned to metabolize vitamin A but rather, they may acquire this property based on local environmental factors like the availability of vitamin A, TLR ligands, or other inflammatory stimuli. Detailed mechanistic studies are underway to further address this question, including a study of the relative expression of RALDH and the effect of adjuvants on RALDH expression by lung DC subsets.

Integrins may be induced via nonspecific stimuli, such as cellular activation (Laudanna et al., 2002). Because our in vivo experimental system involved use of the TLR-3 agonist, poly-ICLC, we tested its effect on the induction of integrin $\alpha 4\beta 7$.

Initially, we used polyICLC alone (without antigen) but could not observe any significant induction of $\alpha 4\beta 7$, whereas OVA alone induced $\alpha 4\beta 7$ on transferred OTII cells. In addition, using the MDA5^{-/-}TLR3^{-/-} mice, we were able to confirm that i.n. immunization-induced $\alpha 4\beta 7$ was not a nonspecific effect of adjuvant. Additionally, we have tested the use of different adjuvants in the induction of $\alpha 4\beta 7$ after i.n. immunization and observed no significant differences between them (unpublished data). Combined, we speculate that in the right cellular context, i.e., in the presence of local availability of RA and TGF- β , the use of an adjuvant amplifies the induction of $\alpha 4\beta 7$ because of DC and T cell activation, as well as DC migration.

Having shown that lung DCs induce $\alpha 4\beta 7$ and induce cellular migration to the GI tract, we wanted to study if pathogen-specific intestinal immunity can be induced by targeting lung DCs. We chose *Salmonella typhimurium*, a pathogen of global significance (Levine, 2006) that is transmitted across the intestinal mucosa and causes a spectrum of diseases ranging from localized intestinal infection to severe systemic illness (Griffin and McSorley, 2011). Notably, although *Salmonella* is not a lung pathogen, we used this model as a tool to examine i.n. vaccine induced protection against *Salmonella* invasion across the GI tract. To confirm protection against enteric challenge with *Salmonella*, we have used three different experimental systems: (1) passive immunization using transferred OT-II cells followed by i.n. immunization, where we demonstrated a modest increase in survival; (2) active prime-boost immunization with OVA showing a significantly increased survival and lack of systemic pathology in the i.n.-immunized mice; and (3) active prime-boost immunization using inactivated *Salmonella* showing reduced systemic burden in the i.n.-immunized mice. Thus, using multiple experimental systems, we were able to show that lung DCs targeted by i.n. immunization are able to induce protective immunity within the GI tract. Notably, in mice that received s.c. vaccine, a nonsignificant increase in survival was also observed. We hypothesize this to be due to the systemic immunity generated by s.c. vaccine and plan to study this further with the use of IgA-deficient, CD103^{-/-} and B7^{-/-} mice.

To conclude, in this manuscript we challenge the dogma that only CD103⁺ gut-resident DCs can recruit T cells to the GI tract. Rather, we provide compelling evidence that lung DCs targeted by i.n. immunization are capable of inducing robust levels of gut-homing integrin $\alpha 4\beta 7$ and licensing T cells to migrate to the GI tract. Pathways of recruitment of antigen-specific immune cells to the gut are therefore much more promiscuous than previously appreciated. There appears to be considerable, albeit hitherto unrecognized, DC-orchestrated, mucosal cross talk that can inform the rational design of novel vaccines.

MATERIALS AND METHODS

Mice. C57BL/6 (B6), C57BL/6-Tg(Tcr α Tcr β)425Cbn/J (OT-II), and C57BL/6-Tg(Tcr α Tcr β)1100MjbJ (OT-I) mice were purchased from Taconic Farms or bred at The Rockefeller University. 6–8-wk-old CD11c-DTR

