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Treg cells require Izumo1R to regulate γδT cell-driven inflammation in the skin

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Izumo1R is a pseudo-folate receptor with an essential role in mediating tight oocyte/spermatozoa contacts during fertilization. Intriguingly, it is also expressed in CD4$^+$ T lymphocytes, in particular Treg cells under the control of Foxp3. To understand Izumo1R function in Treg cells, we analyzed mice with Treg-specific *Izumo1r* deficiency (Iz1rTrKO). Treg differentiation and homeostasis were largely normal, with no overt autoimmunity and only marginal increases in PD1$^+$ and CD44^{hi} Treg phenotypes. pTreg differentiation was also unaffected. Iz1rTrKO mice proved uniquely susceptible to imiquimod-induced, γδT cell-dependent, skin disease, contrasting with normal responses to several inflammatory or tumor challenges, including other models of skin inflammation. Analysis of Iz1rTrKO skin revealed a subclinical inflammation that presaged IMQ-induced changes, with an imbalance of Rorγ+ γδT cells. Immunostaining of normal mouse skin revealed the expression of Izumo1, the ligand for Izumo1R, electively in dermal γδT cells. We propose that Izumo1R on Tregs enables tight contacts with γδT cells, thereby controlling a particular path of skin inflammation.

Foxp3 | T regulatory cells | psoriasis

Significance

These results suggest a role for Izumo1R, a molecule known to facilitate egg/spermatozoa interaction at fertilization, in immunoregulatory circuits in the skin. The expression of Izumo1R in FoxP3+ Treg cells has long been an enigma. The phenotype of knockout mice with Izumo1R deficiency in Tregs suggests that Izumo1R is not generically involved in Treg-mediated tolerance system wide, but is revealed by the induction of psoriasis-like inflammation in the skin. Sensitivity to Izumo1R pathways is not manifest in all models of skin inflammation, suggesting that it is more specific to pathogenesis loops in psoriasis.

FoxP3+ regulatory T cells (Tregs) are gatekeepers of a balanced immune system, playing key roles in the maintenance of tolerance to self and the prevention of runaway immune responses (1, 2). A subset of these cells, dubbed "tissue-Tregs," resides in parenchymal tissues and helps control tissue homeostasis and orderly wound repair (3, 4). The transcription factor Foxp3 plays an important part in determining Treg identity and stability; its absence or dysfunction results in multiorgan autoimmunity, in humans with IPEX syndrome and in *scurfy* mutant or FoxP3-deficient mice (2, 5–9). FoxP3 does so by regulating, at least in part, expression of the canonical Treg signature that distinguishes Tregs from other CD4+ T cells (Tconv), including functionally important genes such as *Il2ra* (CD25), *Tnfrsf18* (GITR), or *Clta4*.

Izumo1R (aka JUNO or FOLR4, encoded by *Izumo1r*) is one such member of the Treg signature. Its expression is higher in Treg than in Tconv cells, correlating with FoxP3 levels in several conditions and in single-cell RNAseq analyses, and is activated by transfection of *FoxP3* into CD4+ Tconv cells (10–15). Izumo1R is also induced in chronically activated Tconvs and has been proposed as a marker of hyporesponsiveness and anergy or of long-lived T follicular helper cells (16–18). Unlike most proteins expressed across immunocytes and whose functions are at least partially understood, Izumo1R is a mysterious molecule. From its sequence and domain composition, it was originally thought to be a receptor for folic acid (hence its early Folr4 name), but recent evidence suggests that it has no real ability to bind folate (19). Much of what is known about Izumo1R derives from studies of fertilization, where it plays an essential role (19–21). Izumo1R is expressed on oocytes, while its ligand Izumo1 is expressed on spermatozoa, and *Izumo1r*$^{-/-}$ females are infertile (19). Structural analysis of Izumo1/Izumo1R interaction has suggested that their contact enables cell–cell adhesion, enabling sperm binding and fertilization (20, 22), a conformational change in Izumo1 upon binding Izumo1R possibly facilitating a zipper-like multimer formation that would stick spermatozoa and oocytes into tight contact.

The function of Izumo1R in Treg cells, and in the immune system in general, remains elusive. Here, we investigated its role through analysis of mice lacking Izumo1R specifically in Tregs. Ablation of Izumo1R in Tregs had few or no consequences on systemic Treg pools and function, but led to focused dysregulation in the skin, where subclinical inflammation arose through dysregulation of γδT cells. In turn, this sets the stage for heightened responsiveness to some, but not all, inflammation-inducing agents in the skin. Hence, our work points to a tissue- and context-specific role for Izumo1R in controlling inflammation in the skin.

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Results

Variegated Expression of Izumo1R in Tregs. Izumo1R is a hallmark of the Treg transcriptome. For first clues to its function, we investigated more deeply its expression pattern in Treg cells across various tissues and over time. Izumo1R was observed on the surface of many splenic $CD4^+$ T cells, with Tregs showing the highest levels (Fig. 1*A*). Expression of Izumo1R correlated with that of CD73, splenic Tregs including a subset with the highest expression of both molecules (Fig. 1*B*). Splenic Tregs with the highest expression of Izumo1R belonged to the antigen-experienced ("aTreg") pool, as evidenced by high CD44 and low CD62L expression, and also displayed the highest levels of PD1 and Nrp1 (Fig. 1*C*). The proportion of this $Izumo1R^{hi}CD73^{hi}$ subset increased with age, suggesting a potential time-specific function for Izumo1R (Fig. 1*D*). The expression of these markers on Treg and Tconv $CD4^+$ T cells varied with organismal location: high in lymphoid organs (spleen and lymph node, Fig. 1*B*), also high in mucosal tissues (lung and colon, Fig. 1*E*), but lower in typical tissue-Tregs in the skin and visceral adipose tissue (Fig. 1*E*). This gradient of Izumo1R protein in tissue Tregs (Fig. 1*F*) was also evidenced by analysis of RNAseq data (Fig. 1*G*), confirming the lower expression in skin and VAT Tregs relative to spleen, colon, and injured muscle. A differential distribution of *Izumo1r* transcripts across Treg subsets was also visualized in reanalysis of published single-cell RNAseq datasets: In spleen Tregs (from ref. 23), *Izumo1r* was readily detected across most Treg cells (Fig. 1*H*, *Left*), independently of signatures of resting (*Ccr7, Sell*) or tissue-precursor Tregs. In contrast, *Izumo1r* transcripts were observed only in a small subset of skin Treg cells, characterized by high *Ccr7* expression, and distinct from the predominant Treg subsets with high expression of *Areg* or of a generic "tissue Treg" signature (Fig. 1*H*, Right, data from ref. 24). These results suggested a potential specificity of Izumo1R expression in Treg cells, distinct from that of conventionally defined tissue Tregs.

Previous studies suggest that $Izumo1R^{hi}$ Tconvs are particularly sensitive to conversion into pTregs (16). We sorted four different Tconv subsets based on Izumo1R and CD73 expression and cultured the sorted cells under iTreg conditions. Izumo1R expression by Tconvs was not required for iTreg differentiation as approximately 50% of Izumo1R-CD73- Tconvs became FoxP3 positive and $Izumo1R^{hi}CD73^{hi}$ cells were not much more susceptible to in vitro Treg differentiation (Fig. 1*I*).

