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Neonatal LTβR signaling is required for the accumulation of eosinophils in the inflamed adult mesenteric lymph node

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Although eosinophils are important contributors to mucosal immune responses, mechanisms that regulate their accumulation in mucosal-associated lymphoid tissues remain ill-defined. Combining bone marrow chimeras and pharmacological inhibition approaches, here we find that lymphotoxin-beta receptor (LTβR) signaling during the neonatal period is required for the accumulation of eosinophils in the mesenteric lymph nodes (MLN) during an enteric viral infection in adult male and female mice. We demonstrate that MLN stromal cells express genes that are important for eosinophil migration and survival, such as *Ccl-11* (*eotaxin-1*), *Ccl7, Ccl9*, and *Cxcl2*, and that expression of most of these genes is downregulated as a consequence of neonatal LTβR blockade. We also find that neonatal LTβR signaling is required for the generation of a rotavirus-specific IgA antibody response in the adult MLN, but eosinophils are dispensable for this response. Collectively, our studies reveal a role for neonatal LTβR signaling in regulating eosinophil numbers in the adult MLN.

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INTRODUCTION

Eosinophils contribute to mucosal immune responses and play an important role in allergy and control of helminth infection.[1–3] Eosinophils also play a role in clearance of certain viruses (e.g., respiratory syncytial virus and parainfluenza virus) through innate mechanisms such as Nitric Oxide production and the degradation of viral particles through ribonuclease activity in granules.[1,4–6] Recent studies have also revealed novel nonclassical functions of eosinophils at mucosal surfaces, including the maintenance of IgA^+ plasma cells in the gut.[7–10] However, despite their roles in mucosal immune responses, it remains poorly understood how eosinophils are maintained in both resting and inflamed mucosal-associated lymphoid tissues such as mesenteric lymph nodes (MLN).

Signaling through the lymphotoxin-beta receptor (LTβR), which is expressed by myeloid cells and radio-resistant stromal cells,[11–13] is required for the development of secondary lymphoid organs during early life, and for the optimal function of dendritic cells and T lymphocytes during adulthood.[11,12,14–16] For example, LTβR signaling on both subepithelial and perivascular fibroblastic stromal cells during embryonic development is critical for the formation of Peyer's patches.[17] One striking phenotype of mice deficient in the LTβR or its ligand $LT\alpha_1\beta_2$, which is expressed by T cells, B cells, and $ROR\gamma t^+$ innate lymphoid cells,[11–13] is a severe and selective reduction in fecal IgA. LTβR signaling in myeloid dendritic cells as well as stromal cells has been shown to be important for promoting IgA class switch in gut associated lymphoid tissues.[18–21] However, nothing is known about the influence of LTβR signaling on mucosal eosinophils and whether eosinophils play a role in LTβR-dependent IgA production.

We recently showed that the MLN is the major site for initiating an IgA class switched B cell response to orally administered rotavirus (RV) in mice. Moreover, LTβR signaling in utero was found to program the MLN stromal cell environment in order to facilitate the intestinal anti-RV IgA response in the adult MLN.[21] However, the neonatal period is also an important developmental window that can shape mucosal immune cell phenotypes, which we did not specifically examine.[22–26] In this study, we combined bone marrow chimeras and pharmacological inhibition approaches, and assessed the impact of neonatal LTβR signaling on stromal cell gene expression in the MLN of adult mice. Like the in utero period, we found that neonatal LTβR signaling is required for an optimal mucosal IgA response to RV infection during adulthood. However unlike the in utero period, neonatal blockade of LTβR signaling results in reduced MLN eosinophil colonization, particularly during RV infection. This reduction in eosinophils correlated with reduced expression of several eosinophil chemoattractants by MLN stromal cells. Collectively, our studies show that LTβR signaling during the neonatal period is important for mucosal B cells responses against foreign pathogens and the accumulation of MLN eosinophils in the inflamed MLN during adulthood.

RESULTS

Neonatal LTβR signaling influences stromal cell numbers and gene expression in the adult inflamed MLN

The neonatal period is a critical developmental window that shapes the mammalian immune system.[24–26] During this period,

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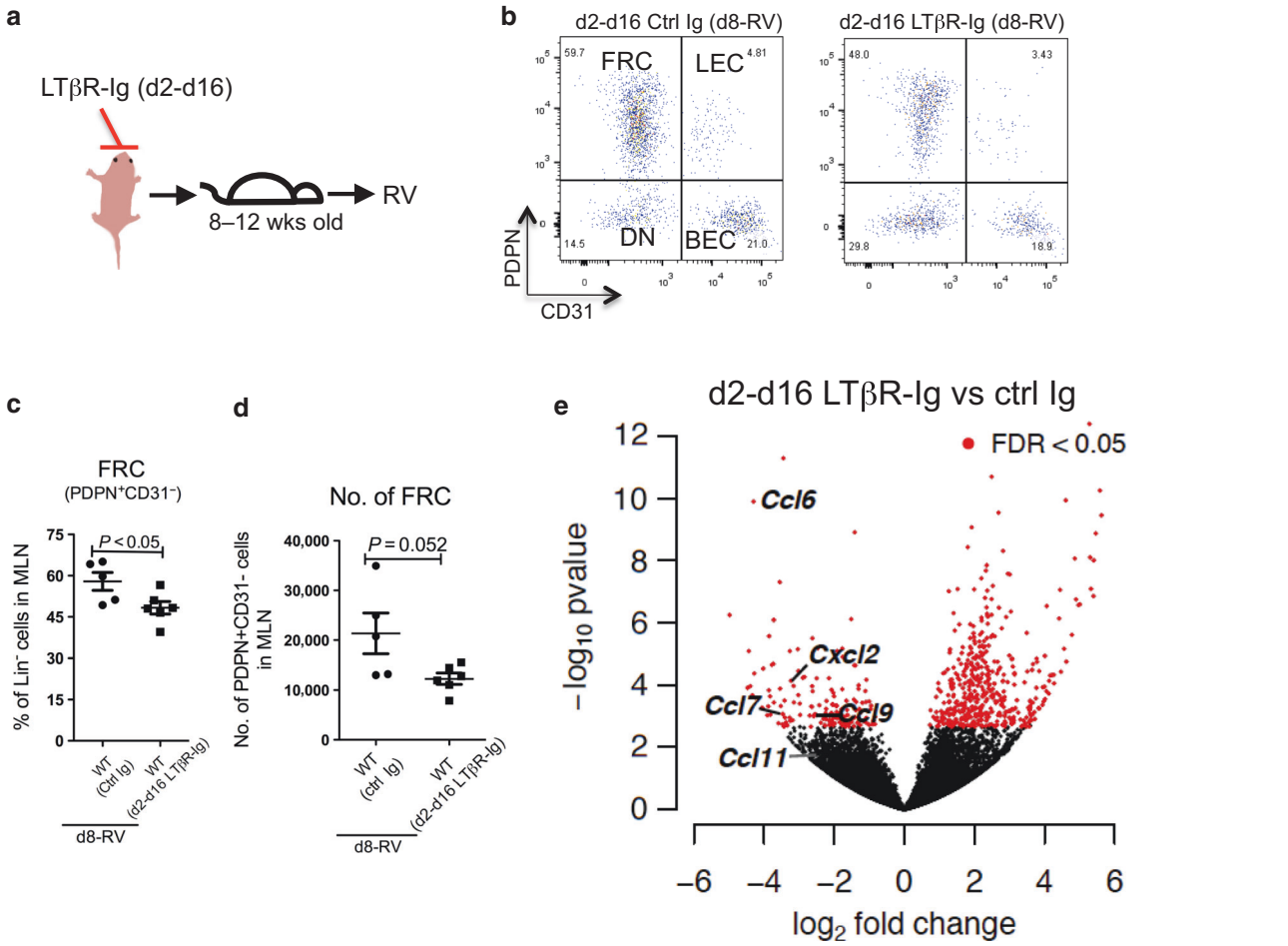