(B6.FVB-Tg(Igax-DTR/EGFP)57Lan/J) mice were purchased from The Jackson Laboratory. CD4^{CRE}TGF- β R^{fl/fl} OT-II mice and the dnRAR-OT-II mice were provided by the Mucida Laboratory (The Rockefeller University, New York, NY). In brief, CD4^{CRE} mice were crossed with TGF- β R^{fl/fl} mice (provided to the Mucida laboratory by the National Cancer Institute, Rockville MD) to specifically deplete TGF- β R from CD4 T cells. To avoid autoimmune disease and premature death in CD4^{CRE} x TGF- β R^{fl/fl} mice, these animals were backcrossed to OT-II Rag^{-/-} animals to obtain TGF- β R^{def} OT-II (TGF- β R^{def}) mice (these mice will be described elsewhere). To analyze RA signaling in CD4⁺ T cells, CD4^{CRE} mice were crossed with dnRAR^{fl/fl} mice (Pino-Lagos et al., 2011; Rajaii et al., 2008) to specifically express the dominant-negative form of RAR in CD4⁺ T cells. To address the importance of RA for mucosal T cell migration after specific T cell activation, these animals were backcrossed to an OT-II Rag^{-/-} background. MDA-5^{-/-}TLR3^{-/-} mice (McCartney et al., 2009) were provided by M. Colonna (Washington University, St. Louis, MO).

Bone marrow chimeras were created by transferring bone marrow from CD11c-DTR, zDC-DTR, and CD11b DTR mice into lethally irradiated WT C57BL/6 mice to avoid lethality associated with DT treatments of CD11c-DTR (Jung et al., 2002; Zammit et al., 2005), zDC-DTR (Meredith et al., 2012a), or CD11b DTR. In brief, C57BL/6 hosts underwent two treatments of 500 rads in an animal γ -irradiator 3-h apart, and 2.5×10^6 bone marrow cells from CD11c-DTR, zDC-DTR, and CD11b-DTR donor, respectively, were transferred i.v. The resulting mice (referred to herein as CD11c-DTR, zDC-DTR, and CD11b-DTR) were housed under immunocompromised mouse conditions and treated regularly with 2 mg/ml neomycin from Sigma-Aldrich in their drinking water. The chimeric mice were fully reconstituted and ready for experimental use after 6 wk.

Mice were maintained in specific pathogen-free conditions. Protocols were approved by the The Rockefeller University Animal Care and Use Committee.

Antibodies. The following reagents were purchased from BD: anti-CD3 (500A2), anti-CD3 (145-2011), anti-CD103 (M290), anti-CD4 (RM4-5), and anti-CD8 (53-6.9). The following reagents were purchased from eBioscience: anti-MHCII (M5/114.15.2), anti-IFN- γ (XMG1.2), anti-CD4 (RM4-5), and anti-CD8 (53-6.9). The following antibodies were obtained from BioLegend: anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 anti-CD11c (N418), anti-CD11b (M1/70), anti-IFN- γ (XMG1.2), anti-CD103 (M290), anti-LPAM-1 (DATK32), anti-CD24 (M1/6a), anti-CCR9 (CW12), anti-CD64 (X54-51711), and anti-TCRV α 2 (B20.1). AQUA (L34957) was obtained from Invitrogen, and CFSE was purchased from Sigma. Anti-CD205 (NLDCC) was produced locally, as previously described (Trumpfheller et al., 2006).

Adoptive cell transfer. OT-I or OT-II mice were used as cell donors for adoptive transfer into syngeneic recipient animals. Lymphocytes were isolated from the spleen and labeled with 5 μ M CFSE (Invitrogen) for 10 min at 37°C. After washing twice with PBS containing 3% FCS, 7×10^6 cells per mouse were injected via the retroorbital venous plexus.

Immunization. CFSE-labeled OT-I or OT-II mice were adoptively transferred into syngeneic age- and gender-matched WT mice and controls. 2 h later, the recipient mice were immunized i.n., i.t., or s.c. with 100 μ g OVA (LPS-free; Seikagaku Corp.) and 50 μ g of poly ICLC (Oncovir, Inc.) using Isoflurane as a short-acting inhalant anesthetic. Additionally, recipient mice were immunized p.o. with 50 mg of OVA (Grade III; Sigma-Aldrich) and 2 μ g of cholera toxin (Sigma-Aldrich). All animals were given food and water ad libitum.