Phenotype of Mice Lacking Izumo1R in Treg Cells. To investigate a role for Izumo1R in Treg cells, we generated conditional knockout mice with a specific deficiency in Izumo1R on Treg cells by intercrossing $Izumo1R^{fl}$ (25) and *Foxp3-Cre* mice. The resulting *Izumo1r*$^{fl/fl}$*Foxp3*$^{-}$Cre mice (hereafter Iz1rTrKO) were born in typical Mendelian proportions and developed normally. Analysis of the Treg compartment in young adults showed Treg frequencies and numbers in the thymus and lymphoid tissues comparable with those of Izumo1R-proficient littermates (Fig. 2*A*). We noted only a modest and inconstant reduction of Treg proportions in the colonic lamina propria (Fig. 2*A*). These colonic Tregs split along the same proportions of Helios+ and RORγ+ subsets, which generally correspond to thymus-derived and microbe-induced pools, as in control littermates (Fig. 2*B*). Overall, splenic Tregs of Iz1rTrKO mice showed normal expression of the typical Treg markers (CD25, CTLA4, GITR, Nrp1, CD73, ICOS), with only slightly higher proportions of $CD44^{hi}CD62L^{lo}$ aTregs (and correspondingly of $PD1^+Nrp1^+$ Tregs; Fig. 2*C*).

To ascertain potential changes in Treg phenotype resulting from the Izumo1R deficiency, we sorted $CD4^+CD25^+YFP^+$ Treg cells from Iz1rTrKO and control littermates for RNAseq profiling (in biological triplicates). Aside from the predicted reduction in *Izumo1r* transcripts themselves, very few changes were noted (Fig. 2*D*; 7 and 36 up- and down-regulated transcripts, respectively, at a FoldChange threshold of 2 and *t* test *P* value of 0.01, which is very close to noise levels in such profiling). Key Treg transcripts such as *Foxp3*, *Ctla4, IL2ra, Lrrc32, Pde3b, Satb1, Klrg1,* and *Ikzf2* were not markedly shifted, and there was only a modest downregulation of the TregUp signature (ref. 12) as a whole.

Compensatory pathways can sometimes mask phenotypes in knockout mice, but true effects can be revealed in competitive settings in which mutant and wild-type cells cohabit. To this end, we generated mixed bone marrow chimeras with equal amounts of bone marrow progenitors from *Izumo1r*$^{fl/fl}$*Cd4-Cre* and *Cre*-negative control littermates, carrying CD45 congenic markers for identification. (In this instance, we used a CD4-cre driver to ensure that early effects on Treg differentiation, before expression of FoxP3 itself, would be captured.) $Izumo1R^{fl/fl}CD4^{Cre}$ Tregs did not display any competitive disadvantage relative to controls across several tissues analyzed (Fig. 2*E*). To test the ability of Izumo1R-deficient $CD4^+$ Tconv cells to differentiate into pTreg cells in peripheral organs, we used the Treg complementation model in *Foxp3-DTR* mice (26), wherein pTregs differentiate from naïve Tconv cells in Treg-ablated hosts. In this assay, equivalent conversion to FoxP3 positivity was observed for *Izumo1r*$^{fl/fl}$*Cd4-Cre* and control Tconvs (Fig. 2*F*).

Kalekar et al. suggested that Izumo1R-negative T cells do not efficiently give rise to pTreg cells after transfer into *Tcra*$^{-/-}$ T cell–deficient mice (16). Hence, we asked whether Izumo1R itself influenced the efficiency of conversion in this system by transferring $CD25^-CD44^+CD73^+$ "anergic" and $CD25^-CD44^{low}CD73^{low}$ "naïve" T cells from *Izumo1r*$^{fl/fl}$*Cd4*-Cre and control littermates into TCRa-deficient hosts (Fig. 2*G*). The results reproduced the prior observations (16) in that $CD44^+CD73^+$ Tconvs differentiated to Foxp3+ status much more effectively than naïve CD44-CD73- cells. However, the presence or absence of Izumo1R itself made no difference, suggesting that Izumo1R does not directly control pTreg conversion, and is simply a marker of the differential ability in the populations. Overall, Izumo1R-deficient cells populated Treg niches as efficiently as control cells, whether in full or competitive settings, and in thymic and peripheral differentiation pathways, establishing that Izumo1R does not have a broad and nonredundant function in Treg differentiation and homeostasis.

Izumo1R-Deficient Treg Cells Have a Skin-Specific Phenotype. We then assessed whether autoimmune deviation or other defects in Treg function appeared in Iz1rTrKO mice. By 10 wk of age, mutant mice of neither sex showed any of the manifestations observed in Foxp3-deficient *scurfy* mice: no visible lesions externally or at autopsy, body weights comparable with those of control littermates, and no lymphoproliferation as judged from spleen and lymph node weight and cellularity (Fig. 3*A*). There was no indication of gut inflammation, macroscopically or in tissue sections. We did note, in approximately 15% of older Iz1rTrKO mice, the development of spontaneous skin ulcerations, typically on the lower-back skin, histological analysis showing severe dermatitis, accompanied by splenomegaly (Fig. 3*B*). Other tissues (heart, colon, kidney, liver, and lungs) in these aged Iz1rTrKO mice did not show any signs of inflammation (*SI Appendix*, Fig. S1, evaluation by a third-party pathologist), suggesting that this phenotype in aged mice is quite skin specific.

Given the paucity of spontaneous disease, we next searched for a functional role of Izumo1R in Tregs across several models of