Fig. 1 Neonatal LTβR signaling impacts MLN stromal cells during adulthood. a Diagram of neonatal inhibition of LTβR signaling. **b** Representative flow cytometry plots of MLN stromal cells from d8-RV infected WT mice that received LTβR-Ig or control Ig at postnatal day2.5/9.5/16.5. FRC fibroblastic reticular stromal cells, LEC lymphatic endothelial cells, BEC blood endothelial cells, DN PDPN/CD31 double negative stromal cells. **c**, **d** Frequency and absolute numbers of $PDPN^{+}CD31^{-}$ FRC stromal cells in the MLN of d8-RV infected WT mice that received LTβR-Ig or control Ig at postnatal d2–d16. Three independent experiments were performed with $n = 5–7$ mice per group per experiment, and one representative experiment is shown. Data are presented as mean ± SEM and were analyzed using two-tailed unpaired Mann–Whitney test. **e** Whole-genome RNA-sequencing of $CD45^{-}$/Ter-119^{-}/$EpCAM^{-}$/$CD19^{-}$/$CD31^{-}$ stromal cells from MLN of d8-RV infected WT mice that were treated with LTβR-Ig or control Ig at postnatal d2–d16. Points in red depict genes that were significantly differentially expressed (FDR < 0.05 and fold change >1.5). The differential expression was analyzed using DESeq2 ($n = 3$ samples per group, and each sample is pooled from 2 to 3 mice).

murine MLN expand at a faster rate than all other lymph nodes, presumably to cope with an increasing burden of commensal antigens in the intestinal lumen.[27] We previously showed that MLN are the primary site for IgA class switch in response to rotavirus (RV) infection. Moreover, we found that LTβR signaling in utero programs the development of MLN stromal cells such that restored LTβR signaling in adulthood is no longer able to support key gene expression changes in the MLN that promote IgA class switch in response to RV infection.[21] Since the neonatal period has a critical and unique role in shaping the developing immune system independent of the in utero period,[22,23] we explored the impact of neonatal LTβR signaling on adult stromal cells derived from the MLN at day 8 post-RV infection. To do this, we treated WT mice with an LTβR decoy protein (LTβR-Ig) vs. control Ig at postnatal days 2.5, 9.5, and 16.5 to inhibit LTβR signaling (Fig. 1a), and then evaluated MLN stromal cells during adulthood. As previously described,[21] we identified four major subsets of stromal cells among lineage-negative (lin^{-}) cells in the MLN: $PDPN^{+}$/$CD31^{-}$ fibroblastic reticular stromal cells (FRC), $PDPN^{+}$/$CD31^{+}$ lymphoid endothelial cells (LEC), $PDPN^{-}$/$CD31^{+}$ blood endothelial cells (BEC) and $PDPN^{-}$/$CD31^{-}$ double negative cells (DN) (Fig. 1b). At d8-post RV infection, despite a grossly normal appearing MLN (data not shown), we observed a modest reduction in FRC in the MLN of WT mice that received neonatal LTβR-Ig at days 2.5, 9.5, and 16.5 (d2–d16) compared to those receiving control Ig (Fig. 1c, d). To test whether neonatal inhibition of LTβR signaling impacted MLN stromal cell gene expression, we performed RNA-sequencing of purified MLN CD31- stromal cells (FRC plus DN cells—Supplementary Fig. 1a). We found that d2–d16 LTβR-Ig treatment altered gene expression in adult MLN stromal cells at d8 post-RV infection (147 significantly downregulated and 447 upregulated genes—Fig. 1e and Supplementary Fig. 1b–e). Notably, the number of genes altered in response to d2–d16 LTβR-Ig treatment was less pronounced compared to what we previously observed in MLN stromal cells derived from in utero LTβR-Ig-treated mice (1698 significantly downregulated and 447 upregulated genes).[21] In summary, neonatal LTβR signaling blockade results in a reduction of FRC as well as alterations in stromal cell gene expression in the adult MLN at d8 post-RV infection.

Neonatal LTα$_1$β$_2$/LTβR signaling is required for eosinophil accumulation in the inflamed MLN

From our gene expression analysis, we observed that alterations in gene sets implicated in the chemotaxis of monocytes and granulocytes were uniquely impacted by neonatal, but not in utero LTβR signaling (Supplementary Fig. 1c, d). Therefore, we evaluated whether any of these cell types were under-represented in the MLN of mice that lacked LTβR signaling in the first 6 weeks of life. There are two ligands for LTβR: membrane-bound LTα$_1$β$_2$ and LIGHT.[12] To test whether LTα$_1$β$_2$ mediates the potential impact of early life LTβR signaling on MLN monocytes and granulocytes, we utilized WT→LTβ$^{-/-}$ bone marrow (BM) chimeric mice that specifically lack early life LTα$_1$β$_2$/LTβR signaling. In co-caged WT→WT *versus* WT→LTβ$^{-/-}$ chimeric mice, we observed no differences in the frequency of monocytes, differentiating monocytes or neutrophils in the MLN at d8 post-RV infection (Supplementary Fig. 2a, b for gating strategy and Supplementary Fig. 3a, b for quantification—note that the MLN of WT→LTβ$^{-/-}$ chimeric mice are smaller in size,[21] thus a tabulation of absolute numbers was not performed). However, compared to WT→WT controls, the frequency of eosinophils was significantly reduced in the MLN of d8-RV infected WT→LTβ$^{-/-}$ mice. Moreover, the reduction in eosinophil frequency in WT→LTβ$^{-/-}$ mice was unique to the MLN and not observed in the small intestinal lamina propria (SILP) or BM (Supplementary Fig. 2c, d for gating strategy and Supplementary Fig. 3c). Thus, LTα$_1$β$_2$/LTβR signaling from the in utero period to 6 weeks of age is required to render the adult MLN competent for the accumulation of eosinophils during RV infection. Next to test a more defined developmental period, we treated pregnant WT dams and their neonatal offspring with an inhibitor of the LT pathway (LTβR-Ig) from E11 to postnatal d19 (Fig. 2a). We found that early life LTβR-Ig treatment resulted in a very dramatic reduction in the frequency and numbers of MLN eosinophils at day 8 post-RV infection (Fig. 2a, e). Therefore, using bone marrow chimeric mice we demonstrate that LTα$_1$β$_2$/LTβR signaling is required for the accumulation of eosinophils in the inflamed adult MLN, and using LTβR-Ig we identify E11-d19 as an important developmental window for this requirement.

We next endeavored to pinpoint the relevant LTβR-dependent developmental window required to condition the adult MLN for eosinophil colonization. To do this, we first examined the dynamics of eosinophil accumulation in the MLN at various timepoints post-RV infection. Although the relative frequency of eosinophils decreased with infection presumably due to the influx of other leukocytes into the inflamed MLN, we found that the absolute number of MLN eosinophils gradually increased in WT mice after RV infection, reaching a fivefold increase at d8 post-infection (Supplementary Fig. 4a). Focusing therefore on the pre-infection and day 8 post-infection timepoints, we next tested the impact of neonatal (d2–d16) LTβR-Ig treatment on MLN eosinophil accumulation. We observed a mild reduction of MLN eosinophils at the steady state (Supplementary Fig. 4b), which correlated with a reduction in MLN FRC stromal cells (Supplementary Fig. 4c) in mice treated with LTβR-Ig during the neonatal period compared to controls. However, at d8 post-RV infection neonatal LTβR-Ig treatment resulted in a more dramatic reduction in the frequency and numbers of MLN eosinophils compared to control treated mice (Fig. 2b, f). This reduction in eosinophils was unique to the MLN and was not observed in the SILP nor the BM (Supplementary Fig. 4d, e). Moreover, the neonatal period was the critical LTβR-dependent developmental window since inhibition of LTβR signaling only in utero (Fig. 2c, g) or only during adulthood (Fig. 2d, h) did not impact eosinophil numbers in the MLN at day 8 post-RV infection. Interestingly, the number of MLN eosinophils actually increased in LTβR-Ig treated adult micet (Fig. 2h). This may be because blockade of LTβR signaling in adult mice impairs lymphocyte entry into lymph nodes,[28] perhaps providing more "space" for eosinophils to accumulate in the lymph node during RV infection. Taken together, these data indicate that LTβR signaling is uniquely required during the neonatal period to support the accumulation of eosinophils in the adult MLN. As the most dramatic reduction in eosinophil numbers caused by neonatal LTβR-Ig treatment was observed during RV infection, we focused on day 8 post-RV infection for the remainder of the study.

Neonatal LTβR signaling is required for stromal cell expression of eosinophil chemokines in the adult inflamed MLN

There are many potential reasons for the observed reduction of MLN eosinophils in mice treated with LTβR-Ig during the neonatal period. These include fewer eosinophils in the blood (i.e., less generation of eosinophils from the bone marrow), impaired eosinophil proliferation or enhanced eosinophil death. However, when we compared neonatal LTβR-Ig vs. control Ig-treated mice we noted equivalent levels of blood eosinophils (Supplementary Fig. 5), and no significant differences in proliferation or apoptosis of MLN eosinophils at day 8 post-RV infection (Supplementary Fig. 6). We next hypothesized that the reduction in eosinophils in the MLN of neonatal LTβR-Ig-treated mice may be associated with altered chemokine expression by MLN-resident stromal cells, which could result in impaired migration of eosinophils to the MLN at day 8 post-RV infection. Mining our two independent RNA-seq datasets we found that MLN stromal cells extracted at d8 post-RV infection express chemokines such as *Ccl11* (*eotaxin-1*), *Ccl6, Ccl7, Ccl9*, and *Cxcl2*, but not *Ccl24* (*eotaxin-2)* or *Ccl26* (*eotaxin-3*), all known chemoattractants that promote eosinophils recruitment[29,30] (Fig. 3a). Moreover, qPCR analysis showed that the mRNA for *Ccl11* (*eotaxin-1*), *Ccl7, Ccl9*, and *Cxcl2*, but not *Ccl6*, were reduced in mice that received LTβR-Ig during the neonatal period (Fig. 3b). In contrast, the reduced expression of these chemokines by stromal cells was not observed in the SILP of neonatal LTβR-Ig-treated mice (Supplementary Fig. 7a), which is in line with the observed comparable frequencies of eosinophils in both LTβR-Ig and control Ig groups in this location (Supplementary Fig. 4d). These data demonstrate that neonatal LTβR signals program the MLN stroma to express eosinophils chemoattractants at d8 post-RV infection, and the reduced expression of these chemoattractants correlates with fewer eosinophils in the adult inflamed MLN.

Neonatal LTβR signaling influences anti-RV IgA responses during adulthood

We previously showed that while mice that lack LTβR signaling in the first 6 weeks of life exhibit normal RV infection-induced germinal center formation in the adult MLN, stromal cell expression of genes that are associated with IgA class switch recombination are reduced, and consequently IgA class switch is strongly suppressed.[21] We did not examine the impact of LTβR signaling specifically during the neonatal window on IgA class switch in these experiments. Indeed, compared to control treatment, neonatal (d2–d16) LTβR-Ig treatment also results in a reduction in IgA$^+$ RV-specific antibody-secreting cells (ASC) in the MLN and SILP at d8 and d49 post-RV infection, respectively (Fig. 4a, b), and the ratio of IgA$^+$ *versus* IgM$^+$ RV ASC was also diminished in these mice, indicative of a defect in IgA class switching in the MLN (Fig. 4c). Consistent with this observation, expression of mRNA encoding pro-IgA switch factors (*Tnfsf13b, Il6, Il33, Tnfsf12*, and *Aldh1a1*) and matrix metalloproteinases *Mmp2* and *Mmp9* that are responsible for cleavage of the IgA switch factor TGFβ,[31] were likewise reduced in the MLN at day 8 post-RV infection (Fig. 4d). Overall, these data indicate that, like the *in utero* period, neonatal LTβR signaling is essential for an anti-RV IgA response during adulthood.

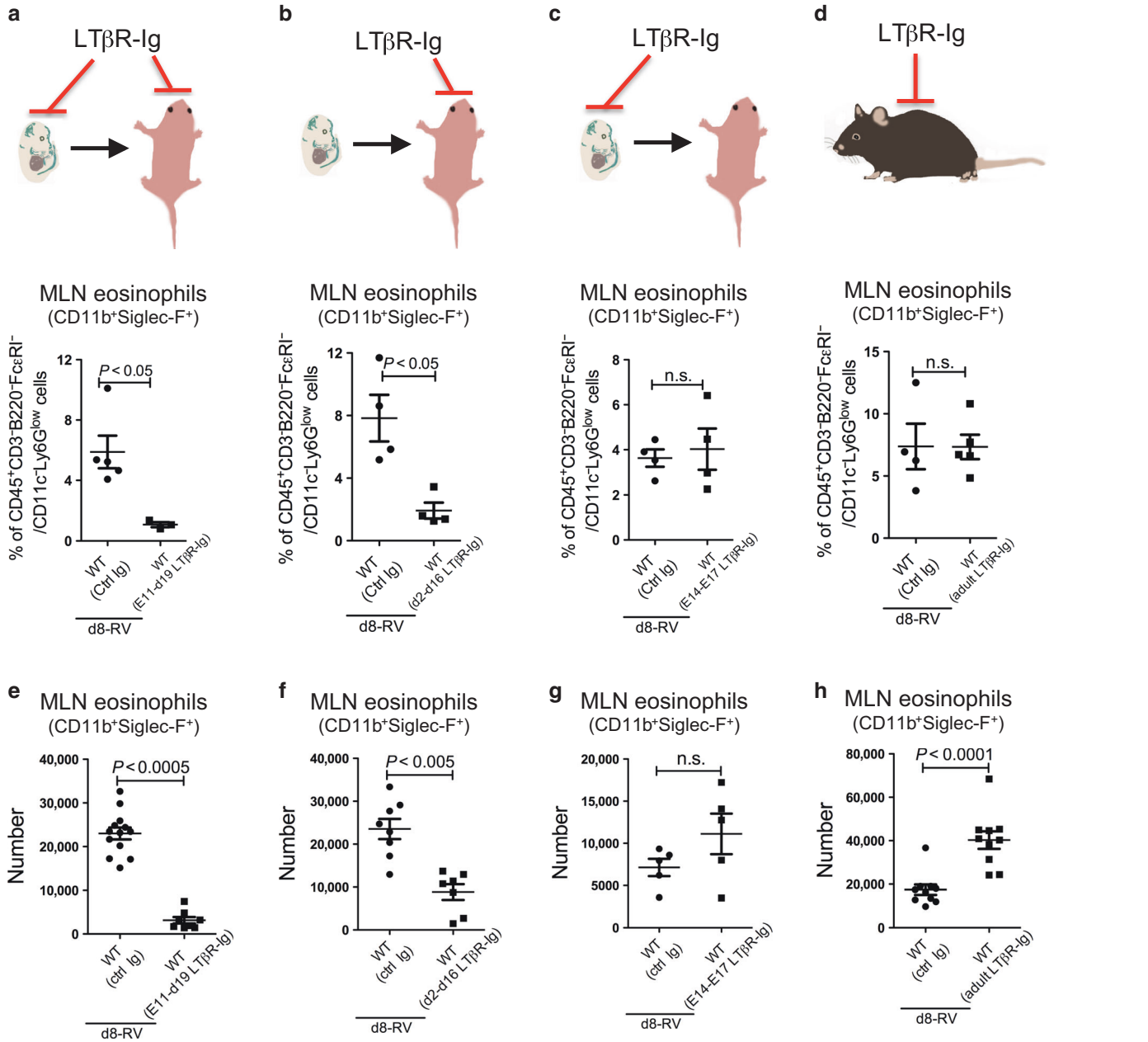

Fig. 2 LTβR signaling during the neonatal period is required for the accumulation of eosinophils in the adult MLN during RV infection. a Depiction of experimental setup for inhibiting LTβR signaling during both fetal and neonatal periods (E11-d19), and corresponding frequency of Siglec-F$^+$CD11b$^+$ cells in the adult MLN at d8 post-RV infection. **b** Depiction of experimental setup for neonatal (d2–d16) inhibition of LTβR signaling in WT mice, and corresponding frequency of Siglec-F$^+$CD11b$^+$ cells in the adult MLN at d8 post-RV infection. **c** Depiction of experimental setup for in utero (E14/E17) inhibition of LTβR signaling in WT mice, and corresponding frequency of Siglec-F$^+$CD11b$^+$ cells in the adult MLN at d8 post-RV infection. **d** Depiction of experimental setup for inhibition of LTβR signaling in adult WT mice, and corresponding frequency of Siglec-F$^+$CD11b$^+$ cells in the adult MLN at d8 post-RV infection. Three independent experiments were performed with $n = 3$–5 mice per group per experiment, and one representative experiment is shown in (**a**, **b**). Two independent experiments were performed with $n = 4$–5 mice per group per experiment, and one representative experiment is shown in (**c**, **d**). Y-axis in the bottom panel of (**a–d**) shows the frequency of CD11b$^+$Siglec-F$^+$ cells among live CD45$^+$/CD3$^-$/B220$^-$/FcεRI$^-$/CD11c$^-$/Ly6G^{low} MLN cells. **e–h** Absolute numbers of MLN Siglec-F$^+$CD11b$^+$ cells for the indicated groups. Data in (**a–h**) are presented as mean ± SEM and were analyzed using two-tailed unpaired Mann–Whitney test.

Eosinophils are not required for the IgA response to RV in MLN during the priming period

Eosinophils have been shown to be important for the generation and maintenance of IgA plasma cells in the SILP,[8] however nothing is known about whether eosinophils impact an antigen-specific IgA response to a mucosal pathogen. Given that we observed a reduction in eosinophils in the MLN concomitant with a defect in the generation of RV-specific IgA in neonatal LTβR-Ig-treated mice, we hypothesized that eosinophils may be involved in the anti-RV IgA response. We first asked whether we could restore eosinophils in mice that lack early life LTβR signaling by introducing supraphysiological levels of IL-5, a major regulator of eosinophil accumulation in tissues.[1,32] To test this, we crossed IL-5 transgenic mice[32] with Blimp-1-YFP mice[33] to track plasma cells. BM from these double transgenic mice was then transplanted into irradiated adult LTβ$^{-/-}$ recipients. We observed that IL-5/Blimp-

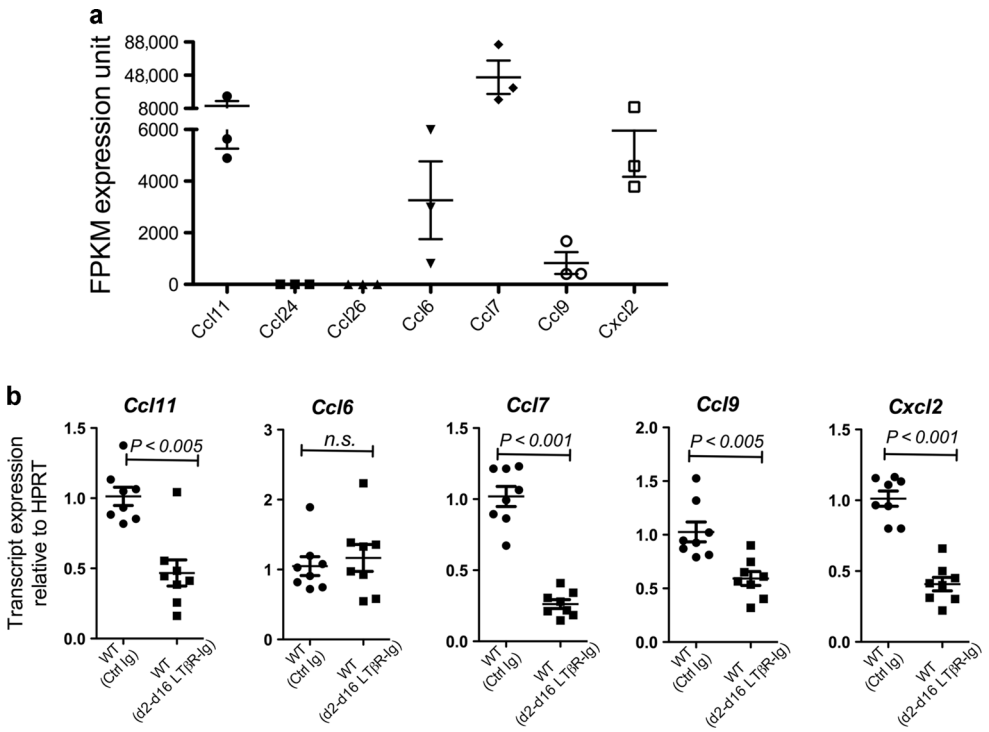

Fig. 3 Impact of neonatal LTβR signaling on the expression of eosinophil chemokine genes in MLN stromal cells from RV-infected adult mice. a RNA-seq data analysis of indicated genes in WT MLN stromal cells ($n = 3$ mice per group) at d8 post-RV infection. Two independent experiments were performed, and one representative experiment is shown. **b** qPCR measurement of *Ccl11*, *Ccl6*, *Ccl7*, *Ccl9,* and *Cxcl2* from MLN stromal cells derived from d8 RV-infected WT mice that were treated with LTβR-Ig *versus* control Ig at postnatal d2–d16 in an independent cohort of mice. Data were combined from two independent experiments ($n = 4$ samples per experiment; each sample was pooled from two mice). Data are presented as mean ± SEM and were analyzed using two-tailed unpaired Mann–Whitney test.

1^{YFP}→LTβ$^{-/-}$ chimeric mice harbored elevated numbers of eosinophils in the MLN at d8 post-RV infection when compared to Blimp-1^{YFP}→WT and Blimp-1^{YFP}→LTβ$^{-/-}$ mice (Fig. 5a, b), demonstrating that impaired eosinophil accumulation in the adult inflamed MLN of mice that lack early life LTα$_1$β$_2$/LTβR signaling is reversible.

We next quantified the generation of MLN-resident Blimp-1^+ plasma cells/plasmablasts in response to RV infection using the Blimp-1 reporter. At the resting state, a greater number of Blimp-1^+ cells was observed in the MLN of uninfected IL-5/Blimp-1^{YFP}→LTβ$^{-/-}$ chimeras compared to the MLN of Blimp-1^{YFP}→LTβ$^{-/-}$ chimeras (Fig. 5c). As we observed before,[21] infection with RV results in an increase in the frequency of YFP^{high} plasma cells/plasmablasts in the MLN compared to uninfected animals. Moreover, the introduction of the IL-5 transgene in IL-5/Blimp-1^{YFP}→LTβ$^{-/-}$ chimeric mice did not further augment the frequency of YFP^{high} plasma cells/plasmablasts in the MLN of d8 RV infected mice (Fig. 5c). In spite of the excessive level of eosinophils in IL-5/Blimp-1^{YFP}→LTβ$^{-/-}$ chimeric mice, the defective anti-RV IgA response in the MLN and SILP was not corrected (Fig. 5d), indicating that an excessive number of eosinophils is incapable of rescuing the poor anti-RV IgA response that is observed in mice that lack early life LTβR signaling.

Since IL-5 induced eosinophils are not sufficient to correct the defective anti-RV IgA response in adult mice that lack early life LTβR signaling, we next asked if eosinophils were necessary for generating this response. As previously described,[8] we injected anti-Siglec-F antibodies into WT mice before and during RV infection (Fig. 6a). Anti-Siglec-F treatment reduced the frequency of CD11b$^+$Siglec-F$^+$ cells in the MLN and SILP (Supplementary Fig. 7b) and the absolute number of CD11b$^+$Siglec-F$^+$ cells in the MLN (Fig. 6b). Given that Siglec-F may be downregulated by the antibody treatment itself, we confirmed depletion of eosinophils by measuring the frequency of CD11b$^+$SSC^{high} cells in the MLN and found a similar result (Supplementary Fig. 7c). However, anti-Siglec-F treatment did not impact the generation of anti-RV IgA secreting cells as measured by ELISPOT in the MLN or SILP at d8 post-RV infection (Fig. 6c). Together, these data demonstrate that eosinophils are dispensable for mounting an anti-RV IgA response in the MLN at d8 post-infection.

To test whether IgA itself impacts eosinophil numbers in the MLN, we utilized mice deficient in activation-induced cytidine deaminase (AID), which lack IgA due to a defect in antibody class switch.[34] We found that AID knockout mice do not exhibit a reduction in MLN eosinophils at day 8 post-RV infection (Supplementary Fig. 7d), although RV-induced accumulation of eosinophils was less obvious in these mice, potentially due to the already large MLN and dysbiosis that have been documented for this strain.[35] Taken together, our study uncouples the IgA response to RV from eosinophil accumulation in the reactive MLN.

Collectively, as illustrated in Supplementary Fig. 8, our studies demonstrate that neonatal LTβR signaling plays an important role in programming gene expression in adult stromal cells in order to maintain both eosinophil accumulation and IgA class switch in the inflamed MLN (see illustrated depiction of these findings in Supplementary Fig. 8).

DISCUSSION

In this study we reveal a novel role for neonatal LTα$_1$β$_2$/LTβR signaling in the accumulation of MLN eosinophils during adulthood during an enteric viral infection. We further show that neonatal LTβR signaling influences adult MLN stromal cell gene expression, in particular genes that are important for the recruitment of tissue-resident eosinophils and for IgA class switch recombination. Combining eosinophil reconstitution and

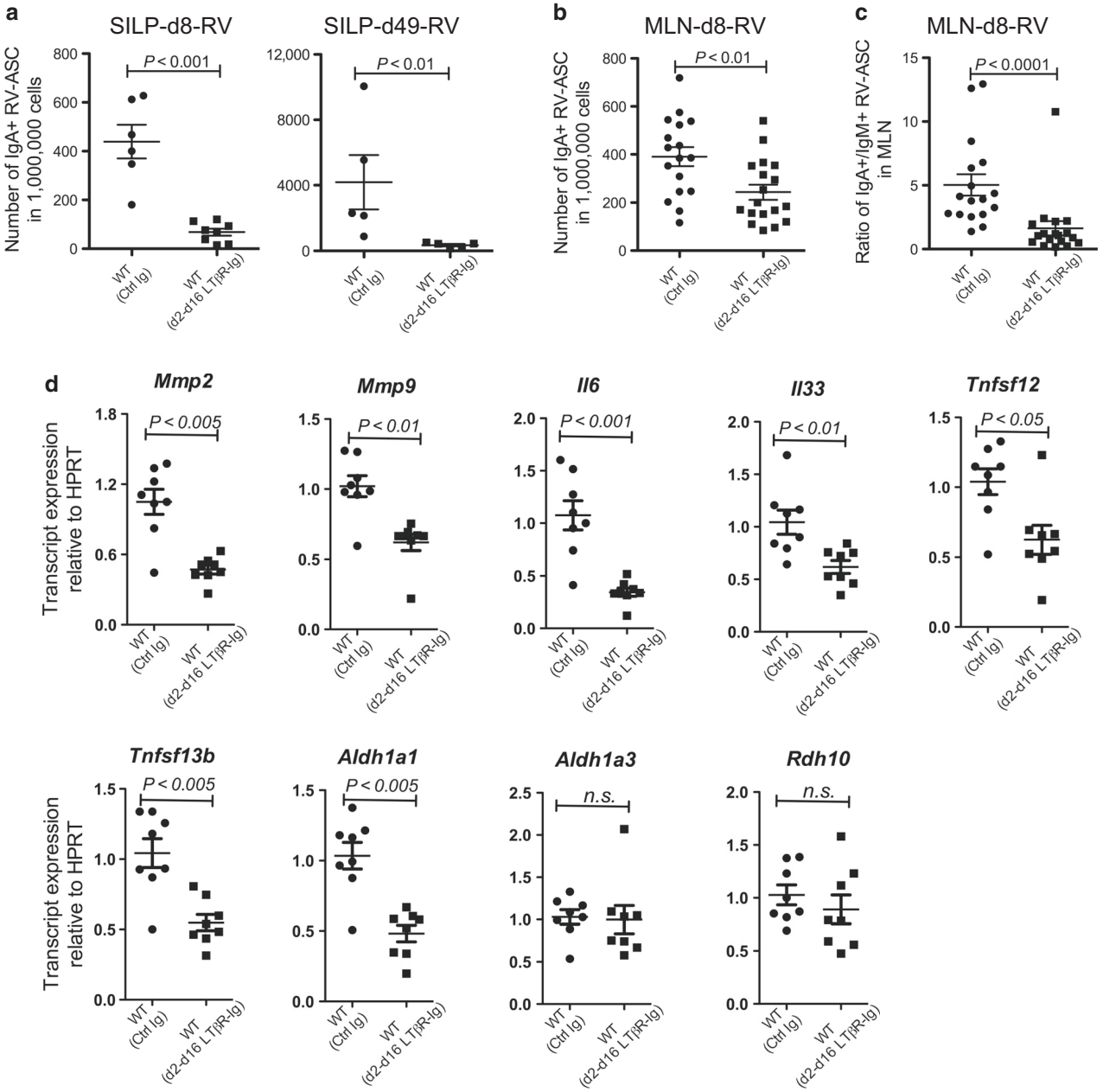

Fig. 4 Neonatal LTβR signaling impacts anti-RV IgA response during adulthood. a Enumeration of IgA^+ RV-ASC in the SILP of d2–d16 LTβR-Ig or control Ig-treated WT mice at d8 (left panel) and d49 (right panel) post-RV infection. Two independent experiments were performed with $n = 4$–8 mice per group per experiment, and one representative experiment is shown. **b** Enumeration of IgA^+ RV-ASC in the MLN of d2–d16 LTβR-Ig or control Ig-treated WT mice at d8 post-RV infection. Data were pooled from four independent experiments ($n = 17$–18 mice per group). **c** The ratio of IgA^+ *versus* IgM^+ RV-ASC in in the MLN of WT mice that received d2–d16 LTβR-Ig *versus* control Ig at d8 post-infection. Data were pooled from four independent experiments ($n = 17$–18 mice per group). **d** qPCR measurement of indicated pro-IgA factors in MLN stromal cells from d8-RV infected WT mice that received LTβR-Ig or control Ig at postnatal d2–d16. Data were combined from two independent experiments ($n = 4$ samples per experiment; each sample was pooled from the two mice). Data are presented as mean ± SEM and were analyzed using two-tailed unpaired Mann–Whitney test.

depletion approaches, we found that eosinophils are dispensable for mounting an IgA response against RV infection in the adult MLN.

Non-hematopoietic stromal cells play a key role in establishing the compartmentalization of lymphoid tissues by segregating T and B lymphocytes into different regions of secondary lymphoid organs.[36,37] Stromal cells also influence antigen trafficking, the migration and survival of lymphocytes, and induction of tolerance. In particular, B cell follicle-resident FRC impact humoral immunity by promoting B cell survival and follicular organization.[38,39] Interestingly, in terms of the total number of genes impacted by early life LTβR-Ig inhibition, we observed differences in the number and categories of genes depending on the developmental window of treatment administration, indicating that while some overlapping functions are imprinted in MLN stroma during in utero and neonatal life, there are also events that are unique to these developmental windows. In particular, while in utero LTβR signaling supports the expression of IgA class switch factors,[21] neonatal LTβR signaling also influences the level of eosinophil chemoattractants expressed in the adult MLN during RV infection.

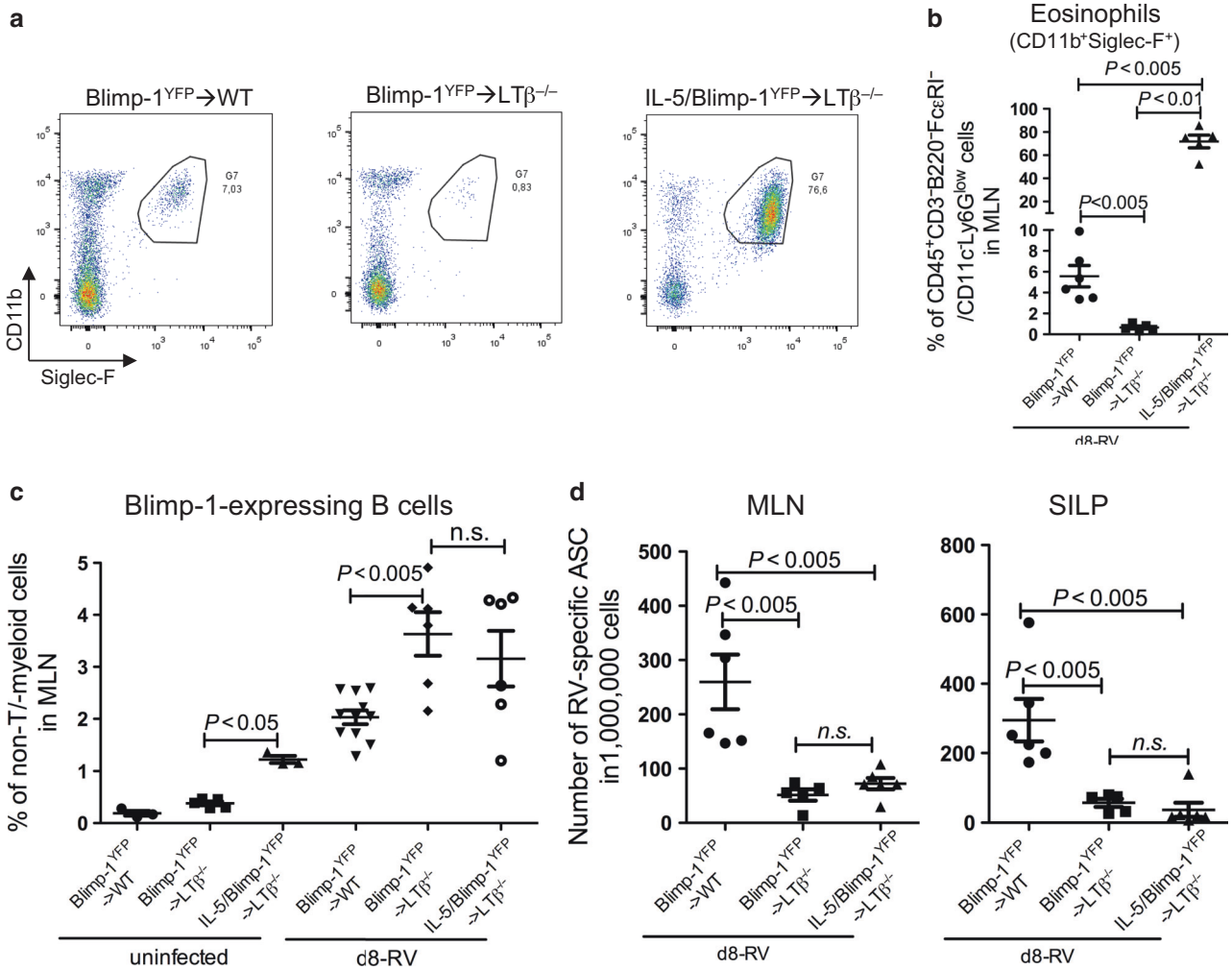

Fig. 5 Restoration of eosinophil deficiency in the MLN of LTβ$^{-/-}$ chimeric mice does not rescue the defective anti-RV IgA responses. a Representative FACS plots of MLN Siglec-F^{+}CD11b^{+} cells from Blimp-1^{YFP}→WT, Blimp-1^{YFP}→LTβ$^{-/-}$ and IL-5/Blimp-1^{YFP}→LTβ$^{-/-}$ chimeric mice at d8 post-RV infection. **b** Frequency of MLN Siglec-F^{+}CD11b^{+} cells from Blimp-1^{YFP}→WT, Blimp-1^{YFP}→LTβ$^{-/-}$ and IL-5/Blimp-1^{YFP}→LTβ$^{-/-}$ chimeric mice at d8 post-infection. Y-axis shows the frequency of Siglec-F^{+}CD11b^{+} cells among live CD45^{+}/CD3^{-}/B220^{-}/FcεRI^{-}/CD11c^{-}/Ly6G^{low} MLN cells. **c** Frequency of YFP^{high} B cells in the MLN of Blimp-1^{YFP}→WT, Blimp-1^{YFP}→LTβ$^{-/-}$ and IL-5/Blimp-1^{YFP}→LTβ$^{-/-}$ chimeric uninfected mice and chimeric mice at d8 post-RV infection, respectively. **d** Enumeration of RV-specific IgA-ASC in the MLN (left panel) and SILP (right panel) of Blimp-1^{YFP}→WT, Blimp-1^{YFP}→LTβ$^{-/-}$ and IL-5/Blimp-1^{YFP}→LTβ$^{-/-}$ chimeric mice at d8 post-infection. Data in (**b–d**) were presented as mean ± SEM and analyzed using two-tailed unpaired Mann–Whitney test.

One limitation of our study is that we were unable to directly link the reduction in eosinophil chemoattractants with impaired migration of eosinophils to the MLN. Reporter mice that follow eosinophils in vivo combined with 2-photon imaging of the MLN may provide an approach to addressing this unanswered question as a future direction.

In addition to their roles in allergy and control of helminth infection, recent studies have uncovered nonclassical roles for eosinophils, particularly their role in the maintenance of IgA plasma cells.[1] Specifically, by using eosinophil-deficient mice and anti-Siglec-F mediated eosinophil depletion, Chu et al. showed that eosinophils contribute to the generation and maintenance of IgA^{+} plasma cells in the intestinal lamina propria.[8] We found that elimination of eosinophils does not impinge on the induction of an RV-specific IgA response. It remains to be tested whether eosinophils are involved in the long-term maintenance of anti-RV plasma cells.

At d8 post-RV infection, we found that the induction of Blimp-1 in MLN B cells was comparable between Blimp-1^{YFP}→LTβ$^{-/-}$ and IL-5/Blimp-1^{YFP}→LTβ$^{-/-}$ chimeras, although MLN of the latter group contained a very high level of eosinophils. Furthermore, the excessive level of eosinophils due to transplant of IL-5 transgenic BM did not rescue the defective RV-specific IgA-ASC response in the MLN and SILP of LTβ$^{-/-}$ mice, indicating that eosinophils are dispensable for the initiation of mucosal IgA responses against RV infection. However, we cannot exclude the possibility that the IL-5 transgene may have provoked an altered array of eosinophils, which may have obscured the ability for "natural" eosinophil recruitment to the reactive MLN to rescue the IgA response in LTβ$^{-/-}$ chimeras. Not only is IL-5 critical for the expansion of eosinophil progenitor cells in the bone marrow,[1,32] IL-5 also promotes the differentiation of IgA-producing B cells.[40] Of note, IL-5 overexpression was found to elevate the frequency of of Blimp-1^{+} B cells in the resting MLN (Fig. 5c). Whether this is due to increased eosinophil numbers (i.e., indirect effect), or due to a direct effect of IL-5 on B cells themselves, remains to be investigated.

In summary, we demonstrated that LTβR signaling during the neonatal period is required for mucosal B cell responses against foreign pathogens and the accumulation of MLN eosinophils in the inflamed MLN during adulthood. In the future, it will be of interest to investigate whether a developmentally programmed specific stromal cell subset provides the foundation for an

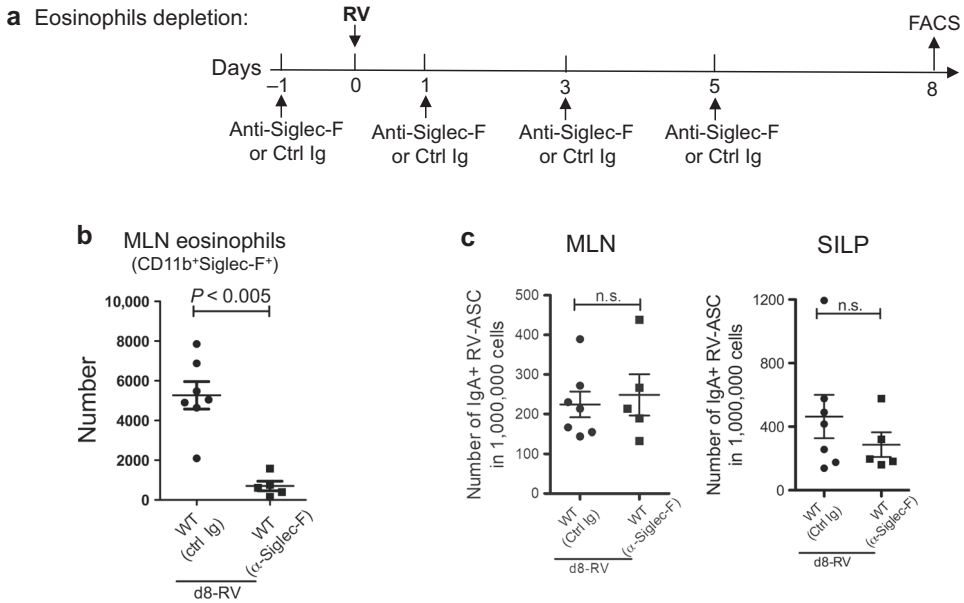

Fig. 6 Depletion of eosinophils in WT mice does not significantly impair the anti-RV IgA response. a Depiction of experimental setup for depleting eosinophils before and during RV infection. **b** Numbers of MLN Siglec-F^{+}CD11b^{+} cells in WT mice receiving anti-Siglec-F depleting antibody versus control antibody at d8-RV infection ($n = 5$–7 mice per group). **c** Enumeration of RV-specific IgA-ASC in MLN (left panel) and SILP (right panel) from control Ig-treated versus anti-Siglec-F treated WT mice at d8 post-RV infection ($n = 5$–7 mice per group). Data in (**b**, **c**) are presented as mean ± SEM and were analyzed using two-tailed unpaired Mann–Whitney test.

eosinophil niche in the inflamed MLN in the context of an allergic and/or anti-parasitic immune response.

MATERIALS AND METHODS

Mice

C57BL/6 WT mice (Charles River Laboratory, St. Constant, QC, Canada) and Blimp-1^{YFP} mice (Jackson Laboratory) were bred in the Animal Vivarium facility at the Division of Comparative Medicine, University of Toronto. LTβ$^{-/-}$ mice were originally from B&K Universal and bred in our animal facility. IL-5 transgenic mice were bred with Blimp-1^{YFP} to generate IL-5/Blimp-1^{YFP} double transgenic mice. AID-deficient mice[41] were obtained from Dr. Alberto Martin. Bone marrow chimeric mice were generated as we previously described.[21] In brief, the adult recipient mice (6–10 weeks old) were irradiated with 2 × 550 rad doses, before receiving the intravenous injection of 2–5 million donor bone marrow cells. In the first 2 weeks post bone marrow engraftment, the recipient mice were provided with water that contains 2 g/L neomycin sulfate to prevent opportunistic infections. 8–12 weeks later, these chimeric mice were infected with rotavirus by oral gavage.[21] The mice were maintained under specific pathogen-free conditions, with the humidity of ~45% and the temperature of around 22 °C. In all cases (pharmacological treatment and bone marrow chimeras), mice from different groups were co-housed to minimize the contribution of the microbiome on our experimental results. This was particularly important because an altered microbiota in eosinophil-deficient mice has been shown to be associated with IgA deficiency in the gut.[42] In addition, we noted that AID-deficient mice, which are known to exhibit dysbiosis, had less pronounced eosinophil accumulation upon RV infection.[35] The experimental procedures were approved by the Animal Care Committee of University of Toronto.

Pharmacological inhibition of LTβR signaling

The pharmacological inhibition of LTβR signaling in vivo was performed as we previously described.[21] To inhibit neonatal LTβR signaling, WT pups were intraperitoneally injected with LTβR-Ig of or control Ig (5 µg Ig per g of pups) at days 2.5, 9.5 and 16.5 post-birth, respectively. After weaning, the d2–d16 LTβR-Ig-treated pups were co-caged with corresponding control Ig-treated pups, respectively.

Eosinophil depletion

The anti-Siglec-F monoclonal antibody (clone: 238047, R & D Systems) or control antibody was injected intraperitoneally (0.5 mg/mouse) at indicated timepoints before and after RV infection. Mice were sacrificed at d8 post-RV infection, and subjected to flow cytometry analysis.

Tissue harvest and cell isolation

As we previously described,[21] all MLNs were harvested from indicated groups of mice, followed by fat tissue removal. Depending on experimental purposes, single-cell suspension of MLN cells were processed in two methods. For MLN stromal cell experiments, MLNs were dissected into small pieces and digested with freshly made enzyme mix consisting of 0.12 mg/ml Collagenase P (Roche), 0.4 mg/ml Dispase II (Roche) and 0.025 mg/ml DNase I (Roche) in RPMI-1640 in a 37 °C water bath, as we previously described.[21] For SILP stromal cell experiments, Peyer's Patches were first removed from the small intestine, and the segmented small intestine was washed with CMF buffer (HBSS + 2% FBS+ 15 mM HEPES), and then vigorously shaken in CMF/EDTA buffer (5 mM EDTA), followed by the digestion with freshly made enzyme mix consisting of 0.2 mg/ml Collagenase P, 0.8 mg/ml Dispase II and 0.1 mg/ml DNase I in RPMI-1640 in a 37 °C water bath for 17 min. For RV-ELISPOT experiments, single suspension of MLN cells were prepared by grinding MLN between glass slides, followed by a 70-µm cell strainer filtration. For BM cell preparation, cells were flushed out from tibia of mice, followed by red blood cell lysis. Lymphocytes from the SILP were prepared as previously described.[21]

RV-specific ELISPOT

RV-specific ELISPOT assays were performed at the indicated days post-RV infection, as we previously described.[21] In brief, MultiScreen-HTS-HA filter plates (Millipore) were coated with inactive RV antigen (Microbix), and then incubated with two-fold serial dilutions of MLN, BM or SILP lymphocytes overnight. The RV-specific antibody-secreting cells were detected by HRP-conjugated goat anti-mouse IgA or IgM (SouthernBiotech). To develop the plates, AEC (Vector Laboratories) was utilized as the colorimetric substrate for HRP.

Flow cytometry of eosinophils

To analyze eosinophils, single-cell suspensions from MLN, SILP, blood or BM were stained with antibodies against mouse CD45.2 (APC-eF780, clone: 104, eBioscience), FITC dump antibodies (CD3, clone: 17A2; B220, clone: RA3-6B2, eBioscience), CD11c (APC, clone: N418, eBioscience), FceRI (eF450, clone: MAR-1, eBioscience), Ly6G (PE, clone: 1A8-Ly6G, eBioscience), Siglec-F (PE-CF594, clone: E50-2440, BD Pharmingen) and CD11b (BV605, clone: M1/70, BioLegend), in the presence of Fc block. The

cells were then stained with 7-AAD (BD Pharmingen) prior to flow cytometry on an LSR X-20.

For the intracellular Ki-67 and cleaved Caspase 3 staining of MLN esoinophils, single-cell suspensions from MLN were labeled with Live/Dead Aqua (Life Technologies), and then stained with antibodies against mouse CD45.2 (PercpCy5.5, clone: 104, eBioscience), FITC dump antibodies (CD3, clone: 17A2; B220, clone: RA3-6B2), CD11c (PE-Cy7, clone: N418, eBioscience), FceRI (eF450, clone: MAR-1), Ly6G (APC-eF780, clone: 1A8-Ly6G, eBioscience), Siglec-F (PE-CF594, clone: E50-2440) and CD11b (BV605, clone: M1/70), in the presence of Fc block. Cells were then permeabilized and fixed using eBioscience perm/fix buffer, followed by overnight staining with rabbit polyclonal antibody against cleaved Caspase 3 (Cell Signaling Technology, Cat. #9661, 1:800 dilution). Then the cells were stained with PE Donkey anti-rabbit IgG (BioLegend, Cat. #406421) and Ki-67 (APC, clone: SolA15, eBioscience).

Flow cytometry of monocytes and neutrophils

Single-cell suspensions from MLN were stained with Aqua and then antibodies against mouse Ly6G (eF660, clone: RB6-8C5, eBioscience), CD11b (BV605, clone: M1/70, BioLegend), Ly6C (PercpCy5.5, clone: HK1.4, eBioscience), CD11c (PE-Cy7, clone: N418, eBioscience) and MHCII (eF450, clone: M5/114.15.2, eBioscience). The stained cells were analyzed on an LSR X-20.

MLN stromal cell analysis

The flow cytometry analysis of MLN stromal cells, and RNA-sequencing of MLN stromal cells were performed as we previously described.[21] Venny plot analysis was employed to compare the list of genes from different treatments, while STRING analysis was used to decipher the functional protein-protein interaction among genes of interest.

Quantitative real-time PCR of MLN and SILP stromal cells

MLN and SILP stromal cells were acquired via magnetic-activated cell sorting, as we previously described.[21] The synthesized cDNA from MLN stromal cells were subjected to qPCR reactions with HPRT as internal control.[43] The qPCR primers used for pro-IgA factors were described in our previous studies.[21] The following primers were used for qPCR analysis of eosinophils-related genes: Ccl11 (F: 5′tccttcatgaccttgtgcag3′; R: 5′ggaata gaagcgctgtggag3′), Ccl6 (F: 5′aagaagatcgtcgctataaccct3′; R: 5′gcttaggcac ctctgaactctc3′), Ccl7 (F: 5′tgaaaaccccaactccaaag3′; R: 5′cattccttaggcgtgac cat3′), Ccl9 (F: 5′ccctctccttcctcattcttaca3′; R: 5′agtcttgaaagcccatgtgaaa3′), Cxcl2 (F: 5′ accccactgcgcccagacagaa3′; R: 5′agcagcccaggctcctcctttcc3′).

Statistical analysis

Data are presented as mean ± SEM. All the data were analyzed using two-tailed Mann–Whitney tests unless noted. $P < 0.05$ was considered significant.

DATA AVAILABILITY

All relevant data are available from the authors. Original unprocessed data can be accessed via Mendeley Data: https://doi.org/10.17632/xy3pssf9c2.1.

MATERIALS AVAILABILITY

All relevant materials are available from the authors.

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AUTHOR CONTRIBUTIONS

C.L. designed the experiments, performed the research, analyzed data, and wrote the paper; L.A.W., A.N., E.L., and D.D. performed the research; M.A. and H.H. analyzed the RNA-seq data; K.H., M.B.B., B.L., and K.M.M. analyzed data and edited the paper; J.L.G. is the principal investigator who designed the experiments, analyzed data, and wrote the paper.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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