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This information is current as of January 15, 2015.

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J Immunol 2013; 191:3337-3346; Prepublished online 14 August 2013;
doi: 10.4049/jimmunol.1300828
<http://www.jimmunol.org/content/191/6/3337>

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Toll IL-1R8/Single Ig IL-1–Related Receptor Regulates Psoriasiform Inflammation through Direct Inhibition of Innate IL-17A Expression by $\gamma\delta$ T Cells

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Expression of the orphan receptor Toll IL-1R8/single Ig IL-1–related receptor has been reported to be reduced in the peripheral blood of psoriatic arthritis patients. However whether TIR8/SIGIRR activity plays a specific role in regulating psoriatic inflammation is unknown. We report that *Tir8/Sigirr*-deficient mice develop more severe psoriatic inflammation in both the chemical (Aldara)- and cytokine (rIL-23)-induced models of psoriasis. Increased disease severity was associated with enhanced infiltration of V γ 4⁺ $\gamma\delta$ T cells that express significantly elevated levels of IL-17A. Critically, we also demonstrate that TIR8/SIGIRR activity directly suppressed innate IL-17A expression by $\gamma\delta$ T cells in vitro and in vivo. Importantly, treatment of *Tir8/Sigirr*^{-/-} mice with an IL-17A neutralization Ab reversed the enhanced disease severity observed in these mice. This study identifies TIR8/SIGIRR as a novel intrinsic negative regulator of innate IL-17A expression and characterizes a novel mechanism involved in the regulation of psoriatic inflammation. *The Journal of Immunology*, 2013, 191: 3337–3346.

Psoriasis is a common chronic immune-driven disease of the skin with incidences of between 1 and 3% in the population of western countries. Although the disease rarely proves fatal, patients suffer significant physical discomfort from the appearance of inflamed psoriatic skin lesions, which can also be associated with an added psychological burden. The pathogenesis of psoriasis involves a complex interplay of immunological, genetic, and environmental factors, all of which can contribute in varying degrees to the specific disease phenotype in humans (1, 2). The condition is commonly characterized by rapid and dysregulated keratinocyte proliferation leading to a thickened epidermis (acantosis). This aberrant keratinocyte behavior often leads to significant retention of nucleated cells within the granular layer (parakeratosis) and a very apparent erythema (redness) because of increasingly dilated blood vessels in the dermis. These manifestations occur in conjunction with a marked immune cell infiltrate composed primarily of dense concentrations of T cells along with

dendritic cells, neutrophils, and macrophages (3). Classically, psoriasis has come to be viewed as a T cell–driven autoimmune disorder due at least in part to its responsiveness to cyclosporine treatment (4) and also as Th1 cells, expressing IFN- γ , were seen as the key drivers of disease pathogenesis (5, 6). However, more recently a deeper characterization of both psoriatic lesional infiltrates and the peripheral blood of patients have uncovered a prominent role for inflammatory cells driven by the IL-23/IL-17 signaling axis as central mediators of disease (7, 8). Such cells include subsets of innate $\gamma\delta$ T cells and lymphoid cells that express abundant amounts of proinflammatory IL-17A, IL-17F, and IL-22 at key skin barrier sites (9, 10). The importance of these cells, along with CD4⁺ Th17 cells that express a similar cytokine repertoire, in psoriasis pathogenesis has underscored the development of a range of mAbs targeting the IL-23/IL-17 signaling axis and, more specifically, IL-17R A (Brodalumab) and one of its ligands, IL-17A (Ixekizumab) (11, 12). These targeted therapies have met with considerable early success in the clinic and will most likely form the next range of front line therapies for psoriasis treatment. However, despite these exciting advances in drug development, significant gaps remain in our understanding of the regulatory mechanisms that underpin the IL-23/IL-17 signaling axis and the cellular and molecular interactions at play in the context of dermal inflammation in psoriasis.

IL-23 is a myeloid-derived cytokine that acts as a key growth factor inducing the expression of a number of type 17 cytokines including IL-17A, IL-17F, and IL-22 from both Th17 cells and innate $\gamma\delta$ cells, which a number of recent studies have implicated as central drivers of psoriatic inflammation (7, 10, 13, 14). IL-23 shares its p40 subunit with the Th1-instructive cytokine IL-12, and an anti-p40 mAb (Ustekinumab) has met with some successes for the treatment of psoriasis in the clinic (15). Although the role IL-23 plays in the development of type 17 responses and associated autoimmunity is well established, a number of other cytokines are also known to contribute to, and indeed compliment, the actions of IL-23 in similar settings. One such example is IL-1, a cytokine that exists in two distinct forms, IL-1 β and IL-1 α . These signaling mediators both share the same receptor, IL-1R1 type 1 (IL-1R1), and elicit remarkably similar effects despite significant differences

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Received for publication March 27, 2013. Accepted for publication July 15, 2013.

This work is supported by Science Foundation Ireland (President of Ireland Young Researcher Award 10/YI/B1827) and the National Children's Research Centre (Paediatric Research in Translational Immunology programme) (to P.T.W.). A.M. is supported by European Commission Project Targeting novel Mechanisms in Resolution of inflammation, Ministero Salute, and Ministero Università e Ricerca.

S.E.R. and P.T.W. conceived and designed the research; S.E.R., A.M.S., M.K., and R.M.H. performed the experiments; S.E.R., A.M.S., M.K., P.G.F., and P.T.W. analyzed the data; C.G., A.M., and P.G.F. contributed reagents/materials/analysis tools; and S.E.R. and P.T.W. wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: IL-1R1, IL-1R1 type 1; mTOR, mammalian target of rapamycin; SIGIRR, single Ig IL-1–related receptor; TIR8, Toll IL-1R8; WT, wild-type.

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in their expression and activity (16). It has long been surmised that IL-1R1 signaling is dysregulated in psoriasis; however, its specific role has proved controversial (17, 18). Recently reports have demonstrated that IL-1R1 signaling is indispensable for acanthosis, neutrophil recruitment, and Munro's abscess formation in the Aldara-induced model of psoriasis (19). These findings coupled with its established role in complimenting the proinflammatory actions of IL-23 highlight the need to further examine the role IL-1R1 signaling plays in psoriasis pathogenesis and in particular investigate the action of signaling mediators that are known to regulate its effects.

One such mediator is Toll IL-1R8 also known as single Ig IL-1-related receptor (TIR8/SIGIRR), which serves as a negative regulator of inflammation driven by the Toll/IL-1R superfamily members (20, 21). TIR8/SIGIRR is currently designated as an orphan receptor for reasons attributed to its unique structure within the Toll/IL-1R superfamily. It possesses a single extracellular Ig domain, distinct from the characteristic three domains of other family members such as the IL-1R1 and IL-18R, and current consensus suggests the small size of this single extracellular portion precludes binding of a cognate extracellular ligand. Furthermore, TIR8/SIGIRR possesses two amino acid substitutions (Ser⁴⁴⁷ and Tyr⁵³⁶ replaced by Cys²²² and Leu³⁰⁵) in its common intracellular TIR signaling domain. Although the functional consequences, if any, of these substitutions are unknown, the observation is considered suggestive of impaired signal transduction ability (21-23). Recently, TIR8/SIGIRR has been identified as a modulator of autoimmune disease through its ability to inhibit the IL-1R1-driven proliferation of Th17 cells (24). Little is known of the impact TIR8/SIGIRR may elicit in human disease; however, it is noteworthy that its expression is significantly downregulated in the peripheral blood of psoriatic arthritis patients (25). This observation coupled with the results of murine studies implicating an important role in the regulation of IL-23/IL-17 signaling strongly indicates a potential role in the regulation of psoriatic inflammation.

In this study, we define a role for TIR8/SIGIRR as a modulator of psoriatic inflammation through direct suppression of innate IL-17A expression by $\gamma\delta$ T cells. *Tir8/Sigirr*^{-/-} mice display an exacerbated disease phenotype in two murine models of psoriasiform inflammation. These events are associated with enhanced infiltration of V γ 4⁺ $\gamma\delta$ T cells with significantly increased expression of IL-17A. Critically, we also demonstrate that TIR8/SIGIRR activity suