**SKIN INFLAMMATION**

RORα-expressing T regulatory cells restrain allergic skin inflammation

Nidhi Malhotra, 1*†‡ Juan Manuel Leyva-Castillo, 1*‡ Unmesh Jadhav, 2,3 Olga Barreiro, 4 Christy Kam, 1 Nicholas K. O’Neill, 5,3 Françoise Meylan, 5 Pierre Chambon, 6 Ulrich H. von Andrian, 4 Richard M. Siegel, 5 Eddie C. Wang, 7 Ramesh Shivdasani, 2,3 Raif S. Geha 1‡

Atopic dermatitis is an allergic inflammatory skin disease characterized by the production of the type 2 cytokines in the skin by type 2 innate lymphoid cells (ILC2s) and T helper 2 (TH2) cells, and tissue eosinophilia. Using two distinct mouse models of atopic dermatitis, we show that expression of retinoid-related orphan receptor α (RORα) in skin-resident T regulatory cells (Tregs) is important for restraining allergic skin inflammation. In both models, targeted deletion of RORα in mouse Tregs led to exaggerated eosinophilia driven by interleukin-5 (IL-5) production by ILC2s and TH2 cells. Expression of RORα in skin-resident Tregs suppressed IL-4 expression and enhanced expression of death receptor 3 (DR3), which is the receptor for tumor necrosis factor (TNF) family cytokine, TNF ligand–related molecule 1 (TL1A), which promotes Treg functions. DR3 is expressed on both ILC2s and skin-resident Tregs. Upon deletion of RORα in skin-resident Tregs, we found that Tregs were no longer able to sequester TL1A, resulting in enhanced ILC2 activation.

We also documented higher expression of RORα in skin-resident Tregs than in peripheral blood circulating Tregs in humans, suggesting that RORα and the TL1A-DR3 circuit could be therapeutically targeted in atopic dermatitis.

**INTRODUCTION**

Atopic dermatitis (AD) is the most common skin inflammatory disease affecting ~17% of children in developed nations (1). AD lesions are characterized by the presence of activated T helper 2 (TH2) cells, as well as by the expansion of type 2 innate lymphoid cells (ILC2s) (2–4). Both TH2 cells and ILC2s may contribute to allergic skin inflammation in AD. Cutaneous inflammation elicited by topical application of calcipotriol (MC903), a low-calcemic analog of vitamin D, has been used as a mouse model of acute AD (5, 6). Allergic inflammation in this model is accompanied by expansion of ILC2s driven by epithelial cytokines (2, 4). More importantly, it is dependent on ILC2s; it is preserved in Rag1−/− mice and is severely attenuated in Tslpr−/− mice, ILC2-depleted Rag1−/− mice, and ILC2-deficient Roraα/wild-type (WT) bone marrow chimeras (2, 4). Cutaneous inflammation elicited by repeated epicutaneous (EC) application of ovalbumin (OVA) or peanut extract to tape-stripped mouse skin provides an antigen-driven mouse model of acute AD (7–9). Allergic inflammation in this model is dependent on T cells, because it is abolished in Rag2−/− mice (9, 10).

CD4+ FOXP3+ T regulatory cells (Tregs) constitute a substantial subset of immune cells residing in murine and human skin (11). Lack of Tregs in humans and mice results in immune dysregulation associated with allergic skin inflammation (12, 13). Treg numbers are unaltered in AD skin lesions (14).

Thus, the role of skin-resident Tregs in controlling allergic skin inflammation is unclear. Here, we have dissected the molecular architecture of skin-resident Tregs and identified retinoid-related orphan receptor α (RORα) as a regulator of genes in Tregs responsible for suppressing allergic skin inflammation.

**RESULTS**

**Skin-resident Tregss exhibit an activated signature and express the transcription factor RORα**

Specialization of tissue-resident Tregs is an important factor in maintaining tissue homeostasis and modulating local immune responses. To investigate whether skin-resident Tregs exhibit a specialized phenotype, we compared the phenotype of skin-resident Tregs and Tregs in skin-draining lymph node (dLN). About 45% of CD4+ T cells in ear skin expressed FOXP3 compared with ~20% of CD4+ T cells in dLN (Fig. 1A). Skin Tregs localized around dermal blood vessels and interfollicular areas (fig. S1A). We compared the transcriptome of CD3+ CD4+ YFP+ Tregs from the skin and dLN of Foxp3GFP-YFP mice. Skin Tregs differed from dLN Tregs by more than 5000 genes (fold change > 2; false discovery rate (FDR) < 0.05). Skin Tregs were enriched for the expression of genes encoding signaling receptors [Icos and Il11l1 (ST2)], activation markers (Cd44 and Klrg1), effector molecules (Il10, Cita4, and Areg), and tissue-homing receptors (Ccr3, Ccr8, and Ccr10) (Fig. 1B).

Flow cytometry demonstrated that the percentage of T cells that expressed ST2, ICOS, and CD44 and the expression levels of these markers were significantly higher in skin Tregs than in dLN Tregs (Fig. 1C). Rora, the gene encoding the transcriptional regulator RORα, was highly up-regulated in skin Tregs (Fig. 1B). This was confirmed by quantitative polymerase chain reaction (qPCR) (Fig. 1D). RORA expression was significantly higher in CD4+ CD25+ CD127low skin Tregs than in circulating Tregs, in humans (Fig. 1E). Human skin Tregs, similar to mouse skin Tregs, display an activated signature with increased expression of ICOS, CTLA4, and CD44 (15).

To examine and map the fate of RORα-expressing Tregs, we bred RoraCre mice to Rosa26Yfp (R26Y) mice. In RoraCreR26Y mice, yellow fluorescent protein (YFP) marks cells that are expressing or previously
expressing skin Tregs are natural Tregs. The percentage of ICOS+ and ST2+ Tregs and the levels of ICOS and ST2 were significantly higher in Rora+ (YFP+) Tregs than in Rora+ (YFP+) Tregs in dLNs (fig. S1C). A negligible subset (<1%) of thymic Tregs were Rora+ (YFP+) (fig. S1D), suggesting that RORA+ Tregs expand and/or are induced in peripheral tissues.

We used Roracre/creR26Y mice to investigate Rora expression by cell subpopulations in the skin. In addition to Tregs, a fraction of CD3+CD4+CD25− T cells, CD3−CD8+ T cells, CD3−TCRγδflow dermal γδ T cells, CD3−TCRγδhigh epidermal γδ T cells, and CD45−Lin+ ILCs in the skin were YFP+ (Rora+) (fig. S2A). In addition, a fraction of CD45 EpCAM+ keratinocytes that are mostly derived from the basal layer of the epidermis and a fraction of CD45 EpCAM− cells, which contain a mixture of mature keratinocytes and fibroblasts in the skin, were YFP+ (Rora+) (fig. S2A). The percentages of YFP+ (Rora+) cells among skin cell subpopulations were not significantly altered following MC903 treatment (fig. S2, B and C). These results show that RORA expression was not restricted to skin Tregs.

**RORA deficiency in Tregs results in exaggerated allergic skin inflammation in response to topical application of MC903**

RORA is necessary for the development of ILC2s (16), promotes Tγ17 cell differentiation, and antagonizes FOXP3 in vitro (17), suggesting a potential pro-inflammatory role. To understand how RORA regulates the function and/or maintenance of skin Tregs, we generated Foxp3egfpRorafl/fl mice. Fluorescence-activated cell sorting (FACS) analysis of skin population of cells from Foxp3egfpRorafl/fl mice for enhanced green fluorescent protein (eGFP) expression revealed that Foxp3 expression was restricted to CD4+ T cells and was not detected in any other additional skin cell population that expressed Rora in the skin, including CD8+ T cells, dermal and epidermal γδ T cells, ILCs, and CD45+ cells (fig. S3). In addition, none of the Foxp3egfpRorafl/fl mice had weight loss or developed the staggerer phenotype observed in RORγt-deficient Rorasg/sg mice (18). Furthermore, the numbers of ILCs and γδ T cells in the skin were not reduced in Foxp3egfpRorafl/fl mice. These results suggest that Rora is deleted specifically in Tregs of Foxp3egfpRorafl/fl mice. RNA sequencing (RNA-seq) analysis of Tregs revealed complete deletion of

Fig. 1. Skin-resident Tregs exhibit an activated signature and express the transcription factor RORA. (A) Representative flow cytometric analysis (left) and quantification (right) of FOXP3+ (CD3+CD4+YFP+) cells among CD4+ T cells in ear skin compared with dLNs from Foxp3egfpcre mice (n = 3 mice per group). (B) Scatterplot of log2 (RPKM + 1) expression by cell subpopulations in the skin. In addition to Tregs, a fraction of CD3+CD4+CD25− T cells, CD3−CD8+ T cells, CD3−TCRγδflow dermal γδ T cells, CD3−TCRγδhigh epidermal γδ T cells, and CD45−Lin+ ILCs in the skin were YFP+ (Rora+) (fig. S2A). In addition, a fraction of CD45 EpCAM+ keratinocytes that are mostly derived from the basal layer of the epidermis and a fraction of CD45 EpCAM− cells, which contain a mixture of mature keratinocytes and fibroblasts in the skin, were YFP+ (Rora+) (fig. S2A). The percentages of YFP+ (Rora+) cells among skin cell subpopulations were not significantly altered following MC903 treatment (fig. S2, B and C). These results show that RORA expression was not restricted to skin Tregs.
the floxed fourth exon of Rora in these mice (fig. S4A). The numbers of YFP^+ Tregs were not altered in the skin or dLNs of these mice (fig. S4B), indicating that RORα is not required for the accumulation or maintenance of Tregs in the skin. The cytokine interleukin-10 (IL-10) is important for Treg function in the gut and lungs (19). There was an increased percentage of IL-10^+ Tregs in Foxp3^creRora^floxed mice compared with controls (fig. S4C).

Topical application of MC903 to ear skin of WT mice results in increased dermal thickness and infiltration of CD45^+ cells that include eosinophils and CD4^+ T cells (5). There was an increased ear thickness, accompanied with an intense cellular infiltrate, and significantly increased dermal thickness in Foxp3^creRora^floxed mice compared with Foxp3^creRora^floxed controls (Fig. 2, A to C). FACS analysis revealed a threefold increase in dermal infiltration by CD45^+ cells in Foxp3^creRora^floxed mice compared with Foxp3^creRora^floxed controls (Fig. 2D). Eosinophils accounted for ~45% of CD45^+ cells in the dermis of MC903-treated Foxp3^creRora^floxed mice, compared with 15% in controls, yielding an eightfold increase in eosinophil numbers (Fig. 2E). The percentages of basophils (ckit^– IgE^+ ), mast cells (ckit^+ IgE^+ ), neutrophils (CD11b^+ Gr1^hi ), T effector cells (Teffs) (CD4^+FOXP3^3), Tregs (CD4^+FOXP3^3), and ILCs (Lin^CD90^) infiltrating MC903-treated skin were comparable in Foxp3^creRora^floxed mice and controls. Nevertheless, the numbers of these cell populations were two- to threefold higher in Foxp3^creRora^floxed mice (Fig. 2, F and G), reflecting the approximately threefold increase in CD45^+ cells in CD45^+5 mice. MC903-driven allergic inflammation in mice of C56BL/6 background is largely dependent on thymic stromal lympho-poitin (TSLP) (2–4). The exaggerated cutaneous inflammatory response in Foxp3^creRora^floxed mice, which are on a C56BL/6 background, was not due to increased Tslp expression (fig. S5A). Serum immunoglobulin E (IgE) levels were higher in MC903-treated Foxp3^creRora^floxed mice than in controls (fig. S5B), indicative of a heightened type 2 response.

**RORα deficiency in Tregs results in increased expression of eosinotaxins and IL-5 in MC903-treated skin**

The proportion of eosinophils in blood was comparable in MC903-treated Foxp3^creRora^floxed mice and controls (fig. S6A), suggesting that the exaggerated eosinophilia in MC903-treated skin of Foxp3^creRora^floxed mice resulted from increased eosinophil recruitment. Eotaxins are the major eosinophil chemoattractants (20). There was increased expression of Ccl11 and Ccl24, which encode for eotaxins 1 and 2, in MC903-treated skin of Foxp3^creRora^floxed mice compared with controls (Fig. 3A). IL-5 plays a critical role in tissue eosinophilia by synergizing with eotaxins and promoting eosinophil survival in tissues (21, 22). IL-5 levels were significantly higher in MC903-treated skin of Foxp3^creRora^floxed mice than of controls (Fig. 3B). IL-4 and IL-13 levels were comparable in the two groups (fig. S6B).

IL-5 is predominantly produced by ILC2s and by a subset of activated T_{H}2 cells (23). ILC2s exist as preactivated resident sentinel cells in the dermis that rapidly release IL-5 and IL-13 upon stimulation (21). In contrast, T_{H}2 cells are recruited to the skin at a later stage of allergic inflammation. MC903 treatment up-regulated IL-33/ST2 and CD69 expression, down-regulated CD25 expression, and had negligible effect on KLRG1 expression on skin Lin^– CD90^ ILCs, but the changes were comparable in Foxp3^creRora^floxed mice and Foxp3^creRora^floxed controls. However, in ILCs, mRNA levels were significantly increased in ILCs from MC903-treated skin of Foxp3^creRora^floxed mice compared with controls (Fig. 3C). There was also a twofold increase in Il4 and Il13 mRNA levels in ILCs from MC903-treated skin of Foxp3^creRora^floxed mice; however, it did not reach statistical significance (fig. S6C). There was a significant increase in CD4^+FOXPO3 IL-5^+, but not CD4^+IL-13^+ or CD4^+IL-4^+, T effs in Foxp3^creRora^floxed mice compared with controls (Fig. 3D). The chemokine CCL8 is up-regulated in AD skin lesions (24) and is critical for the recruitment of CCR8-expressing IL-5^+ T_{H}2 cells to the skin in a mouse model of AD (24). Ccl8 expression was strongly increased in MC903-treated skin of Foxp3^creRora^floxed mice (Fig. 3E). In contrast, Ccl17, Ccl22, Ccl4, and Ccl5 expression was comparable in Foxp3^creRora^floxed mice and controls (fig. S6D). Cxcl11, Ccl2, and Ccl7 expression demonstrated a trend toward an increase in MC903-treated skin of Foxp3^creRora^floxed mice, which could underlie the increased influx of myeloid cells in these mice (Fig. 2F).

**RORα deficiency in Tregs alters the expression of genes involved in Treg cell migration and function and skew Tregs to IL-4–producing effectors**

To gain an understanding of how RORα regulates the function of skin Tregs, we performed next-generation sequencing (NGS) transcriptomic analysis on Tregs isolated from untreated and MC903-treated skin of Foxp3^creRora^floxed mice and Foxp3^creRora^floxed controls (table S1 and fig. S7). We observed a change in ~1700 genes across the four groups examined (fold change > 2; FDR < 0.05) (Fig. 4A). Expression of the central circadian rhythm genes Nr1d1 and Nr1d2 was decreased in skin Tregs from Foxp3^creRora^floxed mice compared with controls, consistent with the role of RORα as a circadian rhythm regulator (25). Genes involved in signaling via transforming growth factor–β (TGFβ) (Smad3), tumor necrosis factor–α (TNFα) (TNfa), nuclear factor κB (NF-κB) (Irk4 and Tira4), and mitogen-activated protein kinase (MAPK) (Fos and Jun) and in cell adhesion (Icam2 and Itga4) were comparably expressed in Tregs from untreated skin of Foxp3^creRora^floxed mice and Foxp3^creRora^floxed controls and underwent comparable changes after MC903 treatment. Genes in the phosphatidylinositol 3-kinase (PI3K)/AKT pathway were down-regulated in RORα-deficient skin Tregs. Dysregulated PI3K/AKT signaling affects Foxp3 and Il2ra expression in Tregs and increases their conversion to T_{H}1 cells (26). We did not observe any effect on Foxp3, Il2ra, or Ilfng expression in our transcriptomic and flow cytometric analyses of skin Tregs from Foxp3^creRora^floxed mice. Several genes encoding chemokines and chemokine receptors (Ccl2, Ccr3, and Ccr5) were up-regulated upon allergic skin inflammation in all mice, but to greater extent in Foxp3^creRora^floxed mice. Up-regulation of these genes is consistent with the increased numbers, and higher velocity, of Tregs in MC903-treated skin of Foxp3^creRora^floxed mice (Fig. 2G; fig. S8, A and B; and movies S1 and S2). Furthermore, Tregs in MC903-treated skin showed less directed movement (fig. S8C). Expression of Ccr6 and CCR6, thought to be important for migration of Tregs into neonatal skin (27), was strongly decreased in skin Tregs from Foxp3^creRora^floxed mice compared with controls both before and after MC903 treatment (Fig. 4, A to C). However, the numbers of skin Tregs in Foxp3^creRora^floxed mice were not reduced (Fig. 3G).

Treg suppressive activity is mediated in part by the nucleotides adenosine and cyclic adenosine 3′,5′-monophosphate (cAMP) (28). Tregs from untreated and MC903-treated skin showed strongly decreased expression of Nt5e, which encodes the 5' ectonucleotidase CD73 that metabolizes AMP to adenosine (28), and reduced surface expression of CD73 compared with controls (Fig. 4, B and D), whereas expression of Pde3b, which encodes the phosphodiesterase 3B that breaks down cAMP (29), was increased (Fig. 4B). Expression of Gemb, which encodes granzyme B that mediates Treg cytotoxic activity, was up-regulated
(Fig. 4B), indicating that not all genes involved in T<sub>reg</sub> function were down-regulated in the absence of ROR<sub>α</sub>.

Expression of IL-4 in T<sub>reg</sub> inhibits their ability to suppress T<sub>H2</sub> cells and ILC2s (30, 31). IL4 levels were elevated in skin T<sub>reg</sub> from Foxp3<sup>−/−</sup>Rora<sup>fl/fl</sup> mice compared with controls (Fig. 4, A and B). This was confirmed by qPCR (Fig. 4E). Furthermore, the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> among IL-4–expressing CD4<sup>+</sup> cells in MC903-treated skin was significantly higher in Foxp3<sup>−/−</sup>Rora<sup>fl/fl</sup> mice than in controls (Fig. 4F). These results suggest that RORα expression prevents the conversion of T<sub>reg</sub> into IL-4–producing effectors.
factor RUNX1 inhibits IL4 expression in T<sub>reg</sub>s (32). Runx1 expression was decreased in T<sub>reg</sub>s from Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice (Fig. 5B), suggesting that reduced RUNX1 activity may derepress IL4 expression in RORα-deficient T<sub>reg</sub>s. T<sub>reg</sub>s from MC903-treated skin of Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice, but not controls, expressed Ccl8 and Ccl24 transcripts (Fig. 4B), suggesting that these T<sub>reg</sub>s contribute to the exaggerated eosinophil-dominated allergic skin inflammation in Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice.

RORα expression in T<sub>reg</sub>s promotes expression of the TL1A ligand DR3 and restrains TL1A-driven allergic inflammation elicited by cutaneous application of MC903

Tnfrsf25 encodes the TNF receptor family member DR3 (death receptor 3), which is expressed constitutively on T cells, including T<sub>reg</sub>s and ILC2s (33, 34). Tnfrsf25 expression, as determined by RNA-seq analysis, and DR3 surface expression, as determined by FACs analysis, were both strongly reduced in skin T<sub>reg</sub>s from Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice compared with Foxp3<sup>eyfp-cre</sup> controls (Figs. 4B and 5, A and B). In contrast, DR3 surface expression by skin ILC2s was comparable in Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice and controls (Fig. 5C). The DR3 ligand TL1A is released by endothelial and myeloid cells. TL1A synergizes with the epithelial cytokines IL-33, IL-7, and IL-25 to enhance IL-5 expression in human and murine ILC2s and to promote allergic inflammation (33, 35, 36). TL1A also acts on T<sub>reg</sub>s to increase their proliferation and their ability to suppress allergic airway inflammation (34). Skin TL1A levels were not altered after MC903 treatment and were comparable in Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice and Foxp3<sup>eyfp-cre</sup> controls (Fig. 5D). Given this finding, we tested the hypothesis that selective down-regulation of the TL1A receptor DR3 on T<sub>reg</sub>s from skin of Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice may play an important role in the exaggerated MC903-driven allergic inflammation observed in these mice. MC903-mediated eosinophilia was attenuated in Tnfrsf25<sup>−/−</sup> mice (Fig. 5E), demonstrating a role for TL1A in MC903-driven allergic skin inflammation. Intradermal injection of TL1A into ear skin resulted in a significant increase in the percentage and numbers of eosinophils, but not neutrophils, in Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice compared with controls (Fig. 6F). More importantly, local TL1A blockade during MC903 treatment by intradermal injection of neutralizing antibody to TL1A significantly reduced MC903-driven allergic skin inflammation in Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice. This was evidenced by a significant decrease in dermal thickness, infiltration by CD45<sup>+</sup> cells and eosinophils, and expression of IL5 and Ccl8 compared with isotype control antibody–treated mice (Fig. 6, G to J). These results suggest that RORα expression in T<sub>reg</sub>s restrains TL1A-mediated allergic skin inflammation and eosinophilia elicited by cutaneous application of MC903.

RORα deficiency in T<sub>reg</sub>s results in exaggerated skin inflammation in response to EC sensitization

To investigate whether RORα deficiency in T<sub>reg</sub>s plays a role in restraining antigen-driven T cell–dependent allergic skin inflammation, we subjected Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice and Foxp3<sup>eyfp-cre</sup> controls to EC sensitization. EC sensitization was elicited by repeated application of the antigen OVA to tape-stripped skin, as illustrated in Fig. 6A. Skin inflammation in this model shares many characteristics of acute AD skin lesions, including epidermal thickening, dermal infiltration by CD45<sup>+</sup> cells (including eosinophils), and increased expression of type 2 cytokines (8, 9). Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice EC sensitized with OVA exhibited significantly increased epidermal thickness and significantly increased infiltration by CD45<sup>+</sup> cells compared with Foxp3<sup>eyfp-cre</sup> controls EC sensitized with OVA (Fig. 6, B to D). Furthermore, the numbers of all cell populations analyzed, including eosinophils, basophils, neutrophils, mast cells, CD4<sup>+</sup>Foxp3<sup>+</sup> T cells, T<sub>reg</sub>s, and ILCs, were two- to fourfold higher in OVA-sensitized skin of Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice compared with Foxp3<sup>eyfp-cre</sup> controls (Fig. 6, E and F). IL4, but not IL13, mRNA levels in OVA-sensitized skin were significantly higher in Foxp3<sup>eyfp-cre</sup>Rora<sup>fl/fl</sup> mice than in controls (Fig. 6G). IL5 mRNA was not
**Fig. 4.** RORα deficiency in Tregs alters the expression of genes involved in Treg cell migration and function and skews Tregs to IL-4–producing effectors. (A) Heat map showing relative expression of genes clustered by K-mean values in skin Tregs of Foxp3eyfp-cre and Foxp3eyfp-creRorafl/fl mice in the steady state and after MC903 treatment (n = 4 to 5 mice per group). (B) Heat map showing the relative expression of select chemotaxis, function, and inflammation genes in skin Tregs from Foxp3eyfp-creRorafl/fl mice and controls (n = 4 to 5 mice per group). (C and D) RNA-seq tracing of Ccr6 and Nt5e expression (left), representative FACS analysis (middle), and MFIs (right) of CCR6 and CD73 expression in skin Tregs of Foxp3eyfp-cre and Foxp3eyfp-creRorafl/fl mice (n = 4 to 5 mice per group). The numbers in the FACS panels represent the percentage of positive cells relative to fluorescence minus one (FMO) control. (E) Relative Il4 mRNA levels in Tregs from MC903-treated skin of Foxp3eyfp-creRorafl/fl mice and controls (n = 4 to 5 mice per group). (F) Representative FACS analysis of IL-4 expression in CD4+ cells and of FOXP3 versus CD90 expression in IL-4+CD4+ cells (left) and quantitation of the percentage of IL-4+CD4+FOXP3+ cells among IL-4+CD4+ cells in the skin of MC903-treated Foxp3eyfp-creRorafl/fl mice and controls.
Fig. 5. RORγt expression in Tregs promotes expression of the TL1A receptor DR3 and restrains TL1A-driven allergic inflammation elicited by cutaneous application of MC903. (A) RNA-seq tracing of Tnfrsf25 expression in skin Tregs from untreated and MC903-treated skin of Foxp3eyfp-cre and Foxp3eyfp-creRorafl/fl mice. (B) Representative FACS analysis (left) and MFIs (right) of DR3 expression by skin Tregs of Foxp3eyfp-creRorafl/fl mice and Foxp3eyfp-cre controls (n = 3 mice per group). The numbers in the FACS panels represent the percentage of positive cells relative to FMO control. (C) Representative FACS analysis of DR3 expression by ILCs from the skin of Foxp3eyfp-creRorafl/fl mice and Foxp3eyfp-cre controls. Results are representative of three independent experiments. The numbers in the FACS panels represent the percentage of positive cells relative to FMO control. (D) TL1A levels in vehicle and MC903-treated ear skin of Foxp3eyfp-creRorafl/fl mice and Foxp3eyfp-cre controls (n = 4 mice per group). (E) Representative FACS analysis (left) and quantification (right) of CD11b+Siglec-F+ eosinophils in MC903-treated ears of Tnfrsf25−/− mice and WT controls. (F) Representative FACS analysis (left) and quantification (right) of CD11b+Siglec-F+ eosinophils and CD11b+Gr1high neutrophils in TL1A-injected skin of Foxp3eyfp-creRorafl/fl mice and Foxp3eyfp-cre controls (n = 3 mice per group). (G to J) Representative H&E-stained sections (G), quantification of dermal thickness (H), quantification of CD45+ cells (right) and CD11b+Siglec-F+ eosinophils (left) (I), and relative mRNA expression of Il5 (right) and Ccl8 (left) (J) in MC903-treated ears of Foxp3eyfp-creRorafl/fl mice injected with anti-TL1A antibody or isotype control (n = 4 mice per group). Columns and bars represent means and SEM. *P < 0.05, **P < 0.01
detectable in sensitized skin in either group. Nevertheless, intra-cellular FACS analysis revealed that OVA sensitization caused a small but significant increase in the numbers of IL-5+ ILCs and IL-5+ CD4+ FOXP3− T effs in Foxp3eyfp-cre control mice. The numbers of IL-5+ ILCs and IL-5+ CD4+ FOXP3− T effs were four- to fivefold higher in OVA-sensitized skin of Foxp3eyfp-creRorafl/fl mice than in controls (Fig. 6H). OVA sensitization did not result in significant changes in IL-33R/ST2, CD69, CD25, or KLRG1 expression by skin ILCs in Foxp3eyfp-creRorafl/fl or Foxp3eyfp-cre controls. These results suggest that RORα+ T regs play an important role in constraining antigen-driven skin inflammation.

**DISCUSSION**

We show that skin T regs express high levels of the transcription factor RORα. Deletion of Rora in T regs does not alter the number of skin T regs but results in exaggerated type 2 allergic skin inflammation in response to topical application of MC903 or EC sensitization with OVA. Thus,
we have identified RORα as a regulator of T<sub>reg</sub> genes responsible for suppressing allergic skin inflammation.

The vast majority of mouse skin T<sub>reg</sub> expressed RORα and had an activated phenotype. In contrast, a small minority of T<sub>reg</sub> in skin dLNs expressed RORα and had an activated phenotype. It remains to be determined whether RORα<sup>+</sup> T<sub>reg</sub> are specifically attracted to the skin or whether the skin environment drives RORα expression in T<sub>reg</sub>. The numbers of skin T<sub>reg</sub> are not altered in Foxp3<sup>3<sub>gypt CreRora<sub>fl/fl</sub></sup></sup> mice. Furthermore, although the majority of human blood T<sub>reg</sub> expressed the skin-homing receptor CLA (37), human blood T<sub>reg</sub> expressed fivefold less RORA mRNA compared with skin T<sub>reg</sub>. These findings argue for local acquisition of RORα expression by T<sub>reg</sub> in the skin.

We demonstrated that RORα expression in T<sub>reg</sub> restrains allergic skin inflammation induced by topical application of MC903, an AD model dependent on ILC2s (2, 4). This was evidenced by increased ear swelling and dermal thickness in Foxp3<sup>3<sub>gypt CreRora<sub>fl/fl</sub></sup></sup> mice, with a threefold increase in the influx of inflammatory cells that included T cells, basophils, neutrophils, and mast cells, and a selective enrichment in eosinophils that showed an eightfold increase over controls. Type 2 cytokines, such as IL-4, are documented to drive eotaxin expression (21, 38). Increased eosinophils in MC903-treated skin of Foxp3<sup>3<sub>gypt CreRora<sub>fl/fl</sub></sup></sup> mice may be explained by synergy between increased skin IL-5 expression and increased skin and T<sub>reg</sub> eotaxin expression, likely driven by increased expression of IL-4 and IL-13. The exaggerated skin inflammation in Foxp3<sup>3<sub>gypt CreRora<sub>fl/fl</sub></sup></sup> mice was not caused by increased cutaneous expression of TSLP, the epithelial cytokine essential for MC903-driven skin inflammation in mice on the C57BL/6 background, the background of the Foxp3<sup>3<sub>gypt CreRora<sub>fl/fl</sub></sup></sup> mice we used. ROR<sub>α</sub> was essential for repressing IL-5 expression in fast-responding ILC2s and for restricting the CCL8-dependent recruitment of IL-5<sup>+</sup> T<sub>H</sub>2 effector cells to the skin, likely by dampening the expression of CCL8 in the skin, and particularly in skin T<sub>reg</sub>, RORα also repressed IL-13 and IL-4 expression by skin ILCs, although the effect did not reach statistical significance, but had no effect on IL-4 and IL-13 expression by T cells. We propose that in addition to their role in restraining adaptive immunity, a central function of T<sub>reg</sub> resident in barrier interfaces, such as skin, is to inhibit the rapid activation of innate lymphocytes. The unrestricted activation of these innate sentinels may contribute to acute flare-ups in allergic diseases.

RORα regulated the expression of several genes important for T<sub>reg</sub> migration and function. Changes in chemokine receptor expression may underlie the increased motility of T<sub>reg</sub> in Foxp3<sup>3<sub>gypt CreRora<sub>fl/fl</sub></sup></sup> mice. Our data suggest that decreased expression by RORα-deficient T<sub>reg</sub> of Tnfrsf25 encoding DR3, a gene important for T<sub>reg</sub> function, contributed to the enhanced skin inflammation in Foxp3<sup>3<sub>gypt CreRora<sub>fl/fl</sub></sup></sup> mice. The exaggerated skin inflammation in Foxp3<sup>3<sub>gypt CreRora<sub>fl/fl</sub></sup></sup> mice may be a direct effect of decreased TL1A activation of T<sub>reg</sub> and/or increased availability of TL1A to activate ILC2s. Definitive evidence of the role of DR3 expression on T<sub>reg</sub> in limiting allergic skin inflammation and its mechanism of action awaits the generation and study of mice with selective deficiency of Tnfrsf25 in T<sub>reg</sub> and/or ILC2s. Furthermore, our data indicate that RORα restrains the conversion of T<sub>reg</sub> into IL-4–producing effector cells, possibly because RORα drives the expression of Runx1, which inhibits Il4 gene transcription. Derepression of the Il4<sup>+</sup> proinflammatory genes in RORα-deficient skin T<sub>reg</sub> likely contributes to the enhanced allergic skin response in Foxp3<sup>3<sub>gypt CreRora<sub>fl/fl</sub></sup></sup> mice. Furthermore, IL-10 expression was increased in RORα-deficient skin T<sub>reg</sub>. The transcription factor AhR (aryl hydrocarbon receptor) enhances IL-10 production in T<sub>reg</sub> (39), whereas IL-4 suppresses it (40). We observed increased Ahr and Il4 expression in RORα-deficient skin T<sub>reg</sub>. Increased expression of AhR and IL-4 may underlie the enhanced IL-10 expression by these cells.

In addition to its role in suppressing ILC2-dependent allergic skin inflammation driven by topical application of MC903, RORα expression in T<sub>reg</sub> was important for suppressing T cell–dependent allergic skin inflammation driven by topical application of the antigen OVA to tape-stripped skin, a T cell–dependent mouse model of AD. This was evidenced by increased epidermal thickness, increased dermal infiltration by CD4<sup>+</sup> inflammatory cells (including eosinophils, mast cells, neutrophils, T cells, and ILC2s), increased cutaneous expression of Il4, and increased expression of IL-5 by T cells and ILCs.

We demonstrate significantly higher expression of RORA in human skin T<sub>reg</sub> than in blood T<sub>reg</sub>, suggesting that our results may be applicable to humans. Our results may be particularly relevant to patients with AD, a disease in which both Th2 cells and ILC2s play important roles in allergic skin inflammation. RORA polymorphisms in asthma (41) and Rora down-regulation in dogs with AD (42) further suggest that RORα may play a regulatory role in atopic diseases. Moreover, expression of Rora in T<sub>reg</sub> resident in tissues such as the gut (43) may endow them with the ability to dampen allergic inflammation in organs other than skin.

### MATERIALS AND METHODS

**Mice**

Foxp3<sup>3<sub>gypt CreRora<sub>fl/fl</sub></sup></sup> (C57BL/6), R26R (C57BL/6), Rag1<sup>−/−</sup> (C57BL/6), and Rora<sup>−/−</sup> (C57BL/6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Rora<sup>−/−</sup> (C57BL/6) mice were generated in the laboratory of P. Chambon (France) (44). Rora<sup>−/−</sup> (C57BL/6) mice were generated in the laboratory of D. O’Leary (45). Tnfrsf25<sup>−/−</sup> mice were generated by E. Y. Wang and obtained from the laboratory of R. Siegel. Foxp3<sup>3<sub>gypt</sub></sup> reporter mice were a gift from T. Chatila. All mice were kept in a pathogen-free environment. All procedures performed on the mice were in accordance with the Animal Care and Use Committee of the Children’s Hospital Boston.

**Preparation of skin cell homogenates from mice and human skin**

Dorsal and ventral ear murine skin was separated using tweezers, chopped, and digested in complete Dulbecco’s modified Eagle’s medium containing Liberase TL (2.5 mg/ml, Roche, Life Technologies) and deoxyribonuclease (DNase) I (20 ng/ml, Sigma) for 90 min at 37°C, with vigorous shaking. Digested tissue was mechanically disrupted with a plunger, filtered, washed, and suspended in media for flow cytometric analysis. Human skin surgical discards of facial skin were obtained from Children’s Hospital Boston.

**Flow cytometry**

All antibodies were obtained from eBioscience and BioLegend, except anti-mouse Siglec-F, which was purchased from BD Biosciences. Cells were preincubated with Fcγ receptor–specific blocking monoclonal
antibody (2.4G2) and washed before staining. Staining with CD45 and fixable viability dye (eBioscience) was used for FACS analysis of skin cell homogenates. One hundred twenty-three count beads from eBioscience were used for estimating cell counts. Cells were analyzed on LSRFortessa (BD Biosciences), and the data were analyzed with FlowJo software (v9.7).

**Intracellular staining analysis for cytokines and transcription factors**

LN and skin cell suspensions were incubated with media containing phorbol 12,13-dibutyrate, ionomycin, GolgiPlug, and GolgiStop for 3 hours. Staining for surface markers was performed, followed by fixation and permeabilization using BD Cytofix/Cytoperm buffer. Cells were incubated with antibodies against cytokines, IL-4, IL-5, and IL-13, along with antibodies to FOXP3, overnight in Perm/Wash buffer (BD Biosciences). This protocol was also used to stain cells with anti-FOXP3 and anti-HELIOS markers without quenching the emission of YFP in Rora\(^{-}\) R26R mice.

**MC903 treatment**

MC903 (catalog no. 2700) was purchased from Tocris Biochemicals. The stock was reconstituted in ethanol. MC903 (2 nM) (in a volume of 20 µl) was topically applied on the ears of mice every other day, for a total of four applications. Ethanol (vehicle) was applied on the control background, but similar results were observed using Foxp3\(^{egfp}\) (C57BL/6) mice. Balb/c mice were preferred to avoid autofluorescence from melanin.

**RNA preparation and qPCR**

Cells were sorted directly into the lysis buffer of RNA Isolation Micro kit (Zymo Research), and RNA was prepared on the basis of kit instructions. For analysis of transcripts in skin, skin tissue was stored in RNA later (Ambion) and homogenized using a tissue homogenizer, and RNA was prepared using RNA isolation kits (Zymo Research). Reverse transcription was performed with an iScript cDNA synthesis kit (Bio-Rad Laboratories). PCRs were run on an ABI Prism 7300 (Applied Biosystems) sequence detection system platform. TaqMan primers and probes were obtained from Life Technologies. The housekeeping gene β\(_2\)-microglobulin was used as a control. Relative mRNA expression was quantified using the 2\(^{-}\)\(\Delta\Delta Ct\) method.

**RNA-seq and transcriptomic analysis**

CD3\(^{+}\)CD4\(^{+}\)Foxp3\(^{+}\) (YFP\(^{+}\)) T\(_{reg}\)s from skin and dLNs were sorted on Aria cell sorter into the lysis buffer (PicoPure RNA Isolation kit, Life Technologies). RNA was prepared after DNase treatment (Qiagen) and sent to Dana-Farber Cancer Institute Molecular Biology Core Facility for library preparation and sequencing. Replicates with a minimum RIN (RNA integrity number) score of 7 were processed. Complementary DNA (cDNA) was synthesized using Clontech SMART-Seq v4 reagents from 500 pg of RNA and fragmented to a mean size of 150 base pairs (bp) with a Covaris M220 ultrasonicator. Illumina libraries were prepared from cDNA using Rubicon Genomics ThruPLEX DNA-seq reagents according to the manufacturer’s protocol. The finished double-stranded DNA libraries were quantified and sequenced on a single Illumina NextSeq 500 sequencing system run with single-end 75-bp reads by the Dana-Farber Cancer Institute Molecular Biology Core Facility. TopHat was used to align reads to mouse genome [Mm9, National Center for Biotechnology Information (NCBI)], and HTSeq was used to estimate read counts. Read counts from all experiments are listed in table S1. Highly correlated triplicate samples were used for comparative analysis (fig. S6). DEseq2 was used to normalize data and access differential gene expression with an FDR of <0.05. Expression levels for individual genes are represented as reads per kilobase of transcript per million mapped reads (RPKM). Heat maps were generated using GENE-E software (Broad Institute). RNA-seq raw data can be accessed through accession no. GSE99086.

**Intravital two-photon imaging**

Foxp3\(^{egfp}\) (Balb/c) mice were anesthetized intraperitoneally using ketamine (100 mg/kg) and xylazine (10 mg/kg). One of the ears was gently attached to an aluminum block using double-sided tape. Ear temperature was maintained at 33°C using a heating pad. GenTeal (Novartis) eye gel was spread over the ear to allow immersion of the 20× objective (0.95 numerical aperture). Images were acquired using an upright microscope (Prairie Technologies) coupled to a Mai Tai Ti:Sapphire laser (Spectra-Physics). To visualize vasculature, mice were intravenously injected with Qdot655 (Molecular Probes) diluted in phosphate-buffered saline. Images were acquired with a laser wavelength of 900 nm for optimal GFP excitation and second-harmonic generation. Epidermis and dermis were analyzed by acquisition of ~100-mm optical stacks every 30 to 60 s for 15 to 60 min with 4-mm spacing. Images were transformed into four-dimensional time-lapse movies and analyzed using Imaris software versions 7.4.2 and 8.4.1 (Bitplane). Imaging experiments were performed in the Balb/c background, but similar results were observed using Foxp3\(^{egfp}\) (C57BL/6) mice. Balb/c mice were preferred to avoid autofluorescence from melanin.

**Histology**

Tissue samples were stored in 10% formalin and sent to the histology core at Boston Children’s Hospital for processing and hematoxylin and eosin (H&E) staining. Slides were analyzed on the 20× objective of bright-field microscope (Nikon), and captured images were analyzed using ImageJ software for enumeration of dermal thickness.

**Local treatments by intradermal injection**

Recombinant TL1A (0.9 µg/µl; catalog no. 753008, BioLegend) was injected intradermally into the ear of mice in a total volume of 10 µl every day for 3 days. Isotype antibody or anti-human/mouse TL1A antibody (R&D Systems) was injected intradermally into the ears in a total volume of 10 µl every other day for 3 days. Cells from ears were prepared, and flow cytometric analysis was performed as described earlier.

**Epicutaneous sensitization**

Six- to 8-week-old female mice were epicutaneously sensitized for 2 weeks, as described previously (9). In brief, for each treatment, female mice were anesthetized, and then their back skin was shaved and tape-stripped with a film dressing (Tegaderm, 3M). In brief, for each treatment, female mice were anesthetized, and then their back skin was shaved and tape-stripped with a film dressing (Tegaderm, 3M). EC sensitization consisted of applying a 1-cm\(^2\) gauze containing 200 µg of OVA (Sigma-Aldrich) to the skin after each tape stripping and securing it with a film dressing. Analyses were done at day 12.

**Enzyme-linked immunosorbent assays**

For detection of total IgE levels, mouse sera were prepared and enzyme-linked immunosorbent assay (ELISA) was performed (88-50460-88, eBioscience) as per the manufacturer’s instructions. For quantification of cytokines in the tissue, mouse ears were flash-frozen in liquid nitrogen. Tissue was chopped, lysed, and homogenized in 500 ml of T-PER tissue protein extraction buffer (catalog no. 78510, Thermo Fisher Scientific).
Scientific) in the presence of complete protease inhibitor and phosphatase inhibitors. Total protein was quantified using a bicinchoninic acid protein assay kit (catalog no. 23227, Pierce), and levels of IL-5 were enumerated after normalizing to the total protein content in the tissue. IL-5 levels in ear skin were measured using Quantikine IL-5 kit (M5000, R&D Systems), and TL1A levels were measured using DuoSet ELISA kit (DY1896-05, R&D Systems).

**Statistical analysis**
Statistical significance was determined by the Mann-Whitney test or analysis of variance (ANOVA) analysis using GraphPad Prism. P < 0.05 was considered statistically significant.

**SUPPLEMENTARY MATERIALS**
immunology.sciencemag.org/cgi/content/full/3/21/eaao6923/DC1

Fig. S1. ROR

Fig. S5. Skin TSLP and serum IgE levels in to produce IL-10.

Fig. S1. ROR

Fig. S5. Skin TSLP and serum IgE levels in to produce IL-10.

Fig. S1. ROR

Fig. S5. Skin TSLP and serum IgE levels in to produce IL-10.

Fig. S1. ROR

Fig. S5. Skin TSLP and serum IgE levels in to produce IL-10.

Fig. S1. ROR

Fig. S5. Skin TSLP and serum IgE levels in to produce IL-10.

Fig. S1. ROR

Fig. S5. Skin TSLP and serum IgE levels in to produce IL-10.


Acknowledgments: We thank the flow cytometry core at Boston Children’s Hospital, the sequencing core at Dana-Farber Cancer Institute, and the Human Skin Disease Resource Core at Brigham and Women’s Hospital for their service; T. Chatila for a gift of Fopxp3^{Cre} mice; D. O’Leary for a gift of RorαCre/Cre mice; R. Clark for sharing human skin samples; and T. Chatila, L. M. Charbonnier, H. Oettgen, and J. Chou for reading the manuscript and useful discussions.

Funding: This work was supported by NIH grant AI113294-01A1, HHSN272201000020C, and intramural funding from the National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH. Author contributions: N.M., J.M.L.-C., and R.S.G. designed the experiments; N.M., J.M.L.-C., U.J., O.B., N.K.O., and C.K. performed the experiments and analyzed the data. F.M., P.C., U.H.v.A., R.M.S., E.C.W., and R.S. contributed critical reagents, mice, or analytic tools. N.M., J.M.L.-C., and R.S.G. interpreted the data and wrote the manuscript. N.M. and J.M.L.-C. performed the statistical analyses. Competing interests: The authors declare that they have no competing interests. Data and materials availability: The RNA-seq data reported in this paper are archived at the NCBI Gene Expression Omnibus database (accession no. GSE99086).

Submitted 16 August 2017
Accepted 17 January 2018
Published 2 March 2018
10.1126/sciimmunol.aao6923

RORα-expressing T regulatory cells restrain allergic skin inflammation


DOI: 10.1126/sciimmunol.aao6923

Curbing ILC2 enthusiasm

Atopic dermatitis is an allergic disease driven by type 2 immune responses in the skin. Using mouse models of dermatitis, Malhotra et al. have identified tumor necrosis factor (TNF) family cytokine, TNF ligand–related molecule 1 (TL1A), and its receptor death receptor 3 (DR3) as being critical in regulating cross-talk between skin-resident T regulatory cells (Tregs) and type 2 innate lymphoid cells (ILC2) that drive skin inflammation. They report that retinoid-related orphan receptor α (RORα) drives expression of DR3 in Tregs, and that, upon deletion of RORα, skin-resident Tregs are unable to sequester TL1A that drives effector functions of ILC2s. The studies open up the possibility of targeting the TL1A-DR3 in the context of dermatitis and other skin allergies.

Use of this article is subject to the Terms of Service