# Nonredundant Function of Soluble LT$\alpha_3$ Produced by Innate Lymphoid Cells in Intestinal Homeostasis

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# Nonredundant Function of Soluble LTα$_3$ Produced by Innate Lymphoid Cells in Intestinal Homeostasis

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Immunoglobulin A (IgA) production at mucosal surfaces contributes to protection against pathogens and controls intestinal microbiota composition. However, mechanisms regulating IgA induction are not completely defined. We show that soluble lymphotoxin α (sLTα$_3$) produced by RORγt$^+$ innate lymphoid cells (ILCs) controls T cell–dependent IgA induction in the lamina propria via regulation of T cell homing to the gut. By contrast, membrane-bound lymphotoxin β (LTα$_1$β$_2$) produced by RORγt$^+$ ILCs is critical for T cell–independent IgA induction in the lamina propria via control of dendritic cell functions. Ablation of LTα in RORγt$^+$ cells abrogated IgA production in the gut and altered microbiota composition. Thus, soluble and membrane-bound lymphotoxins produced by ILCs distinctly organize adaptive immune responses in the gut and control commensal microbiota composition.

Production of immunoglobulin A (IgA) at mucosal surfaces contributes to host defense against intestinal pathogens and governs quantitative and qualitative control of commensal microbiota composition by the host (*1*, *2*). IgA can be induced by distinct T cell–dependent or T cell–independent pathways. T cell–dependent regulation of IgA production takes place mainly in Peyer's patches and requires the formation of germinal centers and the interaction of B cells with follicular helper T cells (*3*). T cell–independent mucosal IgA is produced both in isolated lymphoid follicles (ILFs) and in the lamina propria aided by exposure of B cells to various cytokines and growth factors, without the formation of germinal centers (*4*, *5*).

Lymphotoxin α (LTα) and lymphotoxin β (LTβ) are trimeric cytokines of the tumor necrosis factor (TNF) superfamily that are expressed in either soluble (sLTα$_3$) or membrane-bound (LTα$_1$β$_2$) forms by T and B cells, as well as by retinoic acid–related orphan receptor positive (RORγt$^+$) innate lymphoid cells (ILCs) (*6*). Soluble lymphotoxin is a TNF-like cytokine and its signaling is mediated via both TNFR1 and TNFR2, whereas membrane-bound lymphotoxin signals via LTβR (*6*). Ablation of the surface lymphotoxin–driven pathway via inactivation of the genes encoding LTα, LTβ, or LTβR results in block of lymphoid organ development and in diminished IgA plasma cell numbers in mucosal tissues (*7*, *8*), indicating that membrane-bound lymphotoxin is critical for intestinal IgA production. However, the possible contribution of soluble lymphotoxin to this process is not known.

ILCs were recently described as an important subset of innate immune cells that lack specific antigen receptors but are able to produce a range of effector cytokines (*9*). They are predominantly located in mucosal tissues and provide the first line of defense against various mucosal pathogens (*9*–*14*). In particular, RORγt$^+$ ILCs, via LT production, induce the development of gut-associated lymphoid tissues such as lymph nodes, Peyer's patches, and isolated lymphoid follicles (*8*, *15*, *16*) and are critical for protection against intestinal pathogens (*10*, *11*, *17*), for maintenance of the epithelial barrier, and for the prevention of systemic dissemination of commensal microbiota (*16*, *18*). However, the molecular mechanisms that mediate host control of commensals by RORγt$^+$ ILCs remain largely unknown.

