



IMMUNE REGULATION

Cross-talk between ILC2 and Gata3^{high} T_{regs} locally constrains adaptive type 2 immunity

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Regulatory T cells (T_{regs}) control adaptive immunity and restrain type 2 inflammation in allergic disease. Interleukin-33 promotes the expansion of tissue-resident T_{regs} and group 2 innate lymphoid cells (ILC2s); however, how T_{regs} locally coordinate their function within the inflammatory niche is not understood. Here, we show that ILC2s are critical orchestrators of T_{reg} function. Using spatial, cellular, and molecular profiling of the type 2 inflamed niche, we found that ILC2s and T_{regs} engage in a direct (OX40L-OX40) and chemotaxis-dependent (CCL1-CCR8) cellular dialogue that enforces the local accumulation of Gata3^{high} T_{regs}, which are transcriptionally and functionally adapted to the type 2 environment. Genetic interruption of ILC2-T_{reg} communication resulted in uncontrolled type 2 lung inflammation after allergen exposure. Mechanistically, we found that Gata3^{high} T_{regs} can modulate the local bioavailability of the costimulatory molecule OX40L, which subsequently controlled effector memory T helper 2 cell numbers. Hence, ILC2-T_{reg} interactions represent a critical feedback mechanism to control adaptive type 2 immunity.

INTRODUCTION

Unlike epithelial or mesenchymal cells, whose cellular densities are constrained by the structural boundaries of tissues and their micro-environment, immune cell densities are largely dictated by local inflammatory mediators or the abundance of mitogens (1). Hence, inflamed organs can rapidly and profoundly increase immune cell numbers. Whereas this capacity evolved to counter pathogens, concomitant regulatory mechanisms are equally important to maintain a fine balance between protective and destructive inflammation. Regulatory T cells (T_{regs}) are centrally involved in this homeostatic process, although many questions persist about how diverse T_{reg} subsets locally curtail different types of inflammation (2).

T_{reg} deficiency or depletion often results in unrestrained type 2 inflammation (3–5), although it remains unclear whether specific T_{reg} subsets are elicited to suppress CD4⁺ T helper 2 (T_H2) cells. Shared transcription factors between T_{regs} and T_H2 cells, such as Irf4 (6) and Gata3 (7), are important for T_{reg} function in models of type 2 inflammation; however, T_{reg}-specific deletion of these transcription factors is confounded by their graded or low constitutive expression and concomitant homeostatic or developmental roles (8). It is therefore likely

that shared transcriptional programs promote colocalization of effector T_H2 cells and T_{regs} in the type 2 inflamed niche, although this paradigm is better defined for type 1 or type 17 inflammation (9–11). Regulatory-to-effector T cell ratios strongly influence local control of allergic inflammation (12), and whereas allergenic peptides underpin pathogenic T_H2 cell-driven inflammation, less is known about the mechanisms that guide T_{reg}-mediated restraint of adaptive type 2 immunity (13, 14).

Epithelial or stromal cell sensing of tissue stress initiates type 2 immune activation via the release of the alarmin interleukin-33 (IL-33), which acts on both innate and adaptive type 2 immune cells expressing its receptor ST2. Although IL-33 strongly promotes type 2 immunity, it also mediates the suppressive functions of ST2⁺ T_{regs} (15, 16) and organ-homeostatic roles of ST2⁺ tissue-resident T_{regs} (17–19). Whereas IL-33 can directly influence ST2⁺ T_{reg} function, other reports suggest that indirect effects of IL-33 guide the overall T_{reg} response (20–22). Lung-resident group 2 innate lymphoid cells (ILC2s) not only respond to IL-33 and promote rapid innate type 2 inflammation via secretion of inflammatory cytokines but also influence downstream adaptive type 2 immunity (23). Specifically, the expression of the costimulatory ligand OX40L (*Tnfsf4*) by ILC2s is critical for simultaneous T_H2 and T_{reg} expansion in type 2 inflammation (20). It remains uncertain whether OX40 expression by T_{regs} is required for their local expansion and why ILC2s can simultaneously engage T_H2 cell-driven effector immunity and T_{reg}-driven regulatory immunity.

Here, we asked how ILC2s interact with both the effector and regulatory arms of adaptive type 2 immunity, hypothesizing that ILC2-T_{reg} interactions serve as a negative feedback mechanism in the type 2 inflamed niche. Using spatial, cellular, and molecular profiling of the type 2 inflamed niche, we found that both T_H2 and Gata3^{high} T_{regs} localized closely with ILC2s. Gata3^{high} T_{regs} were greatly enriched in type 2 inflammation because of ILC2-T_{reg} dialogue involving CCL1-CCR8 and OX40L-OX40 signaling. Unbiased profiling of OX40L-expressing

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cells using a gene reporter identified ILC2s as the major cellular source in both the type 2 inflamed lung and its draining lymph node. Using OX40 T_{reg} -conditional knockout mice, we found that $Gata3^{high}$ T_{reg} expansion was greatly impaired in response to IL-33-driven allergic inflammation and, moreover, resulted in the profound local expansion of type 2 immune cells. Mechanistically, we found that $Gata3^{high}$ T_{regs} can directly modulate OX40L surface expression on ILC2s; the increased bioavailability of this costimulatory molecule in OX40 T_{reg} -deficient mice underpinned unrestrained expansion of effector memory (EM) T_H2 cells in the lung and associated lymph nodes. Thus, ILC2s serve as a critical local orchestrator of T_{reg} -mediated constraint of adaptive type 2 immunity.

RESULTS

Innate and adaptive $Gata3^{high}$ lymphocytes with effector and regulatory function coexpand and interact in the type 2 inflamed niche

Both innate and adaptive type 2 effector lymphocytes depend on the transcription factor $Gata3$ for their development and function, which can also be expressed by T_{regs} . However, the functional roles and upstream regulation of $Gata3^{high}$ T_{regs} in type 2 inflammation remain poorly understood. To study the type 2 inflamed niche, we exposed mice to IL-33 or allergens, such as papain, house dust mite, or ragweed pollen extract, and found that $Gata3^{high}$ T_{regs} were expanded in the lungs, along with $Gata3^{high}$ T_H2 cells and ILC2s (Fig. 1A and fig. S1, A and B). Focusing on $Gata3^{high}$ T_{regs} , we observed that this subset was largely $CD62L^-CD44^+$ and highly expressed $KLRG1$, a marker associated with tissue-resident T_{regs} (Fig. 1A and fig. S1C). By intravenous administration of anti- $CD45.2$ monoclonal antibody (mAb), we confirmed that $Gata3^{high}$ $KLRG1^+$ T_{regs} showed increased tissue residency compared with $Gata3^{low}$ $KLRG1^+$ T_{regs} , whereas lung ILC2s were almost exclusively tissue resident (fig. S1D). Next, using multiplex immunofluorescence (IF) microscopy of lung sections, we identified $CD3^+Foxp3^+$ T_{regs} , $CD3^+Gata3^+Foxp3^-$ T_H2 cells, and $CD3^-Gata3^+$ ILC2s after IL-33 administration (fig. S1E). Costaining for CD31 and type I collagen confirmed that ILC2s localized close to the adventitial sheath surrounding large vessels and airways (24), where we also observed T_{regs} and T_H2 cells (Fig. 1B). Image analysis indicated that T_{regs} and T_H2 cells localized closer to ILC2s than $CD3^+Gata3^-Foxp3^-$ conventional T (T_{conv}) cells (Fig. 1C). Similarly, two-photon microscopy of $Il5^{tdTomato}Foxp3^{YFP}$ mouse precision-cut lung explants (25) showed that ILC2s and T_{regs} colocalized after IL-33 administration (Fig. 1D and fig. S1F). Hence, we hypothesized that both effector and regulatory $Gata3^{high}$ lymphocytes have transcriptional programs that enforce their colocalization, as well as potential dialogue, in the type 2 inflamed niche.

To isolate innate and adaptive type 2 lymphocytes, we used $Gata3^{YFP}$ reporters [which retain endogenous $Gata3$ expression (26)] and generated $Gata3^{YFP}Foxp3^{RFP}$ mice (Fig. 1E). We could accurately identify distinct $Gata3^{high}$ $CD4^+$ T cell types and ILC2s, which all expanded in the lung after intranasal IL-33 administration; using flow sorting, we purified these and $Gata3^{low}$ T_{reg} and $CD4^+$ T_{conv} cell subsets for bulk RNA sequencing (RNA-seq) analysis (fig. S1, G to J). We found that ILC2s, T_{regs} , T_H2 cells, and T_{conv} cells expressed their respective lineage-associated genes (Fig. 1F) and that the $Gata3$ expression levels matched that of the reporter (Fig. 1E). T_H2 cells and $Gata3^{high}$ T_{regs} expressed high levels of *Tnfrsf4* (OX40), *Klrg1*, and *Cd44*, whereas *Sell* (CD62L) was amplified in $Gata3^{low}$ T_{regs} and T_{conv} cells, matching our

flow cytometry data (fig. S1C). T_H2 cells as well as $Gata3^{high}$ and $Gata3^{low}$ T_{regs} expressed both *Ccr4* and *Ccr8* (Fig. 1F), which are important for T_{reg} and T_H2 cell migration into the type 2 inflamed niche (27–29). $Gata3^{high}$ T_{regs} expressed more *Ccr8* transcript and surface protein compared with the other T cell subsets (Fig. 1G). Moreover, *Ccr7* expression was low on $Gata3^{high}$ T_{regs} , whereas $Gata3^{low}$ T_{regs} and T_H2 cells expressed intermediate levels compared with T_{conv} cells (Fig. 1F). $Gata3^{high}$ T_{regs} , T_H2 cells, and ILC2s all selectively expressed *Cxcr6* (Fig. 1F), which is associated with retention of lymphocytes in the tissue niche (30). Thus, whereas all T_{regs} and T_H2 cells have receptors to enter the type 2 inflamed lung niche, $Gata3^{low}$ T_{regs} do not highly express genes required for retention in the lungs. Conversely, $Gata3^{high}$ T_{regs} (and T_H2 cells to a lesser degree) had a stronger gene signature related to tissue residency (fig. S1K).

We next performed T cell receptor (TCR) diversity analysis on the sorted T cell populations. We found that $Gata3^{high}$ T_{regs} exhibited substantially reduced TCR diversity and increased proportions of hyper-expanded clones compared with $Gata3^{low}$ T_{regs} and T_{conv} cells (Fig. 1H and fig. S1L). T_H2 cells in IL-33-treated mice were clonally hyperexpanded, and $Gata3^{high}$ T_{regs} and T_H2 cells expressed higher levels of *Nr4a1* (Nur77), indicative of increased TCR stimulation (Fig. 1F). These findings suggest that endogenous T_H2 cells expand in response to local antigen or costimulation.

To investigate how type 2 lymphocytes interact in the inflamed niche, we performed in silico receptor-ligand interactome analysis using CellChat (Fig. 1, I and J, fig. S1M, and data file S1) (31). Predicted heterotypic interactions were strongest between ILC2s and T_{regs} or T_H2 cells. *Tnfrsf4-Tnfrsf4* was predicted to preferentially engage $Gata3^{high}$ T_{regs} while also showing an affinity for $Gata3^{low}$ T_{regs} and T_H2 cells. ILC2s were predicted to engage in multiple autocrine interactions, including via CCL1-CCR8 signaling, as previously reported (32), and our results also suggested that ILC2s preferentially engage $Gata3^{high}$ T_{regs} by this interaction. To determine whether this chemokine axis is important for the IL-33-driven expansion of $Gata3^{high}$ T_{regs} , we next used *Ccr8*^{-/-} mice. Whereas $Gata3^{high}$ T_{reg} numbers were similar in both genotypes at rest, IL-33 administration resulted in impaired expansion of $Gata3^{high}$ T_{regs} in *Ccr8*^{-/-} mice compared with wild-type control mice (Fig. 1K). The diminished but significant effect of IL-33 in *Ccr8*^{-/-} mice suggested that other factors contributed.

Last, we asked whether similar T_{reg} subsets were present in human lungs using a publicly available single-cell RNA-seq dataset of patients with lung cancer (fig. S2A) (33). Unsupervised clustering of *FOXP3*⁺ T_{regs} revealed six subclusters (fig. S2B). Clusters 1, 4, and 6 expressed high *TNFRSF4*, *CCR8*, *CXCR6*, and *GATA3* transcripts, whereas clusters 2 and 3 were *GATA3*^{low} and expressed high *CCR7* (fig. S2C), in agreement with our murine observations.

These data suggested that tissue-resident ILC2s rapidly attract and engage T_{regs} after exposure to IL-33. Moreover, whereas chemokines are important, it is likely that other costimulatory ligand-receptor interactions contribute to the local expansion of $Gata3^{high}$ T_{regs} .

ILC2s orchestrate IL-33-dependent expansion of tissue-resident $Gata3^{high}$ T_{regs} in diverse anatomical sites

IL-33 has a profound effect on the local expansion and maintenance of T_{regs} in different organs and tissues; however, it remains unclear whether this is due to direct IL-33 signaling. To address this question, we generated mixed bone marrow chimeras (BMCs) using congenic *Il1rl1*^{-/-} and C57Bl/6 wild-type donors (Fig. 2A and fig. S3A). We found no substantial effect on tissue-resident T_{reg} numbers or

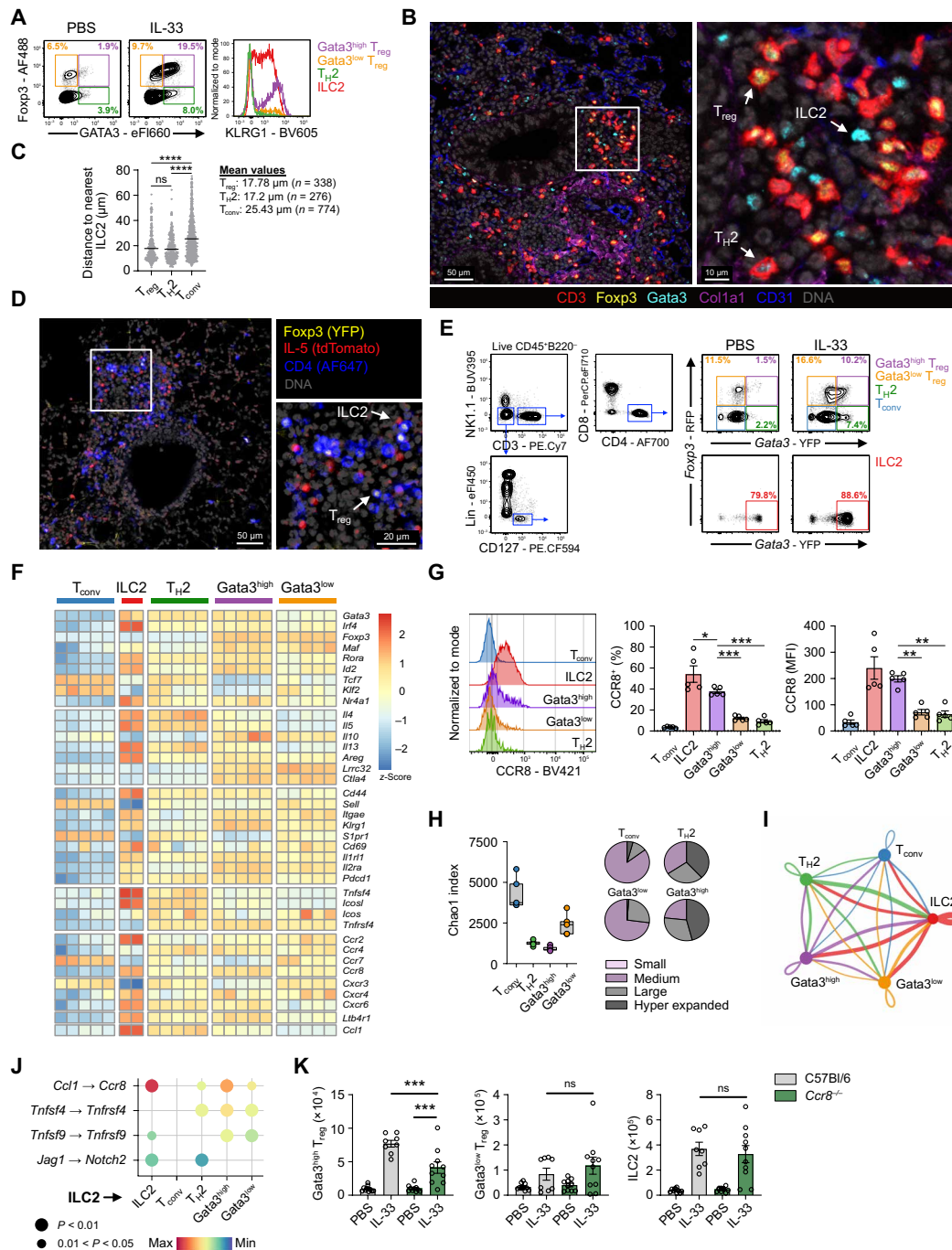


Fig. 1. Type 2 effector and regulatory lymphocytes express gene signatures that enforce localization and enable a cross-talk mechanism in the inflamed lung niche. (A to C) Wild-type mice were treated with PBS or IL-33 (intranasal, days 0 and 1) followed by analysis on day 5 for the indicated lung CD4⁺ T cell subsets (left) and their expression of KLRG1 (right) (A). Multiplex IF microscopy of IL-33-treated mouse lung sections allowed the identification of T_{conv} cells, T_H2 cells, T_{reg}s, and ILC2s (B), followed by distance analysis between the indicated cell types [(C); see fig. S1E]. (D) ILC2s (Il5^{tdTomato} CD4⁺) and T_{reg}s (Foxp3^{YFP} CD4⁺) were visualized in precision-cut lung sections of IL-33-treated Il5^{tdTomato}/Foxp3^{YFP} mice. (E to J) Gata3^{YFP} Foxp3^{RFP} mice were treated as in (A) to identify CD4⁺ T cell subsets and ILC2s (E). Bulk RNA-seq data for the indicated populations (top row), flow-sorted from the lungs of Gata3^{YFP} Foxp3^{RFP} reporter mice (F), and analysis of the percentage expression (middle) and mean fluorescence intensity (right) of CCR8 (G) are presented. TCR diversity analysis of the indicated T cell populations (left) is presented; pie charts show the proportions of the indicated clonal subgroups (right) in the indicated T cell populations (H). CellChat analysis indicates predicted homotypic and heterotypic interactions; the color of the lines represents a direction (i.e., ligand expression by the same color cell), whereas the width represents the number of interactions (I). Several interactions were predicted whereby ILC2s engaged themselves or the indicated T cell population (bottom axis) (J). (K) Wild-type or Ccr8^{-/-} mice were treated with PBS or IL-33 (intraperitoneal, days 0 and 1) followed by the quantification of the indicated lung cell types on day 5. Microscopy images are representative of two (B) or three (D) independent experiments. Histogram images are representative of more than three (A) or two (G) independent experiments. Bar graphs indicate means (±SEM) and show representative data of two (C), three [n = 5, 5, 5, and 5 (G)], and two [n = 6, 4, 6, and 6 (K)] independent experiments. ns, not significant; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001. Statistical analyses: one-way ANOVA with Tukey postanalysis.

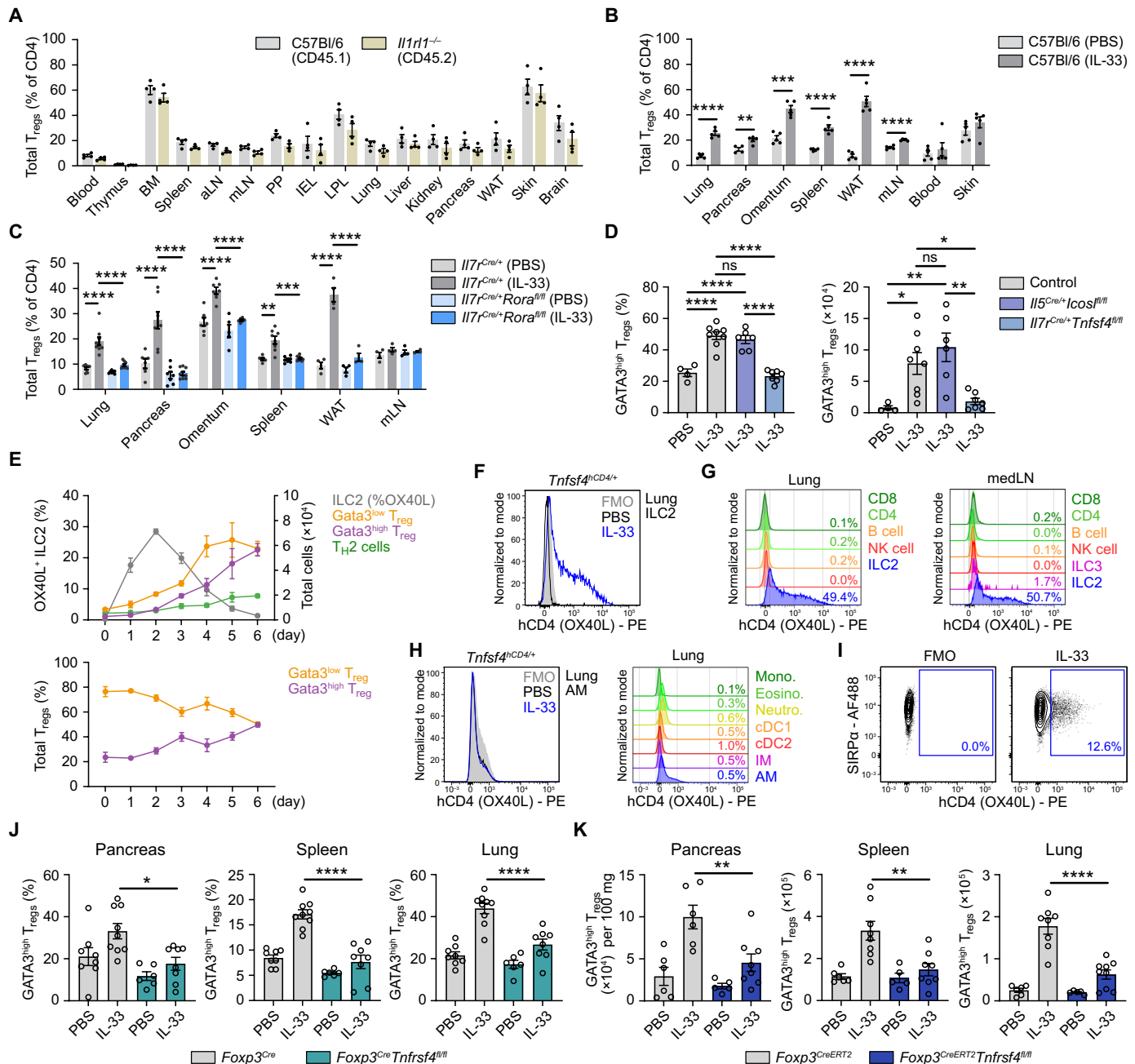


Fig. 2. ILC2s and the OX40L-OX40 signaling axis control tissue-specific $Gata3^{high} T_{reg}$ responses to IL-33. Donor-derived wild-type and $Il1rl1^{-/-}$ T_{reg} percentages in the indicated tissues of untreated BMC mice (A). T_{reg} percentages in the indicated tissues of PBS or IL-33-treated (intraperitoneal) wild-type mice (B) and $Il17r^{Cre/+}$ or $Il17r^{Cre/+} Rora^{fl/fl}$ mice (C). Mice of the indicated genotype were treated with PBS or IL-33 (intranasal, days 0 and 1) followed by the quantification of lung Gata3^{high} percentages (% of T_{reg} s) and numbers on day 5 (D). Wild-type mice were treated with IL-33 (intranasal, days 0 and 1), followed by the analysis of lungs at the indicated time points for OX40L expression by ILC2s (top, left axis), total numbers (top, right axis), or percentages (bottom) of the indicated cell types (E). (F to I) $Tnfrsf4^{hCD4/+}$ mice were treated with IL-33 or PBS (intranasal, days 0 and 1) followed by the measurement on day 3 of hCD4s on lung ILC2s (F), lung and medLN lymphocytes (G), lung myeloid cells (H), AM staining shown left), and medLN cDC2s (I). (J and K) Mice of the indicated genotypes were treated with PBS or IL-33 as in (B), followed by the quantification of Gata3^{high} T_{reg} percentages (% of T_{reg} s) (J) and numbers (K). Dot plots show the representative gating strategy; numbers indicate the percentage of gated cells. Bar graphs indicate means ($\pm$ SEM) and show data from one experiment [$n = 4$ for all (A)], representative data from two independent experiments [$n = 4$ or 5 per group (B)], or pooled data from two independent experiments with [$n = 4$ to 9 (C)], [$n = 4, 8, 6$, and 7 (D)], [$n = 4$ for day 0 and $n = 6$ for all other days (E)], [$n = 8, 9, 6$, and 8 (left); $n = 8, 9, 6$, and 8 (middle); $n = 8, 9, 6$, and 8 (right) (J)], and [$n = 6, 6, 5$, and 8 (left); $n = 6, 8, 5$, and 8 (middle); $n = 6, 8, 5$, and 9 (right) (K)] mice per group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$. Statistical analyses: one-way ANOVA with Tukey postanalysis.

proportions due to cell-intrinsic loss of the IL-33 receptor in many organs tested, including the lungs, but also nonmucosal sites such as the pancreas and adipose tissue (Fig. 2A). Conversely, IL-33 administration resulted in the rapid expansion of T_{regs} in many peripheral sites (Fig. 2B). Nonmucosal sites such as the pancreas also exhibited colocalization of ILC2s and T_{regs} after IL-33 exposure, suggesting that IL-33-responsive ILC2s may coordinate local T_{reg} expansion (fig. S3B). IL-33 also induced the rapid and selective expansion of pancreatic $\text{Gata3}^{\text{high}} T_{\text{regs}}$, as seen in the lungs, which preferentially expressed KLRG1 and were tissue resident on the basis of intravenous CD45.2-labeling experiments (fig. S3, C and D).