Tissue harvesting and cell preparation. Mice were euthanized and blood was obtained from the experimental mice by cardiac puncture. Lung mononuclear cell isolation was performed as previously described (Vermaelen et al., 2001). In brief, after sacrificing the animals, the pulmonary circulation was perfused with saline to remove the intravascular pool of cells. Lungs were carefully separated from thymic and cardiovascular remnants and removed in

toto. Organs were thoroughly minced using iridectomy scissors and incubated for 60 min in digestion medium containing collagenase D in a humidified incubator at 37°C and 5% CO₂. In the last 5 min, 10 mM EDTA was added. Tissue fragments were disrupted mechanically by pipetting and passed through a 70- μ m cell strainer. Mononuclear cells were washed twice in RPMI before use in subsequent experiments as described below.

The spleen and LN were harvested in RPMI media with 5% FCS, mashed between two sterile glass slides, washed with RPMI complete media, and then filtered through a 70- μ m filter. Spleen but not LN samples went through subsequent ACK lysis, were washed twice, and counted. LN single-cell suspensions were prepared by incubation with 400 U/ml collagenase D at 37°C for 30 min.

Intestinal lymphocytes were isolated and prepared as previously described (Mucida et al., 2007). In brief, small and large intestines were removed and placed in cold HBSS media containing 5% FCS. The intestines were carefully cleaned from the mesentery and flushed of fecal content. Intestines were opened longitudinally and then cut into 1-cm pieces. The intestinal tissue was incubated with 1.3 mM EDTA (Cellgro) in HBSS at 37°C for 1 h. The supernatants containing intestinal epithelial cell (IEC) with some superficial villi cells, referred to as the "IEC fraction," were collected and layered over a discontinuous 40/80% Percoll gradient, and centrifuged at 2,800 rpm for 30 min. Cells from the 40/80% interface were collected, washed, and resuspended in complete RPMI media. These purified cells constituted the intraepithelial lymphocyte (IEL) population. To isolate the lamina propria lymphocytes (LPL), the remaining intestinal tissue was minced and transferred to conical tubes. The minced pieces were resuspended in 20 ml of complete RPMI containing 0.125 mg/ml of collagenase (Sigma-Aldrich) and shaken at 200 rpm for 50 min at 37°C. The tissue suspension was collected and passed through a 70- μ m cell strainer and the cells were pelleted by centrifugation at 1,200 rpm. The cells were then resuspended and layered onto a 40/80% Percoll gradient, centrifuged, and processed as described above for the IEL preparation.

DC isolation. Single-cell suspensions were isolated from the murine lung, spleen, mesenteric, mediastinal, and skin-draining LNs as described above. CD11c⁺ cells were isolated as previously described (Ing et al., 2006) using CD11c magnetic beads (Miltenyi Biotec). In brief, the cells were washed in 1 ml of MACS buffer (Miltenyi Biotec), before 30-min incubation on ice with CD11c⁺ beads. The cellular suspensions were washed twice in MACS buffer and passed through a magnetic column. CD11c⁺ cells were isolated by positive selection, washed, resuspended in complete RPMI medium, and counted before co-culture with T cells, as described in the following section.

Lung DC subset isolation. Lung mononuclear cells were isolated as described above. Using magnetic beads, CD11c⁺ cells were enriched and sorted on FACSaria (BD) as B220⁻MHC II⁺CD11c^{hi}CD103⁺CD11b⁻ and B220⁻MHC II⁺CD11c^{hi}CD103⁻CD11b⁺ subsets.

In vitro DC/T cell co-cultures. The culture medium used for DC/T cell cultures was RPMI (Invitrogen) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5 mM β -mercaptoethanol (all from Sigma-Aldrich). Allogeneic T cells were isolated from spleens of C57BL/6 mice by excluding B220⁺, F4/80⁺, CD49b⁺, and I-Ab⁺ cells with anti-rat IgG Dynabeads (Invitrogen). T cells were added to round-bottom microtest wells at 10⁵/well and mixed with isolated DCs at a DC/T cell ratio of 1:2, 1:5, and 1:10 in the presence of OVA (25 μ g/ml final). After 5 d, the expression of α 4 β 7 on proliferating T cells was evaluated by FACS. For the retinoic acid inhibition assays, the DC/T cell co-cultures were incubated with 1 μ M of RAR- β antagonist, LE540 (Wako; dissolved in DMSO at a stock concentration of 1 mM), and added to cultures at final concentration of 1 μ M.