We used mice lacking LTα or LTβ production by RORγt-expressing cells (LTα$^{\Delta ILC,T}$ and LTβ$^{\Delta ILC,T}$ mice, respectively) (*19*, *20*) (fig. S1). Because the transcription factor RORγt is expressed in double positive (CD4$^+$CD8$^+$) thymocytes, these mice also exhibit LT gene deletion in all αβ T cells (*21*) in addition to RORγt$^+$ ILCs. Therefore, to define the role of LT expressed specifically by RORγt$^+$ ILCs, we included mice with T cell ablation of LTα and LTβ, using CD4-Cre transgenic mice (LTα$^{\Delta T}$ and LTβ$^{\Delta T}$, respectively) as controls in all our analyses (fig. S1). Mice lacking expression of either the *LTα* or *LTβ* gene in T cells showed no developmental defects in their secondary lymphoid tissues (*20*), whereas LTβ$^{\Delta ILC,T}$ mice lacked Peyer's patches, isolated lymphoid follicles, and all peripheral lymph nodes except mesenteric (fig. S1), recapitulating the anatomical phenotype of complete LTβ ablation (*22*). LTα$^{\Delta ILC,T}$ mice lacked Peyer's patches, isolated lymphoid follicles, and all lymph nodes (fig. S1). Together, these data demonstrated the role of LT produced by RORγt$^+$ ILCs during embryogenesis for secondary lymphoid organ development (*15*).

Membrane-bound lymphotoxin produced by RORγt$^+$ ILCs is implicated as one of the critical cytokines required for generation of mucosal IgA through the formation of ILFs (*8*). However, LTβ$^{\Delta ILC,T}$ animals that lacked ILFs exhibited normal fecal IgA levels and only slightly diminished blood IgA levels relative to wild-type controls (Fig. 1, A and B). By contrast, concomitant inactivation of surface and soluble lymphotoxins via deletion of the *LTα* gene, in both RORγt$^+$ ILCs and αβ T cells (but not in αβ T cells alone), led to a striking decrease in both blood and fecal IgA levels and was essential for the presence of IgA$^+$ cells in the lamina propria (Fig. 1, A to C). By contrast, LTα expression by T cells was not required either for IgA production or for the recruitment of major immune cell subsets in the small intestine (Fig. 1, A to C, and fig. S2). To rule out a possible contribution from mesenteric lymph nodes that are present in LTβ$^{\Delta ILC,T}$ animals (but not in LTα$^{\Delta ILC,T}$ mice), we established bone marrow transfers into lethally irradiated LTα-deficient recipients that lacked gut-associated lymphoid tissue. In contrast to wild-type, LTα$^{\Delta T}$, and LTβ$^{\Delta ILC,T}$ bone marrow cells, transfer of LTα$^{\Delta ILC,T}$ bone marrow cells failed to induce IgA in recipient mice (Fig. 1D); this result implies a direct role of LTα produced by RORγt$^+$ ILCs in this process, irrespective of the presence of mesenteric lymph nodes and consistent with previous findings in LTα-deficient mice (*7*, *23*).

One of the known functions of IgA in mucosal tissues is to contain and control the composition of commensal microbiota (*24*). Deep sequencing analysis of ileal commensal microflora in wild-type and LTα$^{\Delta ILC,T}$ animals, and further real-time polymerase chain reaction (PCR) of selected intestinal commensals, revealed a marked expansion of segmented filamentous bacteria and a reduction in Bacteroidetes in mice lacking LTα expression by RORγt$^+$ cells (Fig. 1, E and F), implicating LT expression by RORγt$^+$ ILCs in the control of gut microbiota.

Although wild-type, LTα$^{\Delta T}$, LTβ$^{\Delta ILC,T}$, LTα$^{\Delta ILC,T}$, and LTα$^{-/-}$ animals showed similar ILC numbers in the lamina propria (figs. S3 and S4), c-Kit and CCR6 expression by the small intestinal lymphoid tissue inducer-like (LTi) (CD45$^+$Thy1.2$^+$ c-Kit$^{high}$IL-7Rα$^+$CCR6$^+$) cells was controlled by membrane-bound lymphotoxin produced by RORγt$^+$ ILCs (fig. S3). However, comparable phenotypic changes were found in adult LTi cells from LTβ$^{\Delta ILC,T}$, LTα$^{\Delta ILC,T}$, and LTα$^{-/-}$ mice (fig. S4); therefore, this cannot explain the lack

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of intestinal IgA observed in the two latter gene-deficient animals.

Signaling via both TNFR1 and TNFR2 contributed to intestinal IgA production, whereas complete genetic ablation of TNF did not affect fecal IgA levels (fig. S5), consistent with the role for soluble LT produced by RORγt$^+$ ILCs. Furthermore, both TNFR1 and TNFR2 expressed by non-hematopoietic cells contributed to IgA induction, as revealed by reciprocal bone marrow transfer experiments (fig. S5). Therefore, soluble lymphotoxin acts via TNFR1 and TNFR2 expressed by lamina propria stromal cells to promote IgA production.

In the context of intestinal immunity, LTβR signaling is important for B cell homing to the gut (*7*). We found significantly reduced expression of chemokine and adhesion molecules such as CXCL13, VCAM-1, and CCL20 in the small intestine upon ablation of LTα, but not of LTβ, from RORγt$^+$ cells, whereas MAdCAM-1 and CCL21 expression remained unaffected (fig. S5). Moreover, both soluble and membrane-bound lymphotoxins expressed by RORγt$^+$ ILCs facilitated the homing of lamina propria IgM$^+$ B cells (Fig. 2, A and B).

Because intestinal IgA plasma cells can develop from peritoneal IgM$^+$ B cells recruited to the gut (*25*), we assessed the influence of LTα and LTβ expression by RORγt$^+$ ILCs in the peritoneal B cell compartment. Indeed, peritoneal cavity exudate cells from both LTα$^{\Delta ILC,T}$ and LTβ$^{\Delta ILC,T}$ mice contained increased numbers of B1 and B2 B cells (fig. S6), whereas numbers of B cells in the spleen and bone marrow were normal (fig. S6). Additionally, B cells from LTα$^{\Delta ILC,T}$ mice were able to undergo class switching toward IgA in vitro, ruling out a B cell–intrinsic defect in class switch recombination (fig. S6).

Unexpectedly, when LTβ$^{\Delta ILC,T}$ mice were crossed onto a T cell receptor (TCR) αβ–deficient background, we found reduced IgA levels both in the feces and blood (Fig. 2, C and D), which correlated with the absence of IgA$^+$ plasma cells in the lamina propria (Fig. 2, E to G). T cell deficiency in LTβ$^{\Delta ILC,T}$ mice did not further affect homing of B cells to the lamina propria (Fig. 2F and fig. S7). B cell proliferation, activation, and expression of activation-induced cytidine deaminase (AID) are all required for class switch recombination in B cells (*26*), and, indeed, no AID mRNA was detected in freshly isolated lamina propria lymphocytes from LTβ$^{\Delta ILC,T}$ TCRαβ$^{-/-}$ mice, whereas lamina propria lymphocytes from LTβ$^{\Delta ILC,T}$ mice showed AID expression (fig. S7). Moreover, lamina propria B cells in LTβ$^{\Delta ILC,T}$ mice were actively proliferating as revealed by KI67 staining (fig. S7). Taken together, these findings indicate that in the absence of surface lymphotoxin expression by RORγt$^+$ ILCs, IgA class switching can occur in the lamina propria, and that αβ T cells are crucial for this process.

LT expression by RORγt$^+$ ILCs did not affect the development of various dendritic cell (DC) subsets