To ask whether ILC2s are important mediators of local T_{reg} expansion, we used ILC2-deficient $Il7r^{\text{Cre/+}}Rora^{\text{fl/fl}}$ or $Il7r^{\text{Cre/+}}$ control mice. Systemic administration of IL-33 induced the rapid expansion of T_{regs} in most anatomical sites of control mice, which was significantly impaired in ILC2-deficient animals (Fig. 2C). These results demonstrated that the effect of IL-33 on local T_{reg} cell expansion in many anatomical sites can be mediated by ILC2s.

It is known that costimulatory ligands can influence local ILC2- T_{reg} cell interactions, including ICOSL and OX40L (20, 21). To study these costimulatory molecules, we crossed $Il5^{\text{Cre}}$ (34) to $Icosl^{\text{fl/fl}}$ (35) mice or used $Il7r^{\text{Cre/+}}Tnfrsf4^{\text{fl/fl}}$ mice (20), where these ligands are deleted in ILC2s. IL-33 administration to $Il5^{\text{Cre/+}}Icosl^{\text{fl/fl}}$ or $Il5^{\text{Cre/+}}$ control mice resulted in similar expansion of $\text{Gata3}^{\text{high}} T_{\text{regs}}$ in the airways, suggesting that ICOSL-ICOS signaling was not essential (Fig. 2D). Conversely, conditional OX40-deficient $Il7r^{\text{Cre/+}}Tnfrsf4^{\text{fl/fl}}$ mice showed a significant reduction in $\text{Gata3}^{\text{high}} T_{\text{reg}}$ expansion compared with $Il7r^{\text{Cre/+}}$ controls in the lung, as we previously reported (20), as well as in the pancreas, omentum, and adipose tissue after IL-33 administration (Fig. 2D and fig. S3E). $\text{Gata3}^{\text{low}} T_{\text{reg}}$ numbers increased less compared with $\text{Gata3}^{\text{high}} T_{\text{regs}}$, and their expansion was not significantly affected in either $Il5^{\text{Cre/+}}Icosl^{\text{fl/fl}}$ or $Il7r^{\text{Cre/+}}Tnfrsf4^{\text{fl/fl}}$ mice at this time point (fig. S3F). Moreover, OX40L expression was rapidly induced on lung ILC2s after IL-33 administration and preceded the local expansion of $\text{Gata3}^{\text{high}} T_{\text{regs}}$ as demonstrated in a time-course experiment (Fig. 2E and fig. S3G). We also observed a similar induction of OX40L on ILC2 in other anatomical sites, including the pancreas (fig. S3H). To profile cells for their ability to produce OX40L, we used $Tnfrsf4^{\text{hCD4/+}}$ reporter mice, where gene activity is measured instead of transient surface expression of OX40L. We detected robust hCD4 expression on both lung and mediastinal lymph node (medLN) ILC2s after IL-33 exposure but none in phosphate-buffered saline (PBS)-treated mice (Fig. 2, F and G, and fig. S3I); moreover, we did not observe hCD4 expression on other lymphocytes, including lymph node ILC3, at rest or after IL-33 administration (Fig. 2G). We next profiled $Tnfrsf4^{\text{hCD4/+}}$ reporter mice for hCD4 expression on myeloid cells. We did not observe hCD4 expression by myeloid cell types in PBS- or IL-33-treated lungs, including alveolar or interstitial macrophages, granulocytes, or dendritic cells (DCs) (Fig. 2H). We observed some hCD4 expression by cDC2s in the medLN after IL-33 treatment (Fig. 2I). Together, these results suggest that the IL-33–ILC2–OX40L axis serves as an innate checkpoint for local $\text{Gata3}^{\text{high}} T_{\text{reg}}$ expansion.

Intrinsic OX40 signaling is required for IL-33-dependent $\text{Gata3}^{\text{high}} T_{\text{reg}}$ expansion

To investigate whether T_{reg} -intrinsic OX40 signaling could drive IL-33-mediated expansion, we generated T_{reg} -conditional mutants ($Foxp3^{\text{Cre}}Tnfrsf4^{\text{fl/fl}}$, also known as $\text{OX40}^{\Delta\text{Treg}}$) that efficiently and

specifically deleted OX40 on T_{regs} (fig. S4A). $\text{OX40}^{\Delta\text{Treg}}$ mice were born at Mendelian ratios and were phenotypically unremarkable compared to littermate controls. Detailed histological characterization of these mice revealed a minor increase in colonic immune infiltration (fig. S4, B and C). Nevertheless, the lungs of naïve PBS-treated $\text{OX40}^{\Delta\text{Treg}}$ mice were histologically similar to those of control mice (fig. S5B).

Moreover, whereas total percentages of T_{regs} were slightly higher in the spleen, the number and percentage of $\text{Gata3}^{\text{high}} T_{\text{regs}}$ were similar at baseline in the lungs and pancreata of $\text{OX40}^{\Delta\text{Treg}}$ mice compared with $Foxp3^{\text{Cre}}$ control mice, arguing against a substantial role for OX40 in tissue T_{reg} maintenance (fig. S4D). We performed in vitro suppression assays of flow-sorted splenic T_{regs} from $Foxp3^{\text{Cre}}$ control or $\text{OX40}^{\Delta\text{Treg}}$ mice, which showed that OX40 deletion did not impair their suppressive function (fig. S4E). These results were reinforced by transcriptomic analysis of control and OX40 knockout T_{regs} , which showed that besides *Tnfrsf4*, the T_{reg} transcriptome was largely unaffected at baseline, including T_{reg} -associated transcription factors and surface molecules, including *Il1rl1* transcript (fig. S4, F to I), and surface ST2 expression (fig. S4J).

Next, we treated $\text{OX40}^{\Delta\text{Treg}}$ or control mice with IL-33 or PBS followed by immune phenotyping of different organs; IL-33-mediated expansion of $\text{Gata3}^{\text{high}} T_{\text{regs}}$ was significantly reduced in the pancreas and spleen, whereas the proportion of $\text{Gata3}^{\text{high}} T_{\text{regs}}$ was substantially impaired in the pancreas, lung, and spleen (Fig. 2J and fig. S4K). Hemizygous female $Foxp3^{\text{YFP-Cre/+}}Tnfrsf4^{\text{fl/fl}}$ mice were used to further control for cell-extrinsic effects on IL-33-mediated T_{reg} expansion (fig. S4L); we found that YFP⁺ OX40-sufficient T_{regs} expanded, whereas YFP⁺ OX40-knockout T_{regs} failed to respond to IL-33 administration (fig. S4M). Last, we generated $Foxp3^{\text{CreERT2}}Tnfrsf4^{\text{fl/fl}}$ mice to temporally delete OX40 on T_{regs} . Tamoxifen treatment before IL-33 administration resulted in efficient deletion of OX40 and significantly impaired $\text{Gata3}^{\text{high}} T_{\text{reg}}$ expansion in the lung and pancreas, similar to the constitutive knockout (Fig. 2K and fig. S4N). These data demonstrate a critical T_{reg} cell-intrinsic role of OX40 in mediating IL-33-driven $\text{Gata3}^{\text{high}} T_{\text{reg}}$ expansion in diverse anatomical locations.

T_{reg} -intrinsic OX40 is critical for the effective control of type 2 immunity

Because T_{reg} -intrinsic loss of OX40 selectively impairs the expansion of $\text{Gata3}^{\text{high}} T_{\text{regs}}$, which efficiently restrain type 2 immunity (8), we hypothesized that $\text{OX40}^{\Delta\text{Treg}}$ mice would fail to control IL-33-driven type 2 pathologies. We intranasally sensitized and challenged $\text{OX40}^{\Delta\text{Treg}}$ and control mice with *Alternaria alternata* fungal allergen extract or PBS control on days 0, 1, and 14, followed by analysis of airway inflammation on day 20. Whereas allergen exposure provoked a robust type 2 immune response in control mice, $\text{OX40}^{\Delta\text{Treg}}$ mice exhibited profound eosinophil-rich allergic lung inflammation (Fig. 3A and fig. S5A). Moreover, we observed a significant increase in medLN eosinophil numbers in allergen-challenged $\text{OX40}^{\Delta\text{Treg}}$ mice compared with control mice. Similarly, whereas sensitization and challenge induced effector $\text{Gata3}^{\text{high}} T_{\text{H2}}$ cell expansion in control mice, $\text{OX40}^{\Delta\text{Treg}}$ mice exhibited a significant increase in numbers (Fig. 3B). Similarly, ILC2 numbers were increased in inflamed $\text{OX40}^{\Delta\text{Treg}}$ mice (Fig. 3C), although the fold increase in lung T_{H2} cells (33.9-fold) was higher than that in ILC2s (5.4-fold). Moreover, no significant or only minor changes were observed in alveolar macrophage and neutrophil numbers (Fig. 3D). Next, as a measure of lung function, we quantified breadth

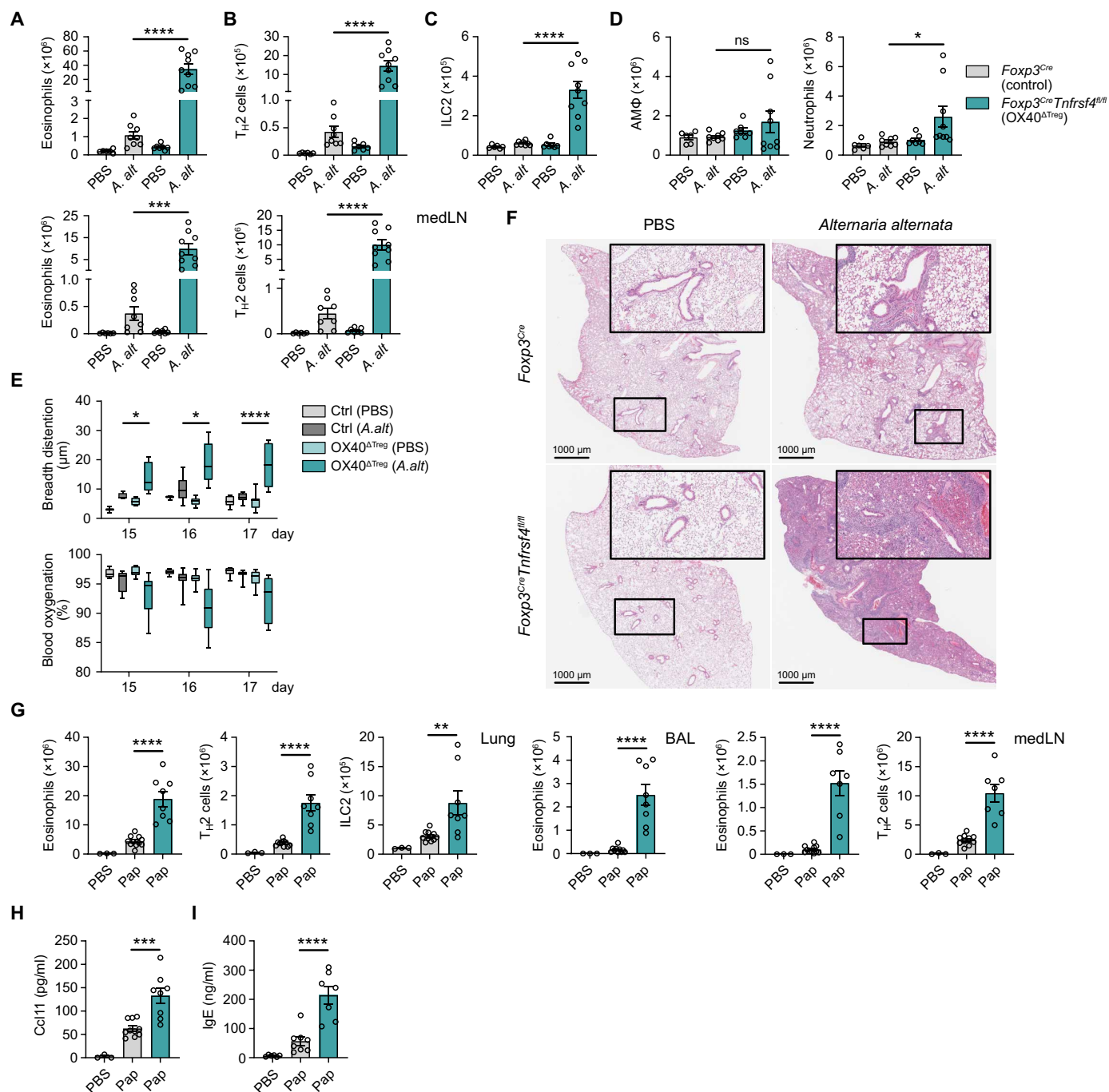


Fig. 3. T_{reg}-intrinsic OX40 is critical for the effective control of type 2 inflammation. (A to F) Mice of the indicated genotypes were treated intranasally with *A. alternata* or PBS on days 0, 1, and 14, followed by euthanasia on day 20. Flow cytometric quantification results are presented for lung (top) and medLN (bottom) eosinophil (A) and T_H2 cell numbers (B). Flow cytometric quantification results are displayed for ILC2 (C), alveolar macrophage (left), and neutrophil (right) cell numbers (D). Mice were analyzed by pulse oximetry for breadth distention (top) and blood oxygenation (bottom) on the indicated days (E). Representative H&E staining of lung sections is presented (F). (G to I) Mice were treated intranasally with papain or PBS on days 0, 1, and 14, followed by euthanasia on day 20. Flow cytometric quantification results are illustrated for lung eosinophil, T_H2 cell, and ILC2 numbers (left); BAL eosinophil and T_H2 cell numbers (middle); and medLN eosinophil and T_H2 cell numbers (right) (G). The BAL concentrations of Ccl11 are depicted (H). The serum concentrations of IgE (I) are presented. Representative histology images of two independent experiments are shown. Bar graphs indicate means ($\pm$ SEM) and show combined data for two independent experiments with [n = 6, 8, 7, and 9 (A to E)] and [n = 3, 10, and 8 (G and H)] or [n = 6, 8, and 7 (I)] mice per group. *P $\leq$ 0.05, ***P $\leq$ 0.001, and ****P $\leq$ 0.0001. Statistical analyses: one-way ANOVA with Tukey postanalysis.

distention and blood oxygenation 3 days after the last allergen challenge (Fig. 3E); whereas control mice showed only minor changes in these parameters after allergen challenge, we observed a notable and persistent increase in breathing effort and reduced blood oxygenation in OX40^{ΔTreg} mice. Histological analysis of lungs on day 20 indicated that naïve mice of both genotypes did not exhibit inflammation; however, allergen-treated OX40^{ΔTreg} mouse lungs were substantially more inflamed compared with those of *Foxp3*^{Cre} control animals (Fig. 3F and fig. S5B). We also assessed lung sections for mucins by periodic acid–Schiff staining and collagens by trichrome staining, which both showed more intense staining in allergen-treated OX40^{ΔTreg} mice (fig. S5, C and D). Next, we used the protease allergen papain as an alternative model of type 2 airway inflammation. Using a similar dosing scheme, we found that OX40^{ΔTreg} mice exhibited significantly more type 2 inflammation, as assessed by quantification of lung ILC2s and T_H2 cells and/or eosinophils in the lungs, bronchoalveolar lavage (BAL), and medLNs (Fig. 3G). Moreover, BAL samples on day 20 were assessed for the eosinophil chemoattractant Ccl11 (Fig. 3H), which was significantly elevated in allergen-treated OX40^{ΔTreg} mice. Similarly, we found that serum immunoglobulin E (IgE) concentrations were higher in OX40^{ΔTreg} mice compared with controls (Fig. 3I). Last, we performed intracellular staining for IL-5 as a measure of the functional capacity of lung T cells or ILC2s after *in vitro* stimulation (fig. S5, E to I). We observed a significant increase in IL-5⁺ T_H2 cell numbers and the percentage of CD4⁺ T cells in OX40^{ΔTreg} mice compared with control mice after allergen stimulation but found no difference in their per-cell capacity to produce IL-5. As expected, ILC2s did not require allergen sensitization for IL-5 production when stimulated with phorbol 12-myristate 13-acetate + ionomycin and, furthermore, did not show differences between genotypes. Hence, we hypothesize that the increase in type 2 inflammation in OX40^{ΔTreg} mice is mainly driven by increased numbers of T_H2 cells, although ILC2s may directly or indirectly contribute. These results suggest that OX40 expression by T_{regs} is critical for restraining airway type 2 inflammation after allergen exposure.

Allergen-exposed OX40^{ΔTreg} mice have impaired Gata3^{high} T_{reg} expansion and uncontrolled effector memory T_H2 cell expansion

Inhaled allergens promote IL-33 release, which is essential for both ILC2 activation and subsequent T_H2 cell responses (36–39). Given that this pathway also engages Gata3^{high} T_{regs}, we assessed the lungs of *Foxp3*^{Cre} control and OX40^{ΔTreg} mice after *A. alternata* challenge by flow cytometry (Fig. 4A and fig. S6A). Gating on CD44⁺CD62L[−] T cells, we identified a significant enrichment of Gata3^{high} T_{regs} in control animals (Fig. 4B); concurrently, Gata3^{high} T_{reg} proportions were significantly reduced in OX40^{ΔTreg} mice compared with control mice after allergen exposure. Conversely, the percentage of lung CD44⁺CD62L[−] effector T_H2 cells was significantly increased in OX40^{ΔTreg} mice (Fig. 4C). Total leukocyte numbers were profoundly increased in allergen-treated OX40^{ΔTreg} mice compared with control animals, resulting in overall increased Gata3^{low} and Gata3^{high} T_{reg} numbers (fig. S6B). However, when we compared the ratio of T_H2 with T_{reg} cells, we found that OX40^{ΔTreg} mice failed to maintain an equilibrium, resulting in significantly higher effector T_H2 cell to T_{reg} proportions (Fig. 4D). Conversely, control mice had constant ratios in naïve and inflamed lungs. We next used multiplex IF microscopy and digital image analysis to determine the densities of ILC2s, T_{regs}, and T_H2 cells in the lungs of *A. alternata*-challenged *Foxp3*^{Cre} control and OX40^{ΔTreg} mice (Fig. 4, E

and F). Mirroring flow cytometric data, we found that OX40^{ΔTreg} mouse lungs showed increased overall cell densities and a marked increase in T_H2 cell to T_{reg} proportions compared with those of allergen-treated control mice (Fig. 4G). Moreover, like IL-33-treated lungs, we found that ILC2s, T_{regs}, and T_H2 cells localized near or within the adventitial cuff in *A. alternata*-treated mice (fig. S6C). Next, using the protease allergen papain, we also observed an impaired relative expansion of Gata3^{high} T_{regs} in OX40^{ΔTreg} mice and amplified T_H2 cell expansion, resulting in significantly higher T_H2 cell to Gata3^{high} T_{reg} ratios in the lung (Fig. 4, H to J, and fig. S6D). These data support our hypothesis that OX40-driven coexpansion of T_{regs} is important for the restraint of T_H2 cell numbers during inflammation.

Given the importance of secondary lymphoid organs for generating both effector and memory T_H2 cell responses, we next focused our attention on the medLN (Fig. 4K). Unlike the lung, we did not observe significant changes in the proportion of Gata3^{high} or total T_{reg} percentages after the allergen challenge or between genotypes (Fig. 4L and fig. S6E). However, the percentage of T_H2 cells increased upon allergen challenge in control mice, and this was significantly amplified in OX40^{ΔTreg} mice (fig. S6F). We then assessed T_H2 cells for the presence of CD44⁺CD62L[−]CD127⁺ EM cells (Fig. 4M) and found that allergen-sensitized OX40^{ΔTreg} mice showed a significant increase in the percentages and absolute numbers of EM T_H2 cells compared with control animals (Fig. 4, N and O). Using a papain allergen, we found a similar significant increase in the percentage of EM T_H2 cells in the medLNs of OX40^{ΔTreg} mice (Fig. 4P). Hence, we conclude that OX40 deficiency on T_{regs} results in a greatly amplified memory T_H2 cell response in the lung-draining lymph node.

Gata3^{high} T_{regs} directly control OX40L availability and preferentially home to the inflamed lung

Given the critical role of OX40L–OX40 signaling in the control of T cell memory (40) and the ability of T_{regs} to control the surface expression of costimulatory ligands (2), we hypothesized that T_{regs} may modulate OX40L bioavailability on lung ILC2s. Using *Foxp3*^{DTR} mice, we asked whether acute T_{reg} depletion could influence OX40L expression upon IL-33 stimulation. We observed a significant increase in the percentage and mean fluorescence intensity of OX40L on ILC2 in IL-33 plus diphtheria toxin (DTX)-treated mice (Fig. 5, A and B). Concomitant analysis of myeloid cell types indicated that T_{reg} depletion did not influence OX40L surface expression on granulocytes or macrophage and DC subsets in IL-33-treated mice (Fig. 5C).

We next used an *in vitro* coculture system to assess the ability of T_{regs} to regulate OX40L expression on ILC2s (fig. S7A). We used *Gata3*^{YFP}*Foxp3*^{RFP} mice to purify viable lung Gata3^{high} and Gata3^{low} T_{regs} and Gata3^{high} ILC2s (Fig. 5D). Lung T_{reg} subsets were able to suppress T_{conv} cells *in vitro*, confirming their conventional suppressive ability (fig. S7B). Culture of ILC2s with IL-33 resulted in the expression of OX40L, which was significantly reduced upon coculture with Gata3^{high} T_{regs}, whereas Gata3^{low} T_{regs} showed reduced capacity to suppress OX40L expression (Fig. 5, E and F); conversely, coculture with T_{conv} cells did not change OX40L expression on ILC2s (fig. S7C). We did not detect OX40L on the surface of T_{regs} *in vivo* after IL-33 administration or *in vitro* after coculture with ILC2s, arguing against trogocytosis-mediated suppression (Fig. 5, A and E); moreover, we confirmed that *Gata3* expression patterns were maintained by T_{regs} in culture (fig. S7D).

We subsequently investigated the mechanism whereby T_{regs} modulate OX40L expression by ILC2s. First, we found that *Tnfrsf4* transcript

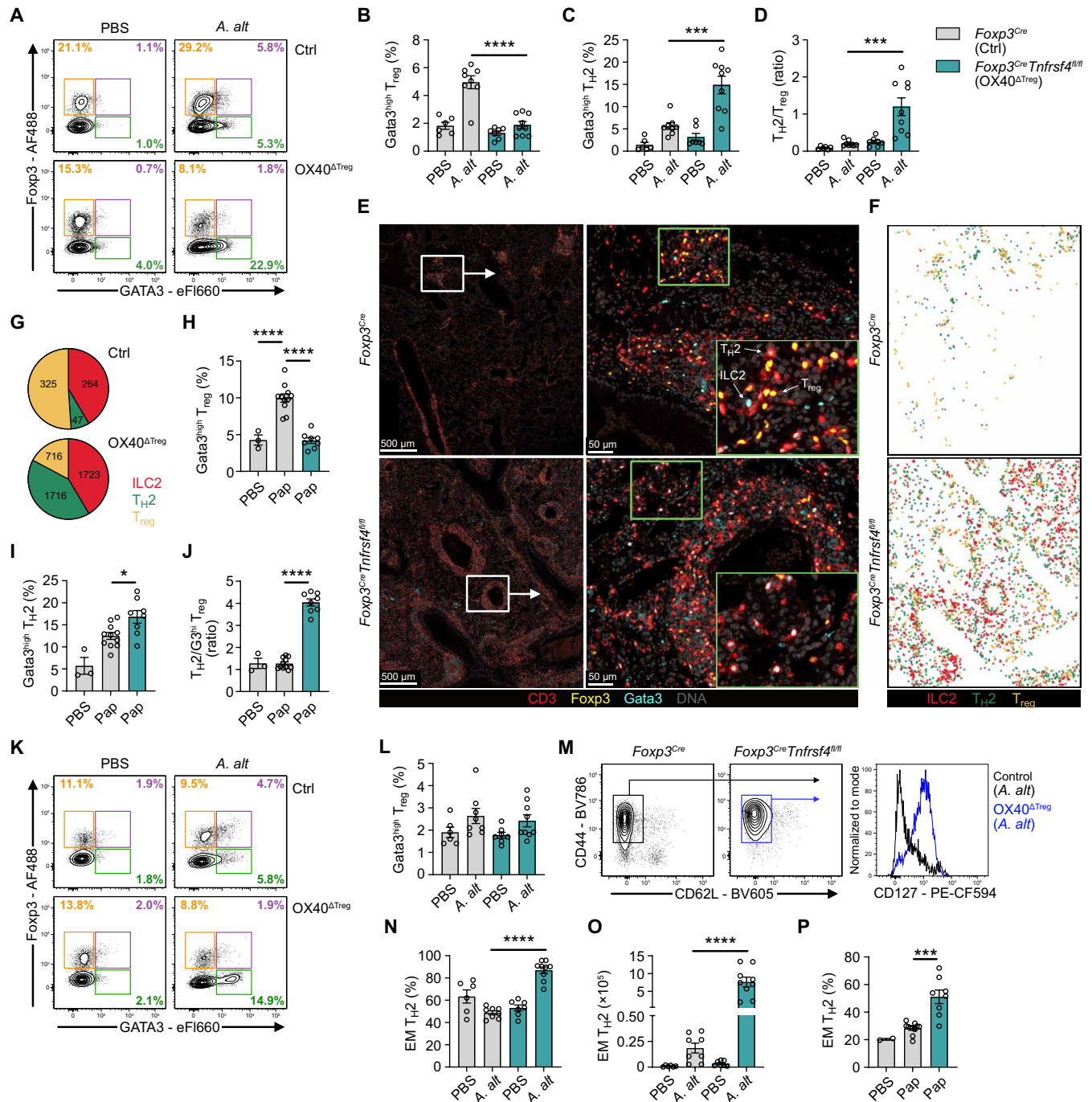


Fig. 4. Allergen-exposed OX40 Δ Treg mice have impaired Gata3^{high} T_{reg} expansion and amplified effector memory T_{H2} cell expansion. (A to G) The lungs of PBS- or *A. alternata*-treated mice of the indicated genotypes were analyzed for percentages of Gata3^{low} and Gata3^{high} T_{reg}s (yellow and purple) or T_{H2} cells (green) by flow cytometry (A), followed by the quantification of the percentage of Gata3^{high} T_{reg}s (B) and T_{H2} cells (C) or indicated cell ratios (D). Multiplex IF microscopy of lung sections allowed the identification of CD3⁺Foxp3⁺Gata3⁺ T_{H2} cells, CD3⁺Foxp3⁺ T_{reg}s, and CD3⁺Foxp3⁻Gata3⁻ ILC2 (E), followed by automated digital image analysis of cell type densities (F and G). (H to J) The percentages of Gata3^{high} T_{reg}s (% of total CD4⁺) (H) and T_{H2} cells (% of total CD4⁺) (I) or indicated cell ratios (J) were measured in the lungs of *Foxp3*^{Cre} or *Foxp3*^{Cre}*Tnfrsf4*^{fl/fl} mice treated with PBS or papain. (K to P) The medLNs of PBS-, *A. alternata*-, or papain-treated mice of the indicated genotypes were analyzed for percentages of Gata3^{low} and Gata3^{high} T_{reg}s (yellow and purple) or T_{H2} cells (green) by flow cytometry (K). The percentages of Gata3^{high} T_{reg}s (% of total CD4⁺) were measured (L). T_{H2} cells were further analyzed for expression of CD127 on CD44⁺CD62L⁻ cells (M), followed by the measurement of CD44⁺CD62L⁻ CD127⁺ EM T_{H2} cell percentages (% of CD44⁺CD62L⁻ T_{H2} cells) (N) and numbers (O). Similarly, EM T_{H2} cell percentages (% of CD44⁺CD62L⁻ T_{H2} cells) were measured in PBS- and papain-treated mice (P). Dot plots show the representative gating strategy; numbers indicate the percentage of gated cells. Bar graphs indicate means ($\pm$ SEM) and show the combined data of two independent experiments with $n = 6, 8, 7$, and 9 ; (B to D, L, N, and O) and $n = 3, 10$, and 8 ; (H to J and P)] mice per group. * $P \leq 0.05$, *** $P \leq 0.001$, and **** $P \leq 0.0001$. Statistical analyses: one-way ANOVA with Tukey postanalysis.

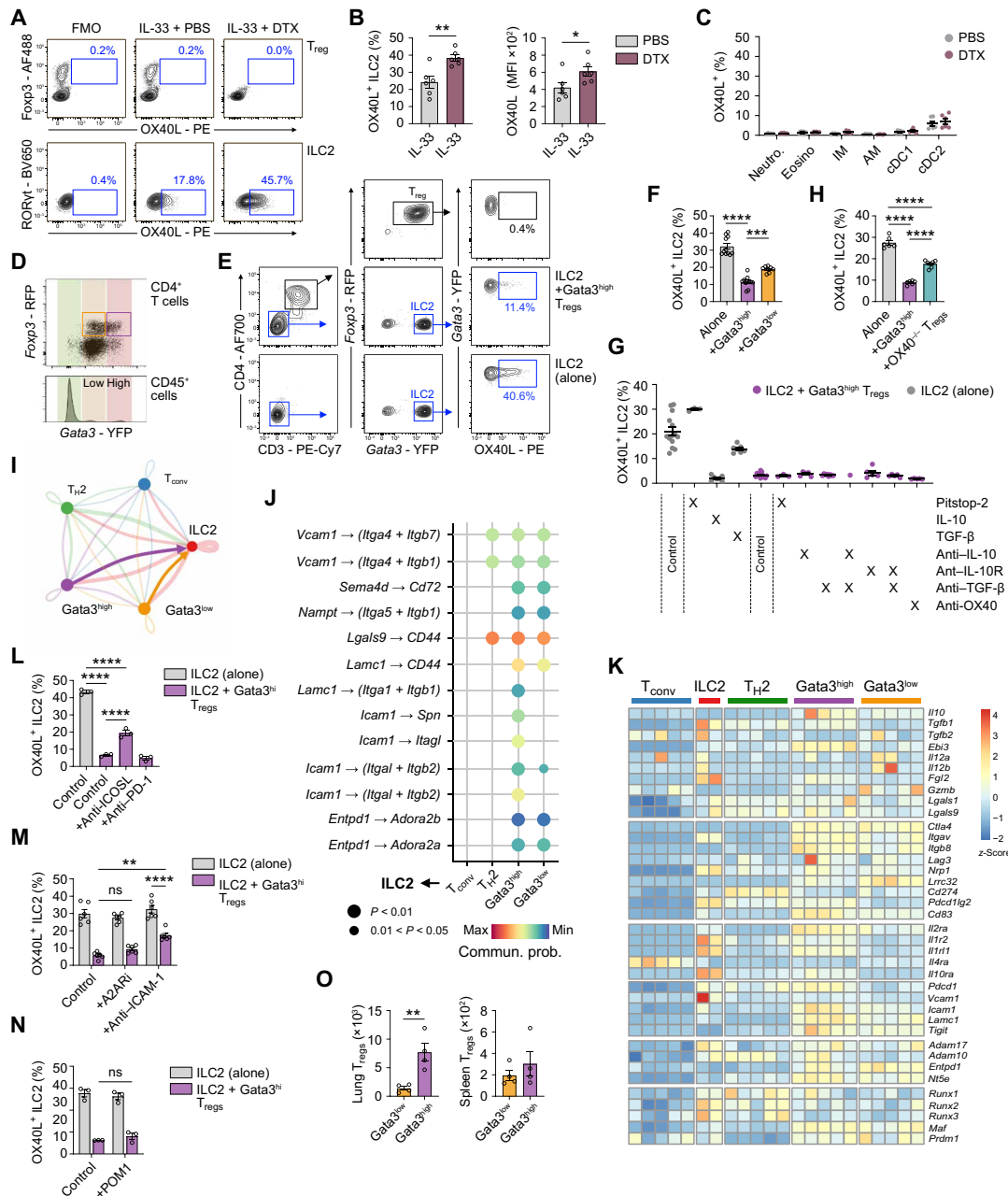


Fig. 5. Gata3^{high} Tregs directly control OX40L availability on ILC2 and preferentially home to the inflamed lung. (A to C) *Foxp3*^{DTR} mice were treated intranasally with IL-33 plus intraperitoneal DTX or PBS on days 0 and 1 and euthanized on day 3. Representative flow cytometric identification of OX40L⁺ lung Tregs and ILC2s (A), quantification of OX40L⁺ ILC2s expressed as a percentage (left) and as the mean fluorescence intensity (right) (B), and the percentage of OX40L⁺ myeloid cells (C) are presented. (D to H) Gata3^{high} (purple) or Gata3^{low} (orange) Tregs were identified and flow-sorted alongside ILC2s from *Gata3*^{YFP} *Foxp3*^{RFP} mouse lungs [(D); see also Fig. S1G]; ILC2s were cocultured alone or with the indicated Treg populations for 20 hours followed by the measurement of OX40L expression on ILC2s or Tregs by flow cytometry [(E) shows representative gating, and (F) shows the percentage of OX40L⁺ ILC2s for each condition]. ILC2 monocultures (gray) or Gata3^{high} Treg cocultures (purple) were supplemented with the indicated reagents, followed by the measurement of OX40L expression on ILC2s (G). OX40-deficient Tregs were flow-sorted from *Foxp3*^{Cre} *Tnfrsf4*^{fl/fl} mice and compared with Gata3^{high} Tregs in their ability to suppress OX40L on ILC2s (H). (I to N) Computationally predicted Treg-to-ILC2 interactions (I) revealed several pathways that were selectively enriched or present in Gata3^{high} Tregs (J). Transcriptome of selected genes involved in immunosuppression in the indicated populations (K). ILC2 monocultures (gray) or Gata3^{high} Treg cocultures (purple) were supplemented with the indicated reagents, followed by the measurement of OX40L expression on ILC2s (L to N). (O) Gata3^{high} or Gata3^{low} Tregs were flow-sorted from IL-33-treated *Gata3*^{YFP} *Foxp3*^{RFP} mouse lungs followed by adoptive transfer of 2.5×10^4 cells into *Rag2*^{-/-} recipient mice. Two days later, the lungs and spleens of recipient mice were analyzed for numbers of *Foxp3*^{RFP} Tregs (O). Dot plots show the representative gating strategy; numbers indicate the percentage of gated cells. Bar graphs indicate means (±SEM) and show representative [n = 3, 3, 3, and 3 (L)] [n = 3, 3, 3, and 3 (M)] or combined [n = 6 and 6; (B and C)] [n = 6, 6, 6, 6, 6, and 6 (M)] data for two independent experiments or combined data from one to three independent experiments [n = 14, 3, 7, 7, 3, 5, 8, 1, 5, 5, and 5 (H)] or combined data more than three independent experiments [n = 11, 11, and 8; (F)], [n = 6, 6, and 7 (G)], and [n = 4 and 4 (O)] (mice or individual cocultures per group). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001. Statistical analyses: unpaired two-tailed Student's t test (B and O) and one-way (F and H) or two-way (M and N) ANOVA with Tukey postanalysis.

levels were unchanged after coculture with T_{regs} , arguing against transcriptional regulation (fig. S7E). We also failed to detect soluble OX40L in cocultures, suggesting that cleavage of membrane-bound receptors did not account for loss of expression (fig. S7F). Furthermore, blocking a disintegrin and metalloproteinase (ADAM) proteases did not affect T_{reg} -mediated suppression of OX40L on ILC2s (fig. S7C). Next, we focused on the internalization of OX40L. We found that blocking endocytosis using Pitstop-2 increased OX40L surface expression on ILC2s in monocultures, whereas T_{reg} -dependent reduction of OX40L expression in coculture was not affected (Fig. 5G). The addition of recombinant IL-10 or transforming growth factor- β 1 (TGF- β 1) to ILC2 monocultures resulted in a significant reduction in OX40L surface expression; however, blockade of either single or both suppressive molecules in ILC2- T_{reg} cocultures did not revert the suppression of OX40L (Fig. 5G). Recombinant IL-10 and TGF- β influenced the *Tnfrsf4* transcript level (fig. S7E), indicating a different mechanism of control. Moreover, we sorted T_{regs} from OX40 $^{\Delta\text{Treg}}$ mouse lungs, which were able to suppress but were less effective than $\text{Gata3}^{\text{high}} T_{\text{regs}}$ at suppressing OX40L on ILC2s (Fig. 5H). Whereas reduced $\text{Gata3}^{\text{high}} T_{\text{reg}}$ numbers in OX40 $^{\Delta\text{Treg}}$ mouse lungs likely contributed to this result, it also demonstrated that the regulation of OX40L on ILC2s could occur independently of receptor-ligand engagement. This finding was further substantiated using anti-OX40 mAb in cocultures, which did not affect OX40L suppression by T_{regs} (Fig. 5G).

We used computational tools to explore alternative regulatory mechanisms. First, we analyzed predicted T_{reg} -to-ILC2 communication, focusing on modules enriched in $\text{Gata3}^{\text{high}} T_{\text{regs}}$ (Fig. 5, I and J, and table S1). These included adhesion molecules (*Vcam1*, *Icam1*, *Lamc1*, and *Lgals9*) and the adenosine 5'-triphosphate hydrolase CD39 (*Entpd1*), among others. IL-10 and TGF- β signaling pathways were not predicted, although this matched our in vitro results and may reflect the lack of TCR stimulation of the T_{regs} in our setting. Our previous ILC2-to- T_{reg} communication analysis also identified ICOSL-ICOS interactions (data file S1); whereas these were not important for mediating T_{reg} expansion, ICOS signaling is known to enhance T_{reg} function (41). We also compared the relative gene expression of known or putative regulatory mechanisms used by T_{regs} in our dataset (Fig. 5K). Besides the secreted factors already investigated, we found that $\text{Gata3}^{\text{high}} T_{\text{regs}}$ expressed *Fgl2*, although this was also highly expressed by ILC2s themselves, as well as *Ebi3*, which together with *Il12a* forms IL-35. We found that *Pdcd1lg2* encoding the inhibitory surface molecule PD-L2 was highly expressed by both $\text{Gata3}^{\text{high}} T_{\text{regs}}$ and ILC2s, whereas the expression of *Cd83* encoding the soluble or membrane molecule CD83 was selective for the former. $\text{Gata3}^{\text{high}} T_{\text{regs}}$ also selectively expressed *Il2ra*, *Il1r2*, and *Il1rl1*, which may act to inhibit IL-2 or IL-33 signaling. Last, genes encoding surface enzymes, including *Entpd1* and *Nt5e* (CD73), were expressed by $\text{Gata3}^{\text{high}} T_{\text{regs}}$, indicating that adenosine-mediated signaling could contribute to reduced OX40L expression by ILC2s. Extending on the computational predictions, we observed that ICOSL was expressed by ILC2s in vitro (fig. S7G); moreover, blocking ICOSL in cocultures resulted in a moderate increase in OX40L on ILC2s (Fig. 5L). Similarly, intercellular adhesion molecule-1 (ICAM-1) blocking mAb in cocultures resulted in a partial rescue of OX40L expression on ILC2s (Fig. 5M). However, blocking PD-1 or components of the adenosine pathway using a CD37 inhibitor (POM-1) or Adora2a antagonist (A2ARi) in cocultures had no effect on OX40L expression (Fig. 5, L to N). Hence, the multifaceted two-way dialogue between ILC2s and T_{regs} appears to coordinate the suppressive function of the latter.

Last, because ILC2- T_{reg} colocalization likely underpins their regulatory function, we asked whether $\text{Gata3}^{\text{high}} T_{\text{regs}}$ preferentially home to the inflamed lung. We adoptively transferred equal numbers of lung $\text{Gata3}^{\text{high}}$ and $\text{Gata3}^{\text{low}} T_{\text{regs}}$ into IL-33-treated *Rag2* $^{-/-}$ recipient mice, which lack T and B cells but have lung ILC2s, followed by the quantification of T_{regs} in the lungs and spleens (Fig. 5O and fig. S7H). We observed significantly more $\text{Gata3}^{\text{high}} T_{\text{regs}}$ in the lungs, whereas equal but greatly reduced numbers of $\text{Gata3}^{\text{high}}$ and $\text{Gata3}^{\text{low}} T_{\text{regs}}$ were recovered in the spleens. Moreover, we found that adoptively transferred T_{regs} maintained their *Gata3* expression pattern (fig. S7I). In summary, these data showed that airway $\text{Gata3}^{\text{high}} T_{\text{regs}}$ are endowed to traffic to the inflamed lung niche while also having enhanced capacity for regulating OX40L expression on ILC2s.

OX40 $^{+}$ T_{regs} control adaptive type 2 immunity by modulating OX40L availability

Our data advance a paradigm whereby OX40 expression on T_{regs} is important for their local expansion and modulation of OX40L expression by ILC2s. We found that OX40L expression by ILC2s was enhanced in OX40 $^{\Delta\text{Treg}}$ mice after administration of papain protease allergen (fig. S8A). Increased OX40L availability in the absence of efficient regulation by $\text{Gata3}^{\text{high}} T_{\text{regs}}$ may therefore explain the unrestrained EM T_{H2} cell formation in OX40 $^{\Delta\text{Treg}}$ mice. To test this hypothesis, we used two approaches: First, we asked whether we could restore T_{H2} cell-driven allergic inflammation in OX40 $^{\Delta\text{ILC2}}$ mice using an OX40 agonist (fig. S8B). As expected, OX40 $^{\Delta\text{ILC2}}$ mice failed to mount an efficient T_{H2} cell response to inhaled allergen papain (Fig. 6A). Agonistic murine IgG1 anti-OX40 administration led to the restoration of both airway T_{H2} cell responses and increased type 2 inflammation while also promoting a trend toward more $\text{Gata3}^{\text{high}} T_{\text{regs}}$. In the medLN, OX40 agonist treatment reversed a significant defect in EM T_{H2} cell formation observed in OX40 $^{\Delta\text{ILC2}}$ mice and rescued the absolute numbers of T_{H2} cells (Fig. 6B).

Next, we asked whether increased OX40L availability was responsible for unrestrained T_{H2} cell-driven inflammation by neutralizing OX40L in OX40 $^{\Delta\text{Treg}}$ mice (fig. S8C). OX40 $^{\Delta\text{Treg}}$ mice treated with papain allergen exhibited amplified type 2 airway inflammation and T_{H2} cell numbers, as expected. $\text{Gata3}^{\text{high}} T_{\text{reg}}$ expansion was impaired (Fig. 6C), and anti-OX40L treatment of OX40 $^{\Delta\text{Treg}}$ mice significantly reduced type 2 inflammation and airway T_{H2} cell numbers. We found no additive effect of OX40L neutralization on $\text{Gata3}^{\text{high}} T_{\text{reg}}$ enrichment in OX40 $^{\Delta\text{Treg}}$ mice. Furthermore, we found that OX40L neutralization reverted the amplified CD127 $^{+}$ EM T_{H2} cell phenotype observed in the medLNs of OX40 $^{\Delta\text{Treg}}$ mice (Fig. 6, D and E), which resulted in significant reductions in EM T_{H2} cell percentages and numbers and an overall reduction in T_{H2} cells (Fig. 6E). Histological analysis of the lungs further indicated that the overt allergic inflammation observed in OX40 $^{\Delta\text{Treg}}$ mice after allergen exposure was reduced by anti-OX40L treatment (Fig. 6F). Last, we leveraged 2W1S peptide-specific tetramers to better understand the effect of T_{reg} -derived OX40 and OX40L neutralization on the clonal dynamics of T_{H2} cells. Papain plus 2W1S peptide administration resulted in the expansion of 2W1S:tetramer $^{+}$ CD4 $^{+}$ T cells, which were skewed toward a $\text{Gata3}^{+} T_{\text{H2}}$ cell fate (Fig. 6G). Mirroring total T_{H2} cell results, we found that OX40 $^{\Delta\text{Treg}}$ mice treated with papain plus 2W1S peptide had significantly more 2W1S:tetramer $^{+} T_{\text{H2}}$ cells, which were reduced with neutralization of OX40L (Fig. 6G). Overall, these data support our hypothesis that OX40L availability is tuned by

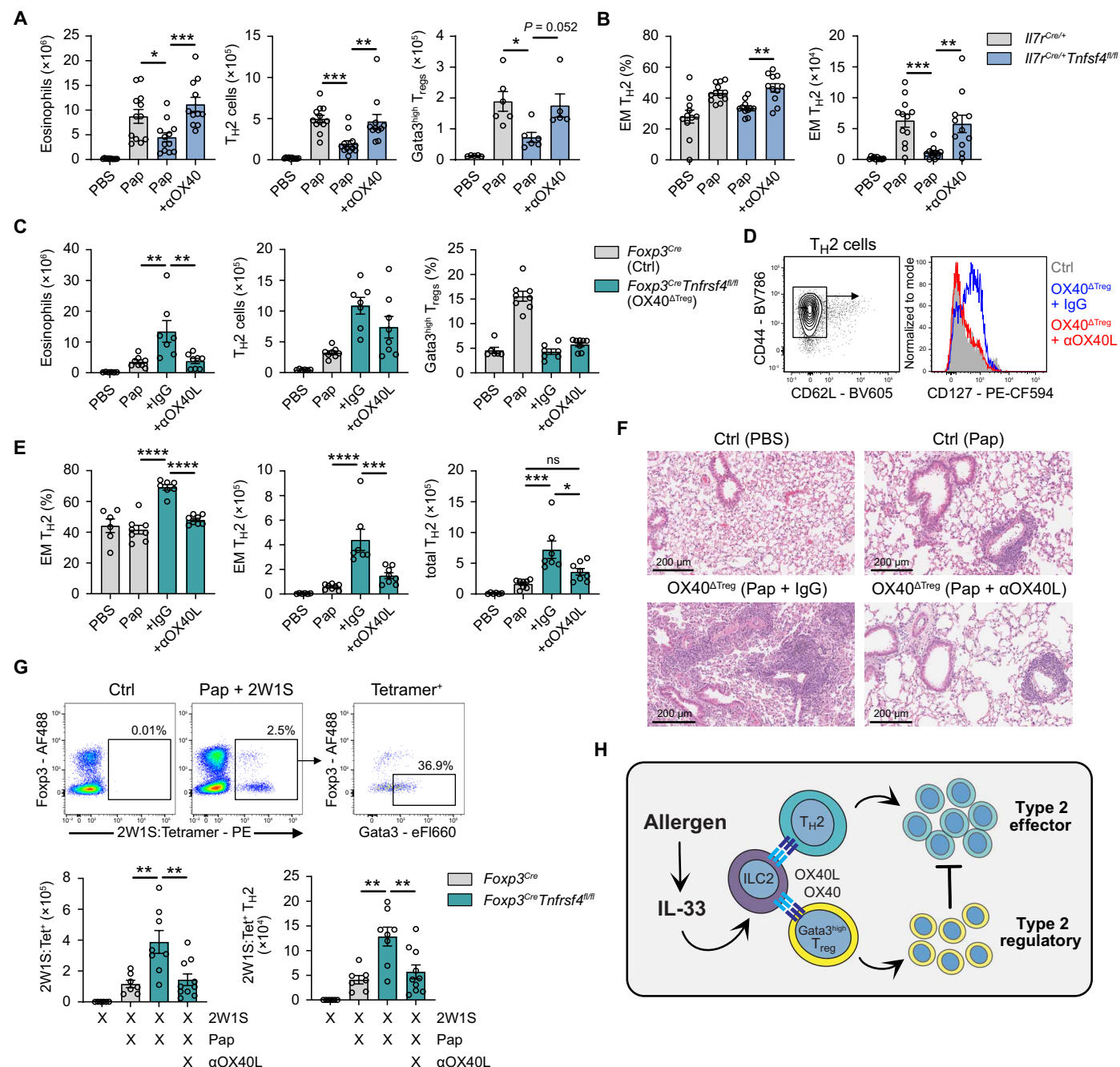


Fig. 6. OX40⁺ T_{reg} control adaptive type 2 immunity by modulating OX40L availability. (A and B) Mice of the indicated genotypes were treated with PBS or papain plus OX40 agonist (see fig. S8B), followed by the quantification of the lung eosinophil number, T_H2 cell number, and Gata3^{high} T_{reg} percentage (A) and medLN CD44⁺CD62L⁺CD127⁺ EM T_{H2} percentage (% of CD44⁺CD62L⁺ T_{H2} cells) (left) and number (right) (B). (C to F) Mice of the indicated genotypes were treated with PBS or papain plus anti-OX40L or rat IgG isotype control (see fig. S8C) and euthanized on day 20. Flow cytometric quantification results are displayed for lung eosinophil, total T_H2 cell, and Gata3^{high} T_{reg} numbers (C). Representative flow cytometric identification (D) and quantification (E) results are presented for total medLN EM T_{H2} (left and middle; as % of CD44⁺CD62L⁺ T_{H2} or cell numbers) or total T_{H2} (right) cell numbers. Representative H&E staining of lung sections (F) is presented. (G) Mice were treated with 2W1S peptide plus PBS or papain (intranasal, days 0, 1, and 14) plus anti-OX40L as indicated (see fig. S8C) and euthanized on day 20. 2W1S:tetramer-positive CD4⁺ cells were detected by flow cytometry (PBS and papain-treated *Foxp3*^{Cre} control mice shown; left), followed by intracellular detection of Foxp3 and Gata3 (right). (G, top) 2W1S:tetramer⁺ total (left) and T_{H2} (right) cells were quantified in the lungs of mice treated as indicated (G, bottom). (H) Graphical abstract. Representative histology images of two independent experiments are shown. Dot plots show the representative gating strategy; numbers indicate the percentage of gated cells. Bar graphs indicate means ($\pm$ SEM) and show combined or representative data from two independent experiments with $n = 11, 12, 12$, and 11 (A and B) and $n = 5, 6, 6$, and 5 (right) (A); $n = 6, 8, 7$, and 8 (C and E); and $n = 6, 7, 8$, and 10 (G) mice per group. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$. Statistical analyses: one-way ANOVA with Tukey postanalysis.

OX40⁺ T_{reg} cells; this mechanism controls the magnitude of adaptive type 2 immunity by regulating immune memory formation.

DISCUSSION

Local immune regulatory mechanisms are fundamentally important for maintaining or restoring tissue homeostasis. Our data support an emerging model whereby ILCs are critical immune regulatory hubs that establish local immune tolerance or immune restraint; specifically, we show that T_{reg}-mediated control of adaptive type 2 immunity is contingent on direct dialogue with ILC2 (Fig. 6H). Whereas ILC2-T_{reg} interactions are multifaceted, OX40L-OX40 signaling was of prime importance and served as a crucial innate immune checkpoint for the local expansion of Gata3^{high} T_{regs}. This T_{reg} subset could efficiently exert feedback control on the local bioavailability of OX40L on ILC2, which profoundly influenced EM T_H2 cell densities and the magnitude of type 2 inflammation.

Whereas ILC2s are emerging as drivers of adaptive type 2 immunity (23), their ability to restrain type 2 inflammation remains unclear. ILC-intrinsic regulatory functions have been reported (42–44), although these do not compensate for the loss of T_{reg} expansion on increased type 2 inflammation in OX40^{ΔTreg} mice. Relatedly, we did not observe a substantial transcriptomic ILC2₁₀ signature (42, 45–47) at the time point where we observed OX40L expression by ILC2s, although it is possible that IL-10 is produced by ILC2s subsequently; ILC2₁₀ cells have been reported to emerge after chronic ILC2 stimulation (48).

In terms of their regulatory function, we argue that ILC2s maintain local parity between T_{reg} and T_H2 cell numbers upon induction of inflammation; this ability to locally coordinate cells is multilayered and involves chemotaxis and costimulatory interactions, among other predicted mechanisms. We found that ICOSL-ICOS signaling was important for T_{reg} function but not IL-33-driven expansion; these and our OX40L-OX40 data reconcile previous findings (20, 21) but also raise questions about how these molecules control T_{reg} biology in other types of inflammation. Similarly, ICAM-1 mediates T_{reg} function (49) and contributes to controlling OX40L expression on ILC2s. Conversely, we found that other known immunosuppressive mechanisms, including TGF-β, IL-10, and adenosine-mediated signaling, were not involved. Moreover, high *Il1rl1* (ST2) expression by Gata3^{high} T_{regs} could act as an IL-33 sink, thereby influencing ILC2 function. We also found that lung Gata3^{high} T_{regs} are functional in standard T cell suppression assays, indicating that direct regulatory mechanisms may contribute to their role in controlling type 2 inflammation. These *in vitro* and *in silico* findings provide a foundation for future investigations into tissue-localized regulatory mechanisms of T_{regs}.

Notably, ILC3s can also express OX40L in the intestines, although direct antigen presentation by ILC3s appears to dominate their interaction with gut T_{regs} (50, 51). We also highlight the unappreciated and divergent effect of the OX40L-OX40 dyad on both the effector and regulatory arms of type 2 immunity and that ILC2s are positioned centrally in this cellular circuit. ILC2s are the primary source of OX40L in our inflammatory model, but the role of other OX40L-expressing cell types should be assessed (52). T_{regs} can modulate OX40L on DCs via trogocytosis (53), although we did not find evidence of this process with ILC2s. Nevertheless, the ability of T_{regs} to modulate OX40L bioavailability on ILC2s fits with our general understanding of how T_{regs} can locally control mitogen levels. Given the broad and generally untargeted therapeutic exploitation of OX40,

our findings may stimulate more refined targeting of either the effector or regulatory arms of this pathway.

The ability of T_{regs} to function depends largely on how they are locally coordinated, and, befitting an emerging framework of inflammation-tailored T_{reg} subsets, we found that Gata3^{high} T_{regs} preferentially engage ILC2s in the type 2 inflamed niche. Nevertheless, Gata3 is constitutively expressed by all T_{regs} and enforces the maintenance and function of Foxp3 (54), whereas Gata3^{high} T_{regs} up-regulate both known and potentially unappreciated target genes that affect their adaptation to the type 2 niche (55, 56). Moreover, whereas Gata3^{low} T_{regs}, which also express lower *Tnfrsf4*, are not preferentially enriched in the type 2 inflamed niche, their expansion is also affected in ILC2-deficient mice. Hence, it is likely that other factors of the type 2 niche collaborate to attract T_{regs}, including ILC2-activated DCs that secrete the type 2 chemokines CCL17 and CCL22 (37, 57) or extracellular matrix components such as hyaluronic acid, which binds to CD44 that is highly expressed on Gata3^{high} T_{regs} (58). Regarding T_{reg} function, we demonstrated their role in a critical checkpoint on the size of the T_H2 EM cell compartment that depended on OX40 expression and local interactions with ILC2.

Whether ILC2 interactions with T_{regs} control type 2 immunity in other anatomical sites remains unknown, although we found that IL-33-driven expansion of tissue T_{regs} is dependent on ILC2s in multiple other organs. Moreover, our study did not explore whether this cellular mechanism operates in other diseases, although our analysis of published single-cell data indicates that similar GATA3^{high} T_{regs} are also present in patients with lung cancer. Also, the relative contribution of ILC2 and T_H2 cells in driving overall type 2 inflammation after an allergen challenge remains incompletely understood. Whereas CD4 T cells are critical mediators of the antigen-recall response and the profound increase in type 2 inflammation in OX40^{ΔTreg} mice, we also observed a significant increase in ILC2 numbers over allergen-stimulated control mice. This finding might be explained by the synergistic effect of T cell-derived factors on ILC2 expansion (59).

In all, this work reveals critical cellular circuitry that underpins T_{reg}-mediated control of adaptive type 2 immunity. We show that the ability of Gata3^{high} T_{regs} to locally constrain adaptive type 2 immunity is contingent on ILC2 cross-talk. Moreover, we find that T_{reg}-mediated modulation of OX40L bioavailability represents a critical regulator of the memory CD4 T_H2 cell response. Hence, ILC2-T_{reg} interactions are a critical node within a homeostatic circuit that controls local type 2 inflammation.

MATERIALS AND METHODS

Study design

Knowing that OX40L-dependent signaling promotes the local expansion of both effector cells and T_{regs}, our study objective was to investigate the role of OX40 on T_{regs} specifically. We hypothesized that direct OX40 signaling was required for local T_{reg} expansion, which subsequently served to restrain adaptive immune responses. We generated T_{reg}-conditional OX40 knockout mice to address these questions in models of IL-33- or allergen-driven airway inflammation.

Mice

Foxp3^{DTR} (60) (JAX, #016958), *Foxp3*^{YFP-Cre} (61) (JAX, #016959), *Foxp3*^{CreERT2} (62) (JAX, #016961), *Foxp3*^{RFP} (63) (JAX, #008374), and *Il5*^{tdTom-Cre} (34) (JAX, #030926) mice were bought from JAX and were maintained in the Cancer Research UK Cambridge Institute

(CRUK-CI) animal facility, under specific pathogen-free conditions along with *Il7r^{Cre/+}* (provided by H.-R.R.), *Il7r^{Cre/+}Rora^{fl/fl}*, *Il7r^{Cre/+}Tnfrsf4^{fl/fl}* [*Tnfrsf4^{fl/fl}* mice (64) provided by T.V. and M. Botto], *Tnfrsf4^{fl/fl}*, *Foxp3^{YFP-Cre}Tnfrsf4^{fl/fl}*, *Foxp3^{CreERT2}Tnfrsf4^{fl/fl}*, *Gata3^{YFP/YFP}* (26) (provided by H.J.F.), *Gata3^{YFP/YFP}Foxp3^{RFP}*, *Il13^{tdTom}* (provided by A.N.J.M.), *Il13^{tdTom}Foxp3^{YFP-Cre}*, *Il5^{tdTom-Cre}Foxp3^{YFP-Cre}*, *Il5^{tdTom-Cre}Icosl^{fl/fl}* (35), and *Tnfrsf4^{hCD4}* mice, which were all in the C57BL/6 background. Wild-type C57BL/6J mice were purchased from Charles River Laboratories. Animal work was conducted under project license PD7484FB9 or PF993249 at the CRUK-CI (with approval from the CRUK-CI, Animal Welfare Ethical Review Body) all in accordance with Home Office regulation. CD45.1 mice and *Il1rl1^{-/-}* mice were maintained in the Medical Research Councils ARES facility (Babraham, UK). *Ccr8^{-/-}* mice were maintained in the Cambridge University Biomedical Services Gurdon Institute animal facility. Experiments involving these strains (all in the C57BL/6 background) were performed in these facilities. Mice were sex- and age-matched whenever possible, and most mice were used at 8 to 12 weeks of age.

Tnfrsf4^{fl/fl} mouse generation

The *Tnfrsf4^{tm1a(EUCOMM)Wsi}* ES cells were purchased from the European Mouse Mutant Cell Repository (Helmholtz Zentrum München, GB) and injected into wild-type CD1 eight-cell-stage embryos. Microinjected embryos were cultured in KSOM + AA medium (KCl, enriched simplex optimization medium with amino acid supplement, Zenith Biotech) at 37°C with 95% humidity and 5% CO₂ until they reached the blastocyst stage. Blastocysts were transferred into pseudopregnant recipients. The resulting F0 mice were bred with C57BL/6 mice, proving germline transmission. Superovulated *Tnfrsf4^{fl/+}* female mice were further in vitro fertilized with C57BL/6 flp sperm to remove the neo cassette.

Tnfrsf4^{hCD4} mouse generation

Stock animals (also known as C57BL/6J-Ox40^{em1H}/H) were generated by the Mary Lyon Centre (MLC) at MRC Harwell via pronuclear injection of CRISPR/Cas9 reagents into one-cell-stage embryos. The MLC generated this mouse strain as part of its commitment to the Genome Editing Mice for Medicine project funded [MC_UP_1502/3] by the Medical Research Council. The research reported in this publication is solely the responsibility of the authors and does not necessarily represent the official views of the Medical Research Council.

In vivo procedures

Recombinant mouse IL-33 [rmIL-33 (0.5 µg, BioLegend)], DTX (10 ng/g, Sigma-Aldrich), 2W1S peptide (25 µg, GenScript), and anti-OX40L mAb (200 µg, clone RM134L, BioXCell) were administered by intraperitoneal injection in 200 µl of PBS. Anti-OX40L mAb was treated beforehand with 0.05 U of PNGase F (Promega, V483A) per microgram of antibody. Samples were incubated at 37°C overnight, and purification of the antibody from the enzyme was achieved through size exclusion dialysis (Pur-A-Lyzer Maxi Dialysis Kit, Maxi 50000, Sigma-Aldrich). Successful deglycosylation was confirmed by SDS-polyacrylamide gel electrophoresis. For intranasal administration, mice were anesthetized by isoflurane inhalation and received rmIL-33 (0.2 µg, BioLegend), papain (10 to 15 µg, Sigma-Aldrich), *A. alternata* (10 µg, Greer Laboratories), house dust mite (100 µg, Greer Laboratories), ragweed pollen extract (300 µg, Greer Laboratories), or anti-OX40 mAb (40 µg, OX86 mIgG1, Antibody and Vaccine Group, University of Southampton) in 40 µl of PBS. For induction of

Cre-ERT2-mediated recombination, mice were fed tamoxifen-containing food (TD.55125; Envigo). Labeling of circulating leukocytes to evaluate tissue residency was achieved by intravenous injection of 3 µg of anti-CD45.2-PE (clone 104, BioLegend) antibody 3 min before euthanasia.

Mixed bone marrow transplantation

Bone marrow (BM) was prepared from B6.SJL-*Ptprca^a Pepc^b*/BoyJ (CD45.1) and *Il1rl1^{tm1Anjm}* (CD45.2 ST2-KO) mice under sterile conditions. Briefly, the hind limb bones (pelvis, femur, tibia, and fibula) were dissected, crushed with a mortar and pestle, and filtered through 100-µm Nitex mesh to remove debris, and erythrocytes were lysed (R7757, Sigma-Aldrich). Cells were counted on a Countess automated cell counter, and a 50:50 mixture of the two sources was injected into recipient mice. The recipient mice were heterozygous B6.SJL-*Ptprca^a Pepc^b*/BoyJ (CD45.1 × CD45.2) and 12 weeks old at the point of transfer. Recipient mice were irradiated with two doses of 5.5 grays (Gy) with 4 hours between doses. BM cells (2 × 10⁶ per recipient) were injected intravenously. Male cells and mice were used. Mice were given Baytril in drinking water for 3 weeks and used for analysis at 8 weeks after transplant. Two micrograms of anti-CD45-biotin was injected intravenously 3 min before euthanasia to label circulating leukocytes. Blood, thymus, BM, spleen, axillary lymph node, mesenteric lymph node, Peyer's patches, intestinal intraepithelial lymphocytes, lamina propria lymphocytes, lung, kidney, pancreas, white adipose tissue, skin, and brain were assessed by flow cytometry.

Pulse oximetry

The right hind legs of mice were shaved using an electric razor and then depilated using hair removal cream before pulse oximetry (MouseOX Plus Pulse Oximeter, STARR Life Sciences Corp., PA, US). Mice were anesthetized (3% isoflurane for induction, followed by 1.5% for maintenance, v/v in supplied air) and kept on a warming pad throughout the measurement. Blood oxygenation and breadth distention were recorded using the ThighClip mouse thigh sensor and analyzed using MouseOX Plus v1.5 software (STARR Life Sciences Corp., PA, US).

Histology

Tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin before sectioning into 3-µm slices. Sample embedding, sectioning, and histological stainings [hematoxylin and eosin (H&E), periodic acid-Schiff, and Masson's trichrome] were conducted by the CRUK-CI Histology Core. Scoring of the colon and lung sections was done by a board-certified veterinary pathologist blinded to the groups and experimental conditions, as previously described (65, 66).

Single-cell preparation

Lung

Cell suspensions were prepared by mechanical dissociation, followed by digest in 4 ml of RPMI 1640 containing collagenase I (563 U/ml) and deoxyribonuclease I (DNase I; 0.225 mg/ml) for 1 hour at 37°C on a shaker (220 rpm), followed by filtration through a 70-µm strainer and red blood cell (RBC) lysis.

Pancreas

Pancreata were weighed and then mechanically dissociated, followed by digestion in 5 ml of Hanks' balanced salt solution (HBSS) containing collagenase I (375 U/ml), DNase I (0.15 mg/ml), and soybean trypsin inhibitor (0.05 mg/ml, Sigma-Aldrich) for 30 min at 37°C on

a shaker (220 rpm), followed by dissociation with a syringe and needle, filtration through a 70- μ m strainer, and RBC lysis.

Omentum and adipose tissue

Tissues were weighed and then digested in 0.5 ml of HBSS containing collagenase I (375 U/ml), DNase I (0.15 mg/ml), and soybean trypsin inhibitor (0.05 mg/ml, Sigma-Aldrich) for 45 min at 37°C on a shaker (1100 rpm), followed by filtration through a 70- μ m strainer. Strainers were further washed with 10 ml of warm RPMI 1640 supplemented with 10% fetal bovine serum (FBS).

Spleen

Spleens were strained through a 70- μ m filter in RPMI 1640 before RBC lysis.

Mediastinal lymph node

Cell suspensions were prepared by mechanical dissociation, followed by digest in 0.5 ml of RPMI 1640 containing collagenase I (375 U/ml) and DNase I (0.15 mg/ml) for 45 min at 37°C on a shaker (1100 rpm), followed by RBC lysis.

Bronchoalveolar lavage

BAL cells and fluid were obtained in 1 ml of PBS.

Flow cytometry

Single cells were incubated with anti-mouse CD16/32 (Thermo Fisher Scientific) to block Fc receptors. For intracellular staining, we used the Foxp3/Transcription Factor Kit (Thermo Fisher Scientific), except when costaining yellow fluorescent protein (YFP) and Foxp3, where the protocol described by Heinen *et al.* (67) was followed. For intracellular cytokine detection, single cells were stimulated with 1 $\times$ protein transport inhibitor and 1 $\times$ cytokine stimulation cocktail (Thermo Fisher Scientific) in culture medium (RPMI 1640 supplemented with 10% FBS) at 37°C for 3 hours before staining. Data were acquired on a BD Fortessa or Symphony instrument. Cells were quantified using CountBright beads. Data were analyzed using FlowJo X (Tree Star).

The following antibodies were used in this study, with clones, vendors, and fluorochrome as indicated: CD8 (53-6.7, eBioscience, PerCP-eFluor710), CD45 (30-F11, BioLegend, BV510), NK1.1 (PK136, BD Biosciences, BV395), CD4 (RM4-5, eBioscience, AF700), human CD4 (RPA-T4, BioLegend, PE), B220 (A3-6B2, eBioscience, APC-eFluor780), CD127 (SB/199, BD Biosciences, PE-CF594), CD44 (IM7, BioLegend, BV785), CD62L (MEL-14, BioLegend, BV605), OX40L (RM134L, BioLegend, AF647 or eBioscience, PE), OX40 (OX-86, eBioscience, PE), Ccr8 (SA214G2, BioLegend, BV421), ICOSL (HK5.3, BioLegend, PE), KLRG1 (2F1, BioLegend, BV605 or eBioscience, PerCP-eFluor710), ST2 (RMST2-2, eBioscience, PE), TCR β (H57-597, BioLegend, PE), Gata3 (TWAJ, eBioscience, eFluor660), Foxp3 (FJK-16s, eBioscience, AF488 or eFluor660), Ror γ T (Q31.378, BD Biosciences, BV650), IL-5 (TRFK5, BD Biosciences, APC), Fc ϵ R1 α (MAR-1, eBioscience, PerCP-eFluor710), CD172 α (P84, BioLegend, AF488), Siglec-F (1RNM44N, eBioscience, SB600), XCR1 (ZET, BioLegend, BV650), CD64 (X54-5/7.1, BioLegend, BV711), CD11b (M1/70, BioLegend, BV785), I-A/I-E (CI2G9, BD Biosciences, BV395), CD11c (N418, eBioscience, AF700), F4/80 (BM8, eBioscience, APC-eFluor780), Ly6G (1A8-Ly6g, eBioscience, PE-eFluor610), and Ly-6C (HK1.4, eBioscience, PE-Cy7). Lineage cocktail contained either CD5 (53-7.3), CD19 (1D3), CD11b (M1/70), CD11c (N418), Fc ϵ R1 α (MAR-1), F4/80 (BM8), Ly-6C/G (Rb6-8C5), and Ter119 (TER-119), or CD3 (145-2C11), NK1.1 (PK136), CD5 (53-7.3), CD19 (1D3), or B220 (RA3-6B2), all on eFluor450 (eBioscience). Dead cells were excluded with the fixable viability dye UV455 or eFluor780 (eBioscience).

ILC2 and T_{reg} cell isolation and coculture

Single-cell suspensions from IL-33-sensitized lungs from *Gata3*^{YFP/YFP} *Foxp3*^{RFP} mice were used to isolate ILC2s [live CD45⁺CD3[−]CD4[−]Foxp3^{(RFP)−}Gata3^{(YFP)high}], and Gata^{high} and Gata^{low} T_{regs} [live CD45⁺CD3⁺CD4⁺Foxp3^{(RFP)+}Gata3^{(YFP)high or low}] by flow cytometry using a BD Melody instrument. Purified cells were cocultured overnight in equal concentrations in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml, Gibco), and streptomycin (100 μ g/ml, Gibco), along with rmIL-33 (10 ng/ml, BioLegend), rhIL-2 (25 ng/ml, PeproTech), rhIL-7 (10 ng/ml, PeproTech), and where indicated additional Pitstop-2 (10 μ M, Sigma-Aldrich), ADAM10/17 inhibitor GW 280264X (0.5 μ M, Tocris), A2A receptor antagonist ZM 241385 (3 μ M, Bio-Techne), or POM-1 inhibitor (50 μ M, Bio-Techne), rhIL-10 (20 ng/ml, BioLegend), rhTGF- β 1 (4 ng/ml, PeproTech), anti-IL-10 mAb (20 μ g/ml, clone JES5-2A5, BioXCell), anti-IL-10R mAb (20 μ g/ml, clone 1B1.3a, BioLegend), anti-TGF- β 1,2,3 mAb (20 μ g/ml, clone 1D11.16.8, BioXCell), anti-OX40 mAb (20 μ g/ml, clone OX-86, Antibody and Vaccine group, University of Southampton), anti-PD1 (5 μ g/ml, clone RMP1-14, BioXCell), anti-ICOSL (10 μ g/ml, clone HK5.3, BioLegend), and anti-ICAM-1 (0.5 μ g/ml, clone YN1/1.7.4, eBioscience). Cells were cultured at 37°C in a humidified, 5% CO₂ incubator for 18 hours, before analysis by flow cytometry, quantitative polymerase chain reaction (qPCR), or detection of soluble OX40L in supernatants.

qPCR analysis for *Tnfrsf4*

RNA was extracted from 5000 to 10,000 cocultured cells (fig. S7A) with the RNeasy Plus kit (Qiagen) and converted into cDNA using the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific), followed by qPCR using the TAKYON Low Rox Probe MasterMix dTTP Blue (Eurogentec) and primers/probe pre-designed assays for *Tnfrsf4* (Mm.PT.58.6713411) (Integrated DNA Technologies). To avoid misinterpretation because of cell number differences between ILC2 monoculture and coculture with T_{regs}, *Tnfrsf4* Ct values were not normalized to a housekeeping gene, having verified beforehand the absence of *Tnfrsf4* transcript in T_{reg} monoculture. The results are expressed as arbitrary units set to 100 for the expression of *Tnfrsf4* in ILC2 monoculture in each independent experiment.

Statistics

Analysis for two groups was calculated using an unpaired two-tailed Student's *t* test; comparisons of more than two groups were calculated using a one-way analysis of variance (ANOVA) with Tukey post-analysis. All data were analyzed using GraphPad Prism 9 (GraphPad software) except for the histology scores, which were analyzed using a permutation test in R (perm.test function of the R package exactRankTests). Results with **P* $\leq$ 0.05 ***P* $\leq$ 0.01, ****P* $\leq$ 0.001, and *****P* $\leq$ 0.0001 are considered significant.

Supplementary Materials

This PDF file includes:

Materials and Methods

Figs. S1 to S8

Table S1

References (68–70)

Other Supplementary Material for this manuscript includes the following:

Data files S1 and S2

MDAR Reproducibility Checklist

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