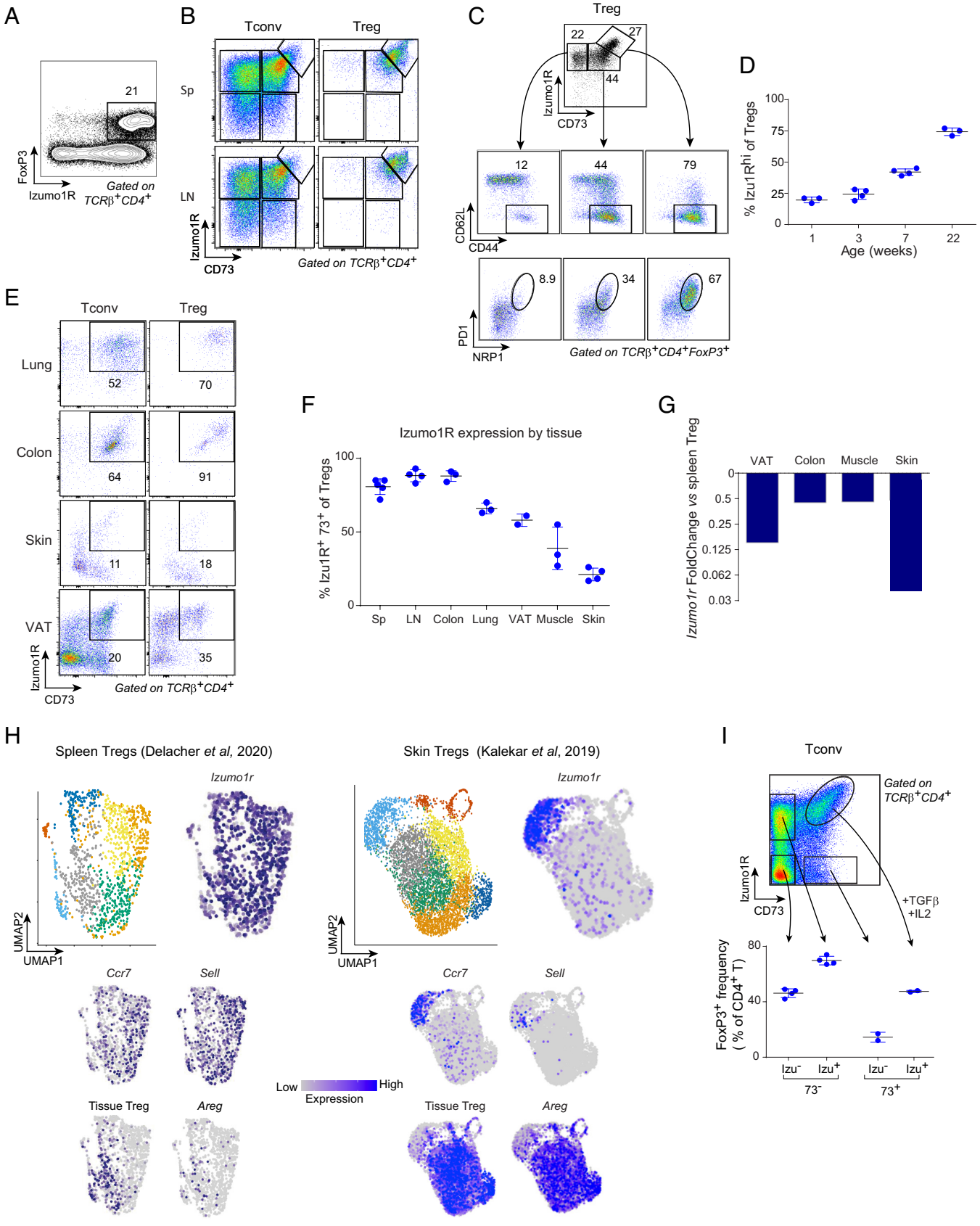

Fig. 1. Izumo1R expression on Tregs varies with tissue and age. (*A*) Representative flow cytometry plot of splenic TCRβ+CD4+ T cells, stained for Foxp3 and Izumo1R. (*B*) Izumo1R vs CD73 in gated FoxP3+ (Treg) or FoxP3- (Tconv) TCRβ+CD4+ spleen or lymph node T cells. (*C*) Representative plots of several markers in TCRβ+CD4+Foxp3+ splenocytes, gated at top. (*D*) Frequency of Izumo1R+CD73+ (Izu1R^{hi}) splenic Tregs over age. (*E*) Representative Izumo1R/CD73 plots of gated Treg or Tconv cells from indicated tissues. (*F*) Quantitation of several experiments as in *E*, each dot an individual mouse. (*G*) *Izumo1r* expression in bulk RNAseq profiling of Tregs from indicated tissues. (*H*) Reanalysis of single-cell RNAseq of spleen and skin Tregs (23, 24), UMAP dimensionality reduction plots color coded for expression of *Izumo1r* (*Top*) or subset-identifying genes (*Bottom*). (*I*) Tconv cells were sorted based on Izumo1R and CD73 expression as indicated, then cultured with TGFβ and IL2, and FoxP3 expression was evaluated at 72 h.

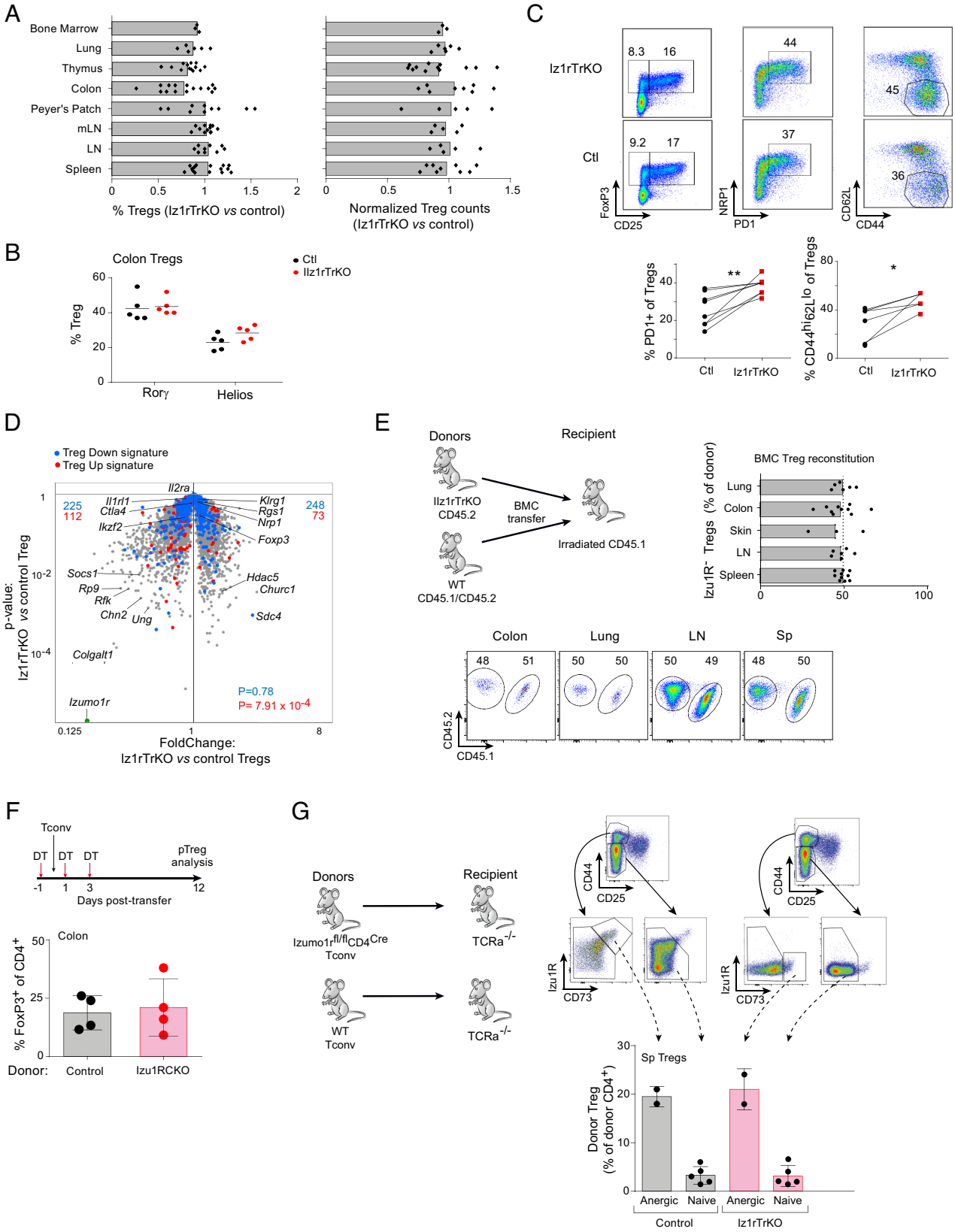

Fig. 2. Mostly normal Treg cells in *Izl1rTrKO* mice. (*A*) Treg frequencies and normalized cell counts in indicated tissues from Izl1rTrKO mice normalized to values in control littermates. (*B*) Frequency of Rorγ^{+} and Helios^{+} among colonic Tregs from Izl1rTrKO mice and control littermates. (*C*) Representative flow cytometry plots and summary dot plots of Tregs from Izl1rTrKO mice and control littermates. (*D*) Volcano plot comparing gene expression in splenic Treg RNAseq profiling (in triplicate) from Izl1rTrKO vs control littermates; highlights: Treg down (blue) and Treg up (red) signatures (12). (*E*) Mixed bone marrow chimeras constructed with Izl1rTrKO and control stem cells (50/50 ratio); representative plots and quantitation of Izl1rTrKO-derived cells (frequency of total donor cells) in chimeric mice analyzed at 10 to 12 wk post transfer. (*F*) pTreg induction in Treg-depleted hosts (*Foxp3.dtr*, treated 3× with Diphtheria toxin), comparing the frequency of resulting FoxP3^{+} pTregs derived from *Izu1Rfl/fl.CD4^{Cre}* or control mice. (*G*) 2 × 10^{5} congenically marked CD4^{+} Tconv cells from indicated donors were transferred to TCRα$^{-/-}$ hosts, with donor Tconv cells being sorted according to CD44 and CD73 expression (*Top*). The frequency of resulting FoxP3^{+} Treg among donor-derived cells was determined 21 d after transfer.