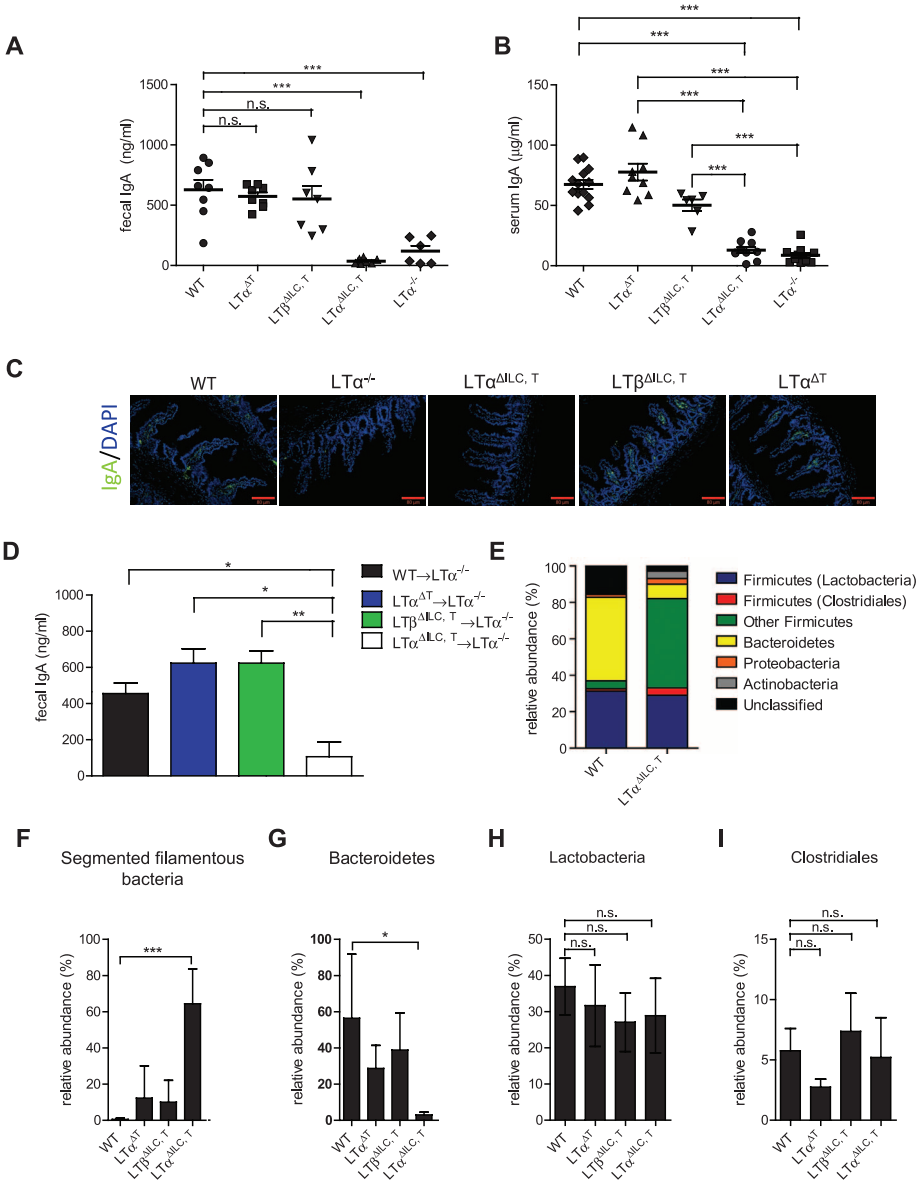


**Fig. 1. Soluble LTα$_3$ produced by RORγt$^+$ ILCs regulates IgA production and microbiota composition in the gut.** (**A**) Fecal IgA levels in naïve wild-type (WT), LTα$^{\Delta T}$, LTα$^{\Delta ILC,T}$, LTβ$^{\Delta ILC,T}$, and LTα$^{-/-}$ animals. (**B**) Serum IgA levels in naïve WT, LTα$^{\Delta T}$, LTα$^{\Delta ILC,T}$, LTβ$^{\Delta ILC,T}$, and LTα$^{-/-}$ animals. (**C**) Immunofluorescence analysis of IgA expression in the small intestine in naïve mice lacking LTα and LTβ expression by RORγt$^+$ cells. Scale bars, 80 μm. (**D**) Fecal IgA levels in LTα$^{-/-}$ recipients, reconstituted with WT, LTα$^{\Delta T}$, LTα$^{\Delta ILC,T}$, and LTβ$^{\Delta ILC,T}$ bone marrow. Feces were collected 2 months after bone marrow transfer; IgA fecal levels were measured as described (*30*). (**E**) Deep sequencing analysis of microbiota composition from terminal ileum of LTα$^{\Delta ILC,T}$ mice and littermate WT controls. Representative microbiota composition in WT and LTα$^{\Delta ILC,T}$ ileum is presented ($n$ = 2 mice per group). (**F** to **I**) Real-time PCR analysis of microbiota composition in terminal ileum of naïve WT, LTα$^{\Delta T}$, LTα$^{\Delta ILC,T}$, and LTβ$^{\Delta ILC,T}$ animals. Error bars, SEM; *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001 (Student's $t$ test). All data, except deep sequencing analysis, are representative of two or more independent experiments with $n \geq 3$ mice.

in mesenteric lymph nodes and in the small intestine (fig. S8), but $LT\alpha_1\beta_2$ expression by $ROR\gamma t^+$ cells did control inducible nitric oxide synthase (iNOS) expression by mesenteric lymph node $CD11c^+$ DCs (Fig. 2H), which is known to be critical for IgA induction (*27*, *28*). Moreover, $CD11c^+$ DCs isolated from mesenteric lymph nodes of $LT\beta^{\Delta ILC,T}$ mice were less potent in inducing IgA in vitro (Fig. 2I). Together, these data indicated that $LT\alpha_1\beta_2$ may control T cell–independent IgA production via regulation of iNOS expression by DCs. Furthermore, our analysis revealed a reduction of CD40L mRNA levels in the small intestine of $LT\beta^{\Delta ILC,T}$ mice after T cell ablation (Fig. 3A). When the T cell compartment in $LT\beta^{\Delta ILC,T}$ $TCR\beta\delta^{-/-}$ animals was reconstituted with wild-type or $CD40L^{-/-}$ αβ T cells, we found that wild-type, but not $CD40L^{-/-}$, T cells could induce IgA production (Fig. 3, B and C). Interestingly, LTα, but not LTβ, expressed by $ROR\gamma t^+$ cells controlled T cell numbers in the lamina propria (Fig. 3, D and E). However, LTα produced by $ROR\gamma t^+$ cells did not affect the numbers of gut-homing T cells on the periphery (fig. S8).

Consistently, we found significant reduction of CD40L mRNA expression in $LT\alpha^{\Delta ILC,T}$ animals relative to littermate controls (Fig. 3F), which led us to hypothesize that soluble lymphotoxin may regulate IgA induction via control of T cell homing to the lamina propria. Indeed, forced activation of CD40 signaling by agonistic antibody in $LT\alpha^{\Delta ILC,T}$ mice resulted in IgA induction (Fig. 3, G and H) without affecting T and B cell homing to the small intestine (fig. S9), further implying that T cells may contribute to the regulation of IgA switching in the lamina propria via the CD40-CD40L pathway. Notably, the induction of unspecific intestinal inflammation, such as dextran sodium sulfate–induced colitis, failed to induce generation of IgA plasma cells (fig. S9). Collectively, these data indicated that $sLT\alpha_3$ derived from ILCs may control IgA induction via regulation of T cell homing to the lamina propria.

Induction of fully competent adaptive immune responses in the intestinal tract is a host defense mechanism directed against potential pathogens, which also allows control of commensal microbiota by the host. Here, we delineated distinct functions for membrane-bound and soluble lymphotoxins expressed by $ROR\gamma t^+$ ILCs in the induction of IgA in the lamina propria (fig. S10). We found that production of membrane-bound lymphotoxin by ILCs regulates T cell–independent IgA induction via iNOS production by $CD11c^+$ DCs. We further demonstrated that ILC-derived soluble LT regulates the