Flow cytometric analyses, intracellular staining and gating schema. Cells were isolated as described in the preceding section. Before staining, cells were washed and resuspended in staining buffer containing 1 \times PBS, 2% BSA, 10 mM EDTA, and 0.01% NaN₃. To block nonspecific staining,

the 2.4G2 anti-CD16/32 antibody was added. Antibodies for cell surface markers were added and cells were incubated for 25 min at 4°C. After the staining, the cells were washed twice and analyzed immediately or fixed in PBS containing 1% paraformaldehyde and 0.01% NaN₃, and analyzed later on an LSR II (BD) using multiparameter flow cytometry.

For intracellular cytokine staining, after surface staining, cells were resuspended in Fixation/Permeabilization solution (Cytotfix/Cytoperm kit; BD), and intracellular cytokine staining was performed according to the manufacturer's protocol. Flow cytometric data were analyzed with FlowJo software (Tree Star). For analysis of the adoptively transferred OT-I and OT-II populations, the transferred cells were identified by forward and side scatter characteristics and dead cells were excluded using Live-dead fixable Aqua (Invitrogen), followed by sequential gating on CD45.1⁺, CD3⁺, CD4⁺Vα2⁺ double-positive populations followed by the expression of α4β7 on CFSE^{lo} cells.

ALDEFLUOR assay. ALDH activity was determined using the ALDEFLUOR staining kit (STEMCELL Technologies) according to the manufacturer's instructions, with some modifications. In brief, LN and lung mononuclear cells were resuspended at 10⁶ cells/ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate (final concentration, 1.5 μM) with or without the aldehyde dehydrogenase inhibitor DEAB (final concentration, 45 μM) and incubated for 30 min at 37°C. For immunophenotyping of ALDEFLUOR-reactive cells, the cells were subsequently stained with PE-, PE-Cy7-, A700-, PerCP Cy5.5-, and Pacific blue-conjugated mAbs in ice-cold ALDEFLUOR assay buffer. ALDEFLUOR-reactive cells were detected in the FITC channel using multi-parameter flow cytometry, as described above.

In vivo DT treatment. DT purchased from Sigma-Aldrich was prepared in a sterile solution of PBS at a concentration of 1 mg/ml. Transient DC depletion was effected in CD11c-DTR chimeras by a single i.p. injection of 200 ng DT; in zDC-DTR mice, a single 100-ng i.p. injection of DT was given i.p.; in CD11b-DTR mice, two 500-ng injections were given on days 0 and 1 i.p.; and in langerin DTR mice, a single dose of 100 ng of DT was given i.p.

Salmonella challenge. Recipients of adoptively transferred OT-II cells, mice immunized i.n. or s.c. with OVA, and mice immunized with heat inactivated, paraformaldehyde-fixed *Salmonella* (10⁶ CFU) were inoculated orally by gavage with WT *Salmonella*-OVA (10⁶ CFU/animal). Mice were monitored with recording of daily weights. Stool culture was performed by culturing pre-weighed stool pellets on days 2, 4, 8, 12, and 40 after challenge (day 40 stool cultures were performed in the long-term survivors to detect for persistence of *Salmonella* in the stool) and expressed as *Salmonella* CFU/g of stool tissue. For splenic cultures, the spleen was harvested from the *Salmonella*-challenged mice on day 6 after challenge, and then weighed, ground in LB broth, and inoculated on Macconkey's Agar in serial dilutions. Kaplan-Meier curves were used to determine the frequency of survival among the challenged mice.

Statistical analysis. Statistical significance was evaluated using a two-tailed Student's *t* test with a 95% confidence interval. Results are expressed as means ± SD. Analysis was performed with a Prism 4 program (GraphPad Software, Inc.).

Online supplemental material. Fig. S1 shows the gating strategy used to identify and sort lung DCs. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20122762/DC1>.

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