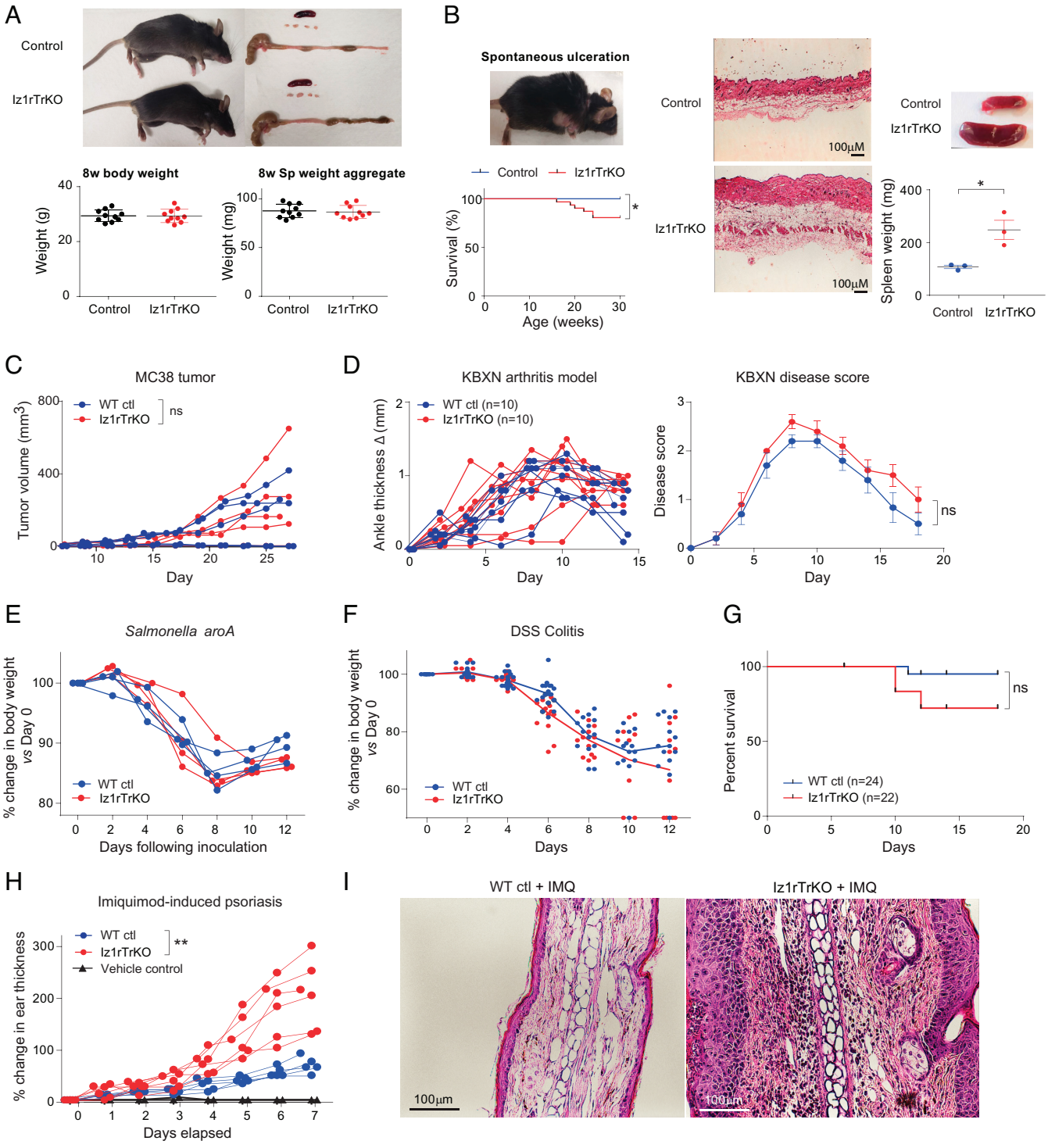

Fig. 3. No widespread autoimmune phenotypes in *Iz1rTrKO* mice. (*A*) Picture of a representative Iz1rTrKO and control littermate with colon, spleen, and lymph nodes, with spleen and body weight at 8 wk (each dot one mouse). (*B*) Representative photograph of aged (24 to 32 wk old) Iz1rTrKO mouse with developed spontaneous ulceration, and Kaplan–Meier survival plot, with representative back skin H&E staining of ulcerated Iz1rTrKO and control mice (*Middle*) and spleen picture and weight from ulcerated Iz1rTrKO or controls (*Right*). (*C*) Tumor rejection: MC38 carcinoma cells were subcutaneously implanted into the flanks of Iz1rTrKO mice and control littermates. Tumor volume measured starting at D7 post injection. (*D*) Arthritis induction: 8 to 10-wk-old Iz1rTrKO mice and control littermates were injected with 200 μL K/BxN serum. Disease severity score and change in ankle thickness were evaluated over time. (*E*) Bacterial gut challenge: Iz1rTrKO and control littermates were gavaged with *Salmonella aroA* and body weight was measured. (*F* and *G*) DSS colitis followed via change in body weight (*F*) and survival (*G*). (*H*) Imiquimod-induced inflammation, evaluated as change in ear thickness after daily application, in a cohort of Iz1rTrKO mice and control littermates. (*I*) Representative H&E staining of IMQ-treated ears.

induced inflammatory disease in which the involvement of Tregs has been demonstrated. No difference was noted between Iz1rTrKO and control littermates in i) the MC38 transplantable adenocarcinoma tumor model: the time-course and tumor growth being comparable in the two groups (Fig. 3*C*); ii) the K/BxN model of serum-induced arthritis (27): the incidence and severity being similar in Izumo1R and control groups (Fig. 3*D*); and iii) intestinal infection by *Salmonella aroA* (28), again showed a similar course and extent of weight loss in the two groups (Fig. 3*E*). In the DSS colitis model, which is also affected by Treg cells (29, 30),

the course of disease, as evaluated by body weight and histology, was again similar in Iz1rTrKO mice and control littermates (Fig. 3*F*), although there was a slightly increased incidence of mortality in the mutant group (Fig. 3*G*). The production of Type 3 cytokines also did not differ between colonic T cells of Iz1rTrKO and control groups, in either the DSS or *Salmonella aroA* models (*SI Appendix*, Fig. S2).

On the contrary, a very clear phenotype was observed in the dermatitis model induced by Imiquimod (IMQ), a Toll-like receptor 7/8 agonist whose repeated administration provokes psoriasis-like lesions (31). Iz1rTrKO mice developed significantly more severe disease, with faster and more pronounced ear swelling (Fig. 3*H*). Hematoxylin/eosin-stained sections of the challenged ear skin at day 7 revealed much more severe inflammation and acanthosis in Iz1rTrKO mice than in controls (Fig. 3*I*). Hence, Izumo1R expression on Tregs appears to be particularly important for modulating inflammation in the skin while largely dispensable for overall Treg homeostasis and function in several other inflammatory contexts.

Iz1rTrKO Mice Harbor a Specific Defect Related to Imiquimod-Induced Pathology. The above phenotypic screen revealed a very specific requirement for Izumo1R in Treg cells in the skin-IMQ model. We then asked what might explain this preferential phenotype, which was unexpected given that Izumo1R is expressed in many lymphoid organs, and actually less in the skin (Fig. 1). The differential sensitivity of Iz1rTrKO mice to IMQ was confirmed over 30 experimental littermate pairs in 4 experiments, typically performed in mice of 8 to 10 wk of age (Fig. 4*A*). We also noted a spreading of the lesions to neighboring areas of the skin, along with splenomegaly in IMQ-treated Iz1rTrKO mice compared with control-littermate or vehicle-treated controls (Fig. 4*B*). To ask whether the restricted Iz1rTrKO phenotype was linked to the location (skin) or to a particular aspect of the IMQ model, we tested Iz1rTrKO mice in two other models of skin inflammation. The MC903 dermatitis model, provoked by the application of the vitamin D3 analog, operates via induction of TSLP in keratinocytes and recruitment of Interferon (IFN)-γ–producing CD4+ T cells. (32). Here, no effect of the *Izumo1r* deficiency was observed (Fig. 4*C*). Second, we assessed the oxazolone model of hapten-specific allergic hypersensitivity, in which inflammation is marked by an increase in Th2 cytokines and TNFa in the skin lesions (33). Again, no differences in disease severity were observed between Iz1rTrKO and control littermates (Fig. 4*D*). Finally, skin Tregs have been shown to regulate hair follicle stem cells and influence follicle development (34). Accordingly, we assessed this function by inducing the anagen cycle by depilation at approximately 56 d of age as previously described (34). We did not observe an impairment in hair growth in CKO compared with control littermates, suggesting that Treg function in regulating hair follicle stem cells is not influenced by Izumo1R (Fig. 4*E*). Thus, the impact

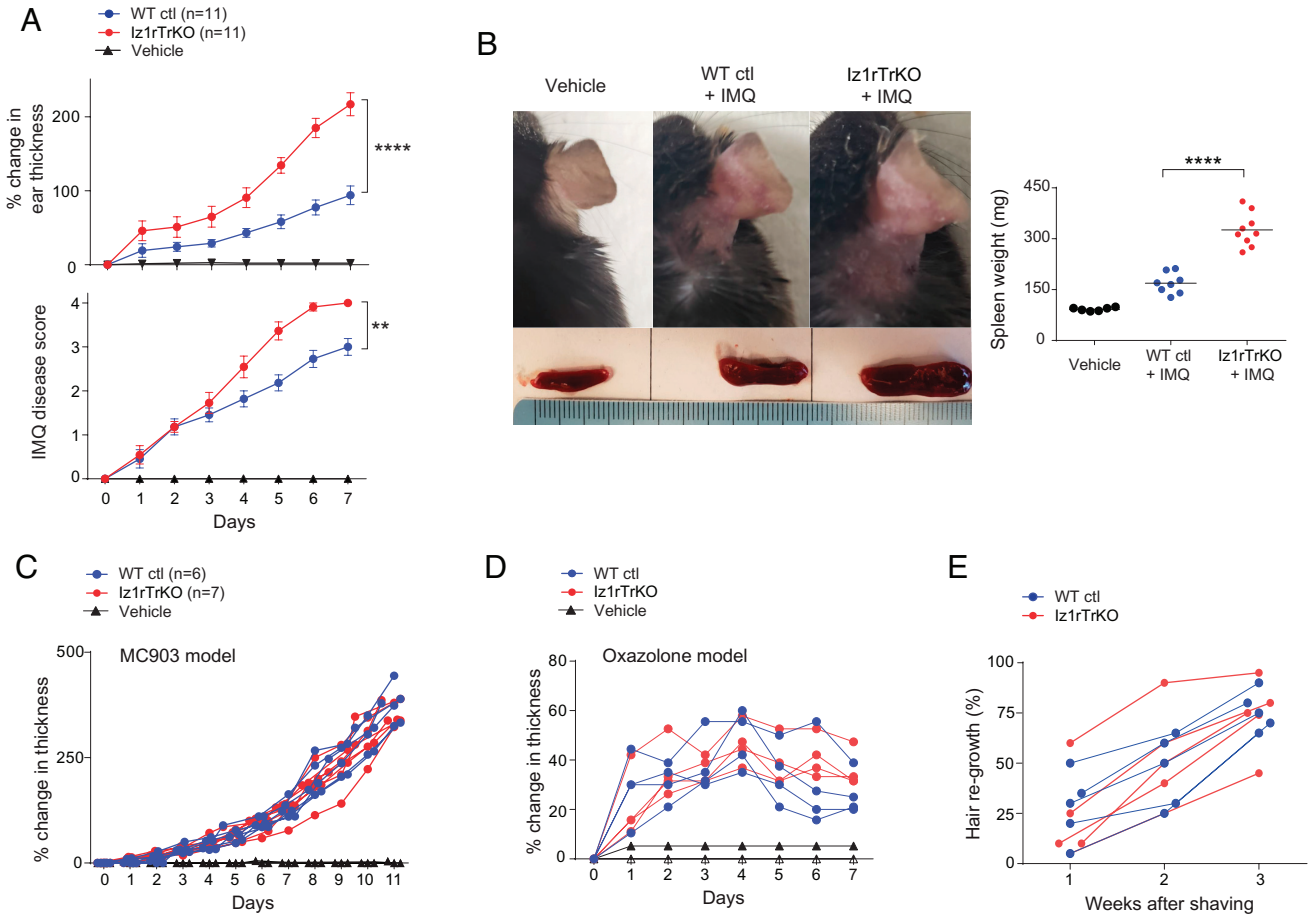

Fig. 4. Treg dysfunction in Iz1rTrKO skin is specific to IMQ-induced inflammation, not a widespread failure of skin Treg function. (*A*) Cumulative results in the IMQ model, averaging ear thickness and pathology disease scores in Iz1rTrKO mice and control littermates. (*B*) Splenic weight and representative ear and spleen images of Iz1rTrKO mice and control littermates after IMQ application – note the spread of inflammation and hair loss beyond the ear proper. (*C*) Calcipotriol (MC903, 4 nmole) was applied daily to Iz1rTrKO mice and control littermates; the results are represented as percent change in ear thickness for each animal. (*D*) Oxazolone was applied to ear of Iz1rTrKO mice and control littermates, and increases in ear thickness are represented as above. (*E*) Kinetics of hair regrowth for Iz1rTrKO mice and control littermates up to 3 wk following depilation. Control littermates of *Izumo1R*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ genotype.

of the Izumo1R deficiency seems to be revealed only in the specific context of skin inflammation in the IMQ model, with the cross talk between keratinocytes, T cells, and neutrophils that this model entails, but does not correspond to a generalized breakdown of Treg functions in the skin.

Enrichment of γδ17 T Cells Leads to Low-Grade Type-17 Inflammation in the Skin of 10-wk-old Iz1rTrKO Mice. Given the very specific requirement for *Izu1R* in the context of IMQ-induced inflammation, we performed a series of experiments aimed at identifying its mechanistic underpinnings. First, analysis of skin Treg frequencies ruled out the trivial explanation of a specific loss of Tregs after IMQ: Treg frequencies were not affected by disease induction, in either control or Iz1rTrKO mice (Fig. 5*A*). Second, to identify cellular intermediates, we performed a broad immunoprofiling of skin immunocytes during IMQ challenge. Most cell types analyzed (B and αβT lymphocytes, myeloid cells) showed no marked change in representation, but two cell types did stand out. First, IMQ-challenged skin from Iz1rTrKO mice contained significantly more neutrophils, commensurate with the stronger inflammation (Fig. 5*B*). In addition, and although the overall proportion of γδT cells did not change, they contained a higher fraction of RORγ+ cells (Fig. 5*C*). To test whether there were a general induction of Type-3 immunocytes in the absence of Izu1R on skin Tregs, we performed RNAseq on purified γδT and $CD4^+$ Tconv cells from the skin of unmanipulated Iz1rTrKO and control littermates. A clear shift of a Th17 gene signature (ref. 35, *Methods*) was observed in both cell types (Fig. 5*D*), suggesting a predetermined bias toward IL17-based inflammation that might lead to more severe responses to IMQ in Iz1rTrKO mice.

For clues to the molecular mechanism of enhanced inflammation, we conducted RNAseq on whole skin from Iz1rTrKO and control mice, analyzing both back and ear skin. Abundant changes were noted (*SI Appendix*, Fig. S3*A*), with a set of genes induced in both ear and back skin of Iz1rTrKO mice (Fig. 5*E*). Suggestively, these transcripts included those encoding members of the IL-1 family cytokines [e.g., *Il1b*, *Il1f9* (encoding IL36G) and *Il18*], chemokines (such as *Ccl3*, *Ccl4*), and inflammatory mediators (*Clec4* family). *S100a8/9* (encoding calprotectin) are Ca-binding inflammatory mediators, very highly expressed in neutrophils, which are highly represented in psoriatic skin lesions of mice and humans (36). Accordingly, signatures of IL18, IL36, Th17, and neutrophil-mediated immunity (35, 37) were also enriched (*SI Appendix*, Fig. S3*B*).

To determine whether the proinflammatory transcripts enriched in the otherwise asymptomatic skin of 10-wk-old Iz1rTrKO mice might be tied to the heightened susceptibility to IMQ-induced disease, we compared these changes to those elicited by IMQ inflammation (Fig. 5*F*). Interestingly, although both conditions showed specific sets of induced and repressed genes, many of the differentially expressed genes identified in Fig. 5*E* proved to be induced by IMQ, in particular the IL1 family, Ccl3/4 chemokines, and S100a8/9 (further quantified in Fig. 5*G*). This concordance suggests that Iz1rTrKO mice are primed for stronger inflammation outcome after IMQ, as they already overexpress inflammatory genes that drive imiquimod-induced psoriasis, predisposing to heightened responses from Rorγ+ T cells and neutrophils in the skin.

To see whether these presymptomatic effects of the Izumo1R deficiency in Tregs were influenced by the microbiota, we treated 8-wk-old Iz1rTrKO mice and controls with an antibiotic cocktail for 2 wk before analyzing skin immunocytes. The elevated frequencies of neutrophils and Rorγ+ γδT cells were brought back to control levels in antibiotic-treated Iz1rTrKO mice (*SI Appendix*, Fig. S4*A*), suggesting that microbial input may partake in the dysregulation. This notion was further substantiated in *SI Appendix*, Fig. S4*B*, as antibiotic treatment greatly reduces the hyperresponsiveness to IMQ in Iz1rTrKO mice. We suspect that these effects result from perturbations of the skin microbiota, but it is also possible that gut microbes, and thence migration of IL17-producing cells from the gut, are responsible for this reduction.

Izumo1R Tregs Maintain Homeostasis in the Skin through Regulation of Rorg+ γδ T Cells. To try to understand the sequence of events in the skin of Iz1rTrKO mice, we performed a time-course analysis, focusing on the cells and genes found to be dysregulated in the evaluations discussed above. First, skin Treg frequency and numbers were comparable in Iz1rTrKO mice and control littermates at time points between 14 and 70 d of age, which showed that the expected fluctuations linked to anagen and telogen phases of hair follicles (34) were similar in the two genotypes (Fig. 6*A*). The previously noted increase in the frequency of Rorγ+ γδT cells in the skin was a relatively late event, manifest only around 56 d of age (Fig. 6*B*), but slightly before the increase in neutrophils at day 70, consistent with the notion that production of IL17 by γδT cells recruits neutrophils to inflammatory sites. No increase in Rorγ+ αβT cells was noted at any time point (Fig. 6*B*). Second, we traced by RNAseq profiling of whole back skin the induction of the IL1 family members and inflammatory mediators overexpressed in the skin of Iz1rTrKO mice. In accordance with the timing of cellular changes, these mediators and markers of inflammation were only up-regulated after a comparatively long time, with their induction becoming clear at only 56 or 70 d of age (Fig. 6*C*). Hence, the onset of skin inflammation in Iz1rTrKO mice overlaps with a resurgence in the frequency of Rorγ+ γδ T cells, suggesting an important regulatory nexus involving Izumo1R on Tregs at this time.

During fertilization, Izumo1R expressed on oocytes interacts with Izumo1 on spermatozoa, and this interaction is hypothesized to act as an anchor that brings cells closer together (20). We thus searched for Izumo1 expression in immunocytes and stromal cells of the skin. Only trace levels of Izumo1 were detected by RNAseq, here, in ImmGen data (www.immgen.org); mostly in plasma and thymic epithelial cells), or in single-cell atlases of skin tissue (38, 39). On the contrary, immunostaining for Izumo1 in the skin revealed rare $Izumo1^+$ cells, which counterstained with anti-γδTCR and CD45 (Fig. 6*D*). These Izumo1+ γδT cells were localized in the lower dermis, in the vicinity of the hair follicle base, where Tregs have been previously reported to congregate (24, 34). This observation opens the possibility that Izumo1R/Izumo1 interaction channels Treg suppression to γδ T cells in the skin, thereby controlling downstream inflammatory mediators that recruit neutrophils (Fig. 6*E*).

Discussion

The functional heterogeneity of the Treg pool, with diverse roles depending on developmental timepoints, tissular location, and type of regulatory pathway, is well recognized (3). Here, by investigating Izumo1R, a classic Treg signature gene and a direct transactivation target of FoxP3, but of unrecognized function, we may have uncovered an unrecognized facet of Treg biology. Mice with Izumo1R-deficient Tregs showed no defect in thymic or peripheral differentiation, no dysregulated homeostasis, even in competitive settings, no systemic autoimmunity, and no increased susceptibility to inflammatory challenge in several tissues. Only a subclinical skin inflammation appeared past 2 mo of age and led to a strongly exacerbated response to challenge by the TLR agonist Imiquimod, which brings forth two key questions: why the skin specificity, and why the late-acting timeframe?

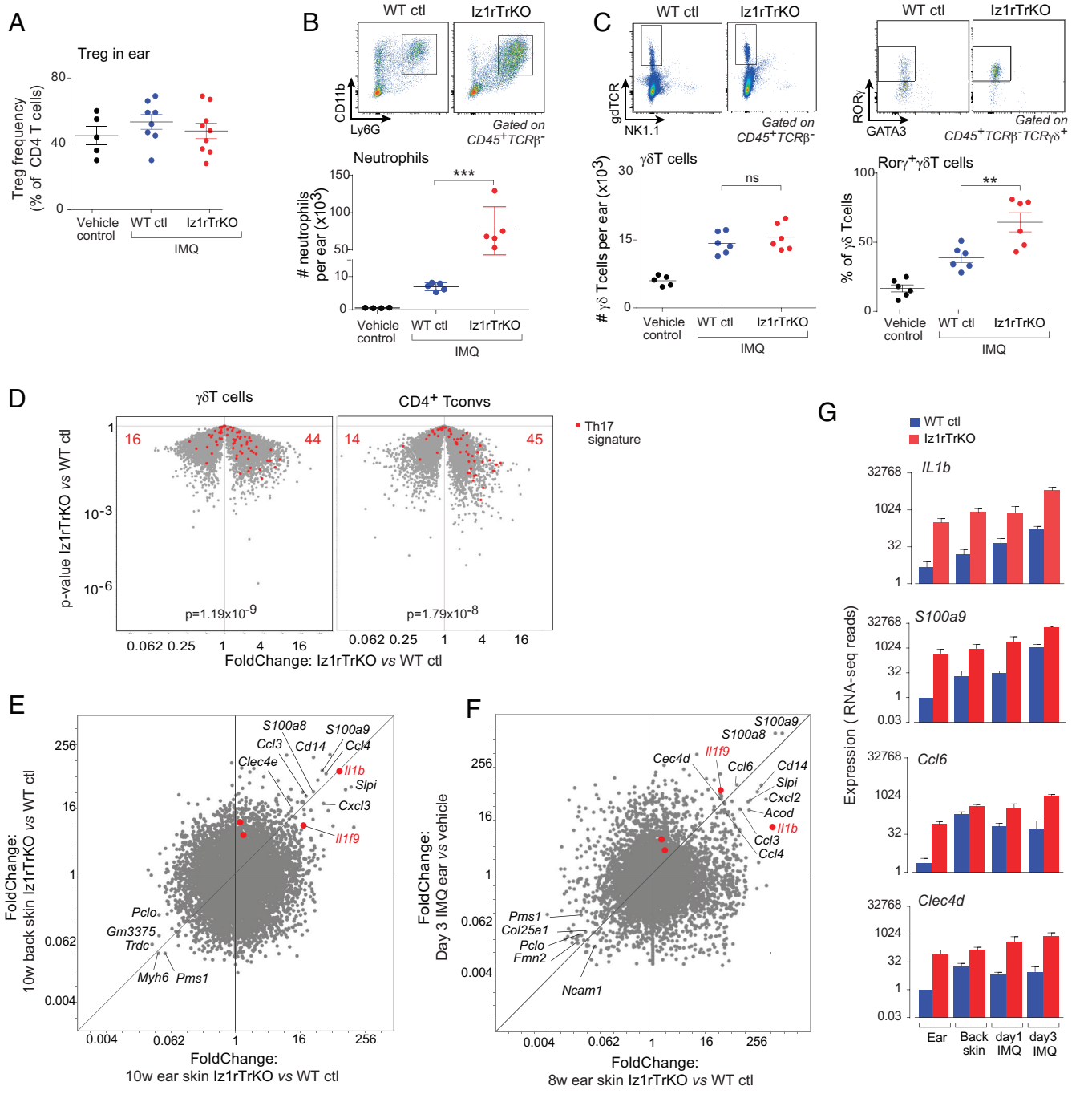

Fig. 5. Subclinical inflammation in Iz1rTrKO mice is amplified by imiquimod. (*A*) Treg frequencies in ear tissue skin after IMQ application. (*B* and *C*) Representative plots and quantitation of ear skin neutrophils (*B*) and γδT cells and Rorγ^{+} γδT cells (*C*) from indicated 8 to 10 w.o. mice. (*D*) Volcano plots of back skin γδT cell and CD4+αβT transcriptomes, determined by RNAseq from Iz1rTrKO vs control littermates (in triplicate). Red highlights: Th17 (red) upsignature (35). (*E*) FoldChange-FoldChange (FC-FC) plots relating changes in ear and back skin transcriptomes, in whole-tissue RNAseq from Iz1rTrKO vs control littermates. IL1 family members detected highlighted in red. (*F*) FC-FC plot relating changes in Iz1rTrKO vs control ear skin (*x*-axis, 8-10 w.o. mice, whole tissue, untreated) to changes elicited by IMQ in WT mice (*y*-axis, Day 3 vs control). (*G*) RNAseq reads for *IL1βS100a9CCL6,* and *Clec4d* in indicated tissues and treatment groups (averages from n = 2 to 3 mice per group). Control littermates were of *Izumo1R*$^{wt/wt}$*Foxp3*$^{Cre/wt}$ genotype.

The overexpression of inflammatory cytokines and the related changes in skin immunocytes appeared only by 50 to 70 d of age in Iz1rTrKO. Several time-dependent Treg functions have been described, but this timeframe is quite different from these precedents. It occurred well after the manifestations of full FoxP3 deficiency, which begin at 12 to 18 d of age in mice (40); much later than the particular Treg pool selected in an Aire-dependent manner during the first few days after birth (41); later than the dramatic impact of microbial colonization of the gut on Treg pools (42, 43) and later than the colonization of parenchymal tissues by tissue-resident Tregs in the first weeks of life (44, 45). Intriguingly, this time element was also seen in the expression of Izumo1R itself, which only stabilizes after 2 or 3 mo. One might speculate that a regulatory loop is being tuned, the upregulation of Izumo1R on Tregs responding to inductive cues on the cell it regulates.

Why the skin? Importantly, the Izumo1R deficiency did not affect all aspects of skin homeostasis: MC903- and oxazolone-induced skin inflammation were not worsened; hair regrowth after shaving, which skin Tregs influence (34), was normal. This dichotomy suggests a selective dysregulation: not a specific requirement for Izumo1R by

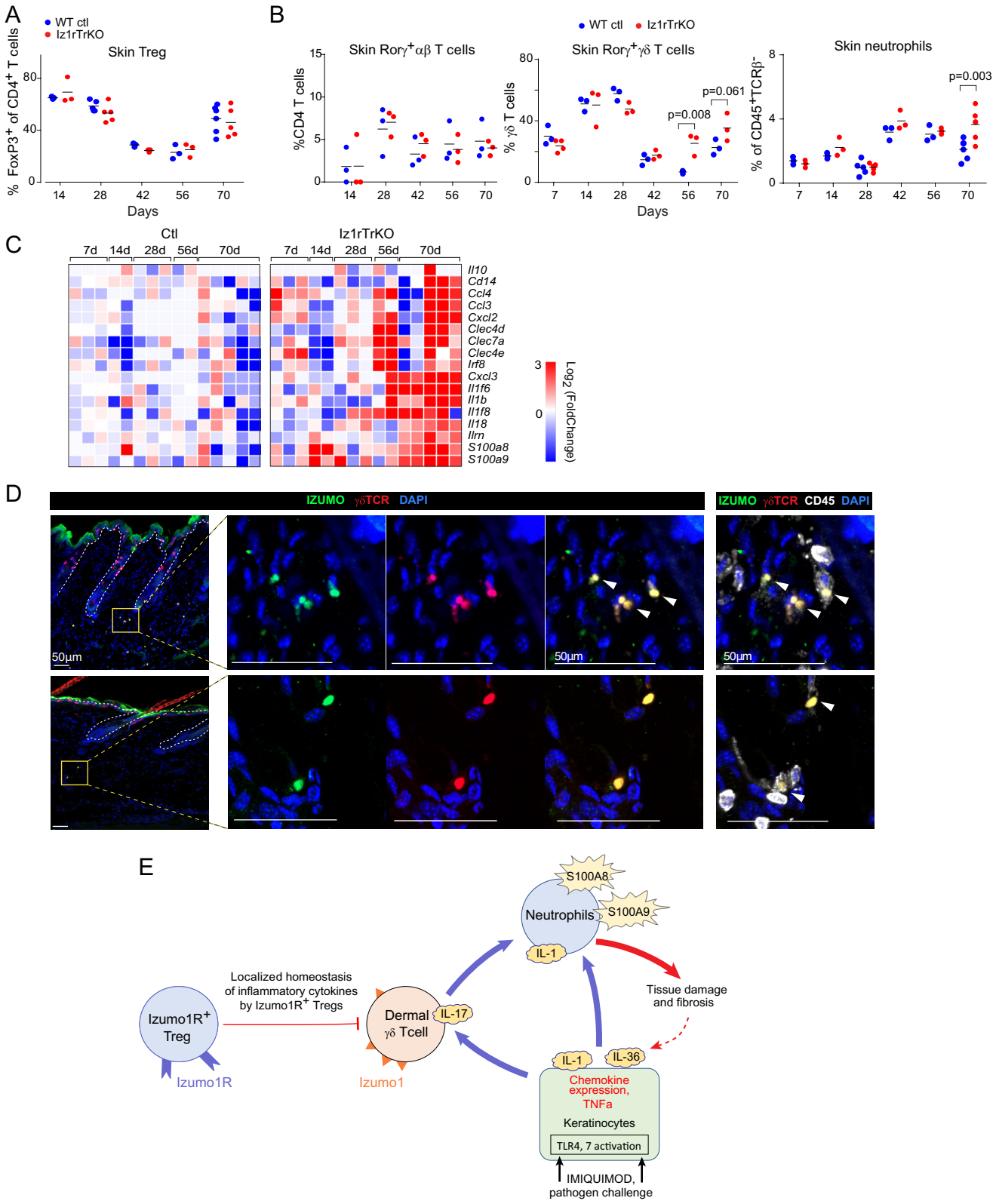

Fig. 6. Izumo1R in skin Tregs operates via Rorγ+ γδT cells. (*A* and *B*) Frequency of indicated immunocytes in the back skin of Iz1rTrKO mice and control littermates during the first months of age. (*C*) RNAseq analysis of whole skin during the same period. The expression of the key inflammatory genes dysregulated in Iz1rTrKO skin (selected from Fig. 5*E*, including Il1 family members) is shown for each sample as the FoldChange vs the mean of identically aged control littermates. (*D*) Representative images showing confocal microscopy of dorsal skin sections (8 w.o. B6 mice) stained for Izumo1 and γδ TCR. (*E*) Model of the inflammatory circuit in mouse skin influenced by Izumo1R on skin Treg cells.

skin Tregs in general (which would be paradoxical, given its relative underexpression there), but rather that the inflammatory/regulatory loop revealed by IMQ challenge, but not by other local challenges, depends on Izumo1R. There is an interesting clinical correlate here: Psoriasis and atopic dermatitis have generally different predisposing genetics and cellular agents, and generally affect different skin regions. Previous studies have established a role for Rorγt+ γδ T cells, in particular Vγ4 subsets, in orchestrating skin inflammation during

IMQ-induced psoriasis-like disease, with relevance to psoriasis patients (46–48). Inflammation elicited by IMQ via TLR signals has been shown to depend on a circuit between inflammatory cytokines of the IL1 family produced by keratinocytes, which in turn induce IL22 and IL17 in γδT cells, with induction of IL23 on myeloid cells that amplifies the circuit, finally recruiting neutrophils that further boost the inflammation (46) (Fig. 6*E*). The actors revealed by immunophenotyping and gene expression profiling were precisely those: RORγ+, IL17-producing γδT cells, neutrophils, IL17, and IL1 family members. Despite the enrichment of the Th17 gene signature in CD4 T cells from Iz1RTrKO mice, we did not observe a clear increase in Th17 cells in Iz1RTrKO mice at steady state, contrasting with their γδT homologs. While we cannot rule out a contribution to the pathology from Th17 abT cells, γδT cells do appear as the main drivers of Iz1RTrKO pathology (Figs. 4*D* and 6*B*), in keeping with their demonstrated role (46). The subclinical inflammation evidenced by whole-skin RNAseq would jump-start the response to IMQ, but not to MC903 or Oxazolone sensitization which operate via different mechanisms. Among the IL1 family members, *Il1f8* (encoding IL36b) was the earliest induced in the time-course analysis (Fig. 6*C*). IL36 is made by stressed keratinocytes, and one might hypothesize that it is involved in the initial stage of the subclinical inflammation, before IL1 produced by neutrophils establishes the full inflammatory state. The inflammatory loop that Izumo1R helps control may thus specifically consist of the keratinocyte/γδT pair, with the IL1 family and IL17 as key cytokine intermediates (Fig. 6*E*). TLR activation would also condition the poised subclinical state, consistent with the effect of antibiotic treatment on the hypersensitivity to IMQ. Within this framework, a finding that a subset of skin γδT cells expressed Izumo1, the cognate ligand for Izumo1R, was the clue that γδT cells may be the point of action through which Treg-borne Izumo1R dampens this loop.

Specific regulation of γδT cells by Treg cells at mucosal surfaces has been reported previously, in multicell models of allergy or inflammation in which γδT cells have a determining role (49–52). Conversely, Treg cells dependent on γδT cells have been reported (53), suggesting a complex interplay. More specifically, $ST2^{+}Gata3^{+}$ Tregs cells have been reported to control γδT cells during allergic challenge of the airways (house dust mite system), with IL35 being a key mediator in this pathway. Hemmers et al. also uncovered an ST2-dependent role for Tregs in controlling IL-17A-producing γδT cells during experimental autoimmune encephalomyelitis (52). These regulatory circuits would appear to be different from that involving Izumo1R, as neither *Il1rl1* (ST2), *Gata3,* nor *Ebi3* expression overlapped with *Izumo1r* in skin Tregs.

Does Izumo1R-dependent regulation of γδT cells solely take place in the skin? Given Izumo1R's widespread expression, and the ubiquitous nature of γδT cells, this would seem an unlikely proposition. The reason we observed significant manifestations of the deficiency only in the skin may be because we missed testing similar circuits in other organismal locations, or because redundant regulatory pathways can complement the lack of Izumo1R elsewhere, but not in the skin. It is also possible for perturbations that start in the skin can spread elsewhere, such as the splenomegaly observed following spontaneous skin ulceration and imiquimod-induced skin lesions (Figs. 3*B* and 4*B*). Similarly, we cannot rule out that the regulation of other physiological circuits involving IL17 and related cytokines might also involve Izumo1R, although we did not see such dysregulation in other tissues in spite of an extensive search.

What type of interaction is mediated by the Izumo1R/Izumo1 pairing? The only precedent wherein this interaction has been studied in some detail is that which occurs between oocytes (expressing Izumo1R) and spermatozoa (expressing Izumo1). During fertilization, this ligand/receptor pair is thought to serve mainly as an adhesion anchor, with increased specificity and affinity resulting from a conformational change that brings the hinge region of Izumo1 into a "locked" position (20). Spermatozoa/oocyte binding during fertilization clearly constitutes a vastly different context from the interactions between immunocytes, and it is interesting to consider how this primordial mode of cell interaction might have been repurposed by the immune system. Treg cells are typically highly mobile cells in lymphoid organs or tissues (54–57). On the contrary, skin Tregs clustered at the base of the hair follicles are relatively immobile under normal conditions (58–60). Layilin has recently been proposed as a molecular anchor that limits Treg mobility in tissues (60), but we speculate that the Izumo1/Izumo1R pair also partakes in this phenomenon. In its absence, the lack of strong tethering of Tregs to γδT cells would free the latter to promote low-grade inflammation centered around the IL1 and IL17 axes, in turn enabling an explosive inflammation upon related challenge.

In conclusion, this study provides leads into the function of Izumo1R on Treg cells. Tregs have pleiotropic functions, some of which are affected by broadly acting mediators such as IL10 or Areg. Instead, Izumo1R may be one of those focused effector molecules that underlie cell-specific control, like neuritin in the control of germinal center B cells (61). It supports a focused interaction involving Tregs and γδT cells, by borrowing a tight ligand/receptor pair from a radically different physiological system.

Materials and Methods

Mice. The following mice were obtained from the Jackson Laboratory and bred in our facility: $Foxp3^{tm3(DTR/GFP)Ayr/J}$ (62) and $Tg(Cd4\text{-}cre)^{1Cwi/BfluJ}$ (63). $Izumo1R^{Flox}$ (B6.129P2-$Izumo1r^{tm1Salb/Mmmh}$) mice were obtained from the University of Missouri Metagenomics Center. $Foxp3^{Cre\text{-}YFP}$ mice were obtained from Dr. A. Rudensky (64). All mice were backcrossed and maintained on the B6 background in our SPF facility at Harvard Medical School. Unless otherwise indicated, experiments were performed on mice at 6 to 8 wk of age, and littermate controls were used in all experiments. For imiquimod challenge, 125 μg 5% Imiquimod cream (Taro) was administered topically to the dorsal and ventral aspects of ear skin of adult mice for up to 7 d. Mice were monitored at least once per day and ear thickness was measured using a caliper, and local inflammation was scored based on tissue thickness, acanthosis, skin scaling, and formation of abscess-like lesions. For MC903 challenge, 4 nmol calcipotriol dissolved in 70% ethanol (10 mg stock, MedChemExpress) was administered topically to the dorsal and ventral aspects of ear skin of adult mice daily for up to 12 d. All experiments were performed under approved protocol IS00001257.

RNA-Seq Library Preparation and Sequencing. For population-level RNA-seq, cells were double-sorted by flow cytometry into TCL buffer (Qiagen) containing 1% 2-mercaptoethanol (Sigma) and libraries were constructed according to the Immgen protocol for low-input RNA-seq analysis as described previously (44), as detailed in *SI Appendix, Materials and Methods*.

Immunofluorescence Histology. Dorsal skin samples were fixed for 15 min in 4% paraformaldehyde (PFA) at room temperature, washed with PBS, immersed in 30% sucrose overnight at 4 °C, and embedded in OCT (Sakura Finetek). 50-μm sections were blocked for 1 to 2 h (5% Donkey serum, 1% BSA, 2% Cold water fish gelatin, and 0.3% Triton in PBS), prior to sequential staining with primary and secondary antibodies. All images were acquired using a Zeiss 980 confocal microscope at ×20 or ×63 magnification. The pictures are presented as maximum intensity projections.

Data, Materials, and Software Availability. Sequencing data can be found in the GEO database under accession number GSE193572 (65). All data needed to evaluate the conclusions in the paper are present in the paper and/or *SI Appendix*.

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