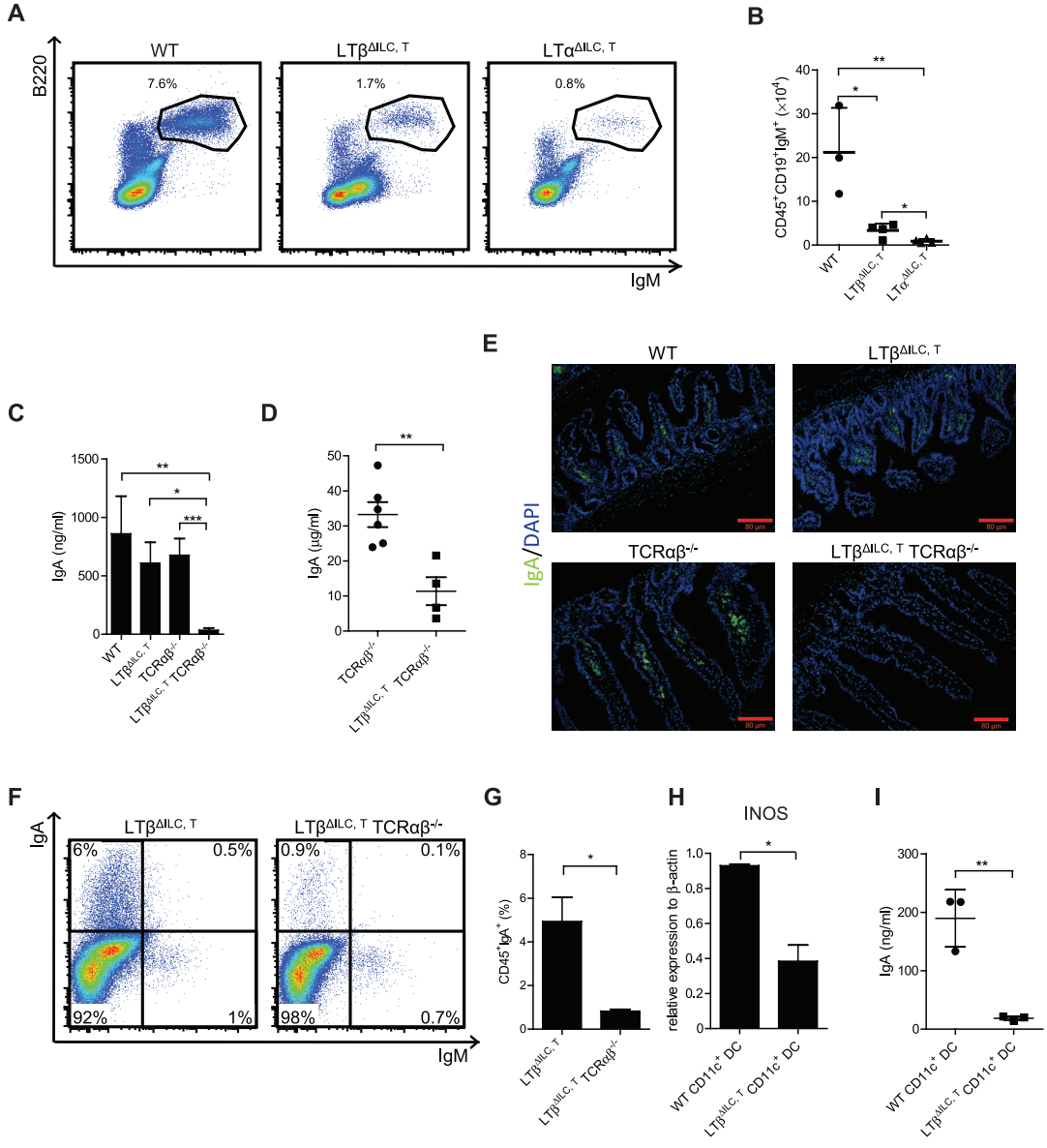


**Fig. 2. Membrane-bound $LT\alpha_1\beta_2$ produced by $ROR\gamma t^+$ cells controls T cell–independent IgA induction in the lamina propria in the absence of organized gut-associated lymphoid tissue.** (**A**) B cell frequencies in the lamina propria of WT, $LT\beta^{\Delta ILC,T}$, and $LT\alpha^{\Delta ILC,T}$ mice. (**B**) Numbers of $CD45^+CD19^+IgM^+$ cells in the lamina propria of WT, $LT\beta^{\Delta ILC,T}$, and $LT\alpha^{\Delta ILC,T}$ mice. (**C**) Fecal IgA levels in WT, $LT\beta^{\Delta ILC,T}$, $TCR\alpha\beta^{-/-}$, and $LT\beta^{\Delta ILC,T}$ $TCR\alpha\beta^{-/-}$ mice. (**D**) Serum IgA levels in $TCR\alpha\beta^{-/-}$ and $LT\beta^{\Delta ILC,T}$ $TCR\alpha\beta^{-/-}$ mice. (**E**) Immunofluorescence analysis of IgA expression in the small intestine in WT, $LT\beta^{\Delta ILC,T}$, $TCR\alpha\beta^{-/-}$, and $LT\beta^{\Delta ILC,T}$ $TCR\alpha\beta^{-/-}$ mice. Scale bar, 80 μm. (**F** and **G**) Representative fluorescence-activated cell sorter (FACS) dot plots (F) and frequencies (G) of $CD45^+IgA^+$ cells in lamina propria of $LT\beta^{\Delta ILC,T}$ and $LT\beta^{\Delta ILC,T}$ $TCR\alpha\beta^{-/-}$ mice. (**H**) iNOS mRNA expression levels in $CD11c^+$ cells sorted from mesenteric lymph nodes. (**I**) IgA levels in 5-day cultures of WT splenic $IgM^+$ B cells together with $CD11c^+$ DCs isolated from mesenteric lymph nodes of WT or $LT\beta^{\Delta ILC,T}$ mice. All data are representative of two or more independent experiments with $n \geq 3$ mice. Error bars, SEM; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test).

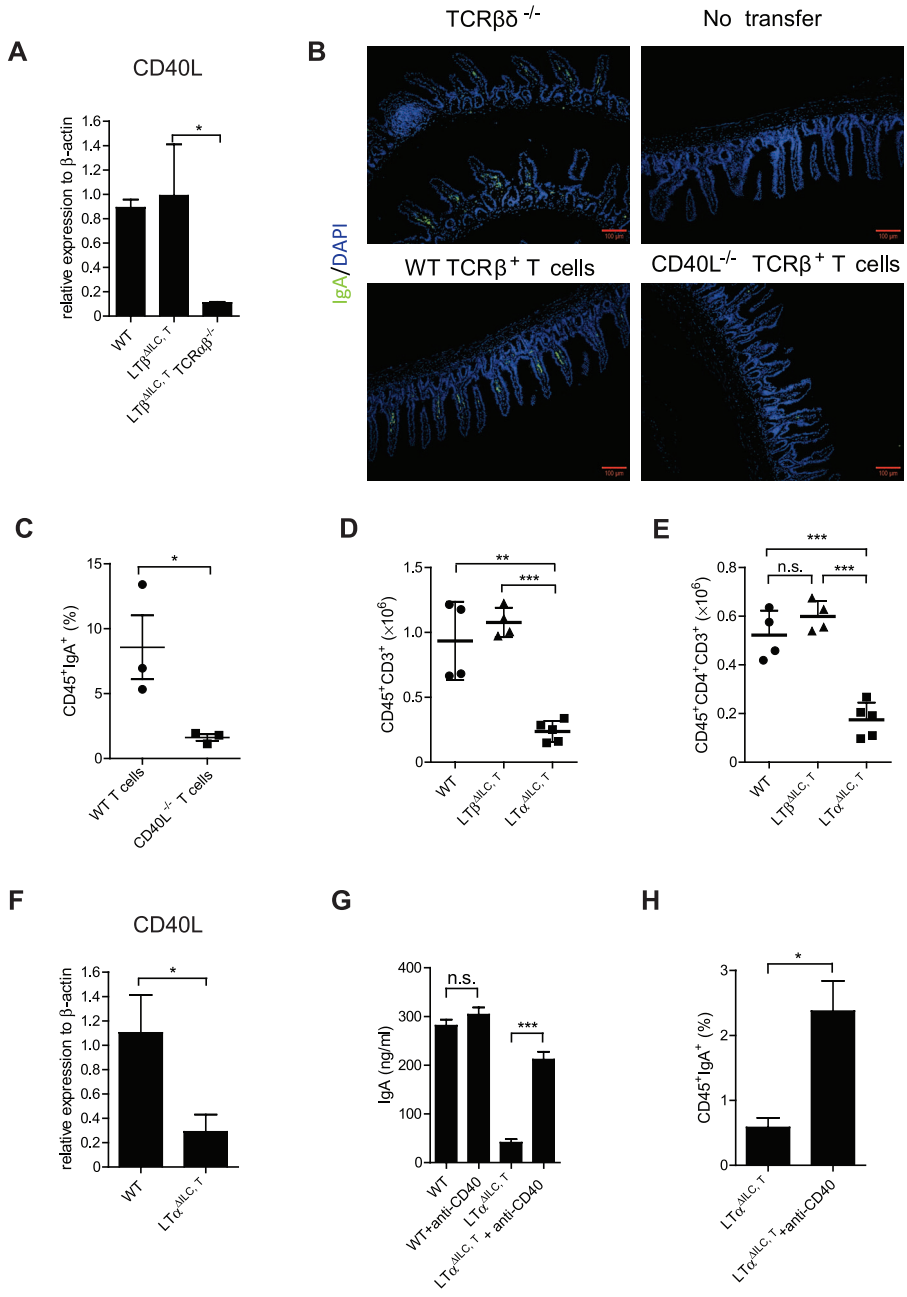


**Fig. 3. Regulation of T cell–dependent IgA production by soluble LTα$_3$ produced by RORγt$^+$ ILCs.** (**A**) CD40L mRNA levels in jejunum of naïve WT, LTβ$^{\Delta ILC,T}$, and LTβ$^{\Delta ILC,T}$ TCRαβ$^{-/-}$ mice. (**B**) Immunofluorescence analysis of IgA expression in the small intestine in naïve TCRβδ$^{-/-}$, LTβ$^{\Delta ILC,T}$ TCRβδ$^{-/-}$ mice, as well as in LTβ$^{\Delta ILC,T}$ TCRβδ$^{-/-}$ mice, reconstituted with WT or CD40L$^{-/-}$ αβ T cells. Scale bar, 100 μm. (**C**) Frequency of CD45$^+$IgA$^+$ cells in the lamina propria of LTβ$^{\Delta ILC,T}$ TCRβδ$^{-/-}$ mice 2 weeks after adoptive transfer of WT or CD40L$^{-/-}$ αβ T cells. (**D** and **E**) Numbers of CD45$^+$CD3$^+$ cells (D) and CD45$^+$CD3$^+$CD4$^+$ cells (E) in the lamina propria of WT mice and mice with LTα or LTβ ablation in RORγt$^+$ cells. (**F**) CD40L mRNA levels in jejunum of WT and LTα$^{\Delta ILC,T}$ animals. (**G**) In vivo induction of IgA in LTα$^{\Delta ILC,T}$ mice by agonistic antibody to CD40. (**H**) CD45$^+$IgA$^+$ cells in the lamina propria of LTα$^{\Delta ILC,T}$ mice after anti-CD40 treatment. All data are representative of two or more independent experiments with $n \geq 3$ mice. Error bars, SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's $t$ test).

T cell–dependent pathway of IgA production via control of T cell homing to the gut. Through these processes, lymphotoxins produced by RORγt$^+$ ILCs control microbiota composition in the host and may influence various pathophysiological processes. Our findings highlight a rare nonredundant function of soluble lymphotoxin and may be relevant for anti-TNF therapy using etanercept, as this drug can block not only TNF but also soluble lymphotoxin (*29*), and thus the effects of such treatment may affect IgA levels and gut microbiota in patients.

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30. See supplementary materials on *Science* Online.



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## Supplementary Materials

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Materials and Methods
Figs. S1 to S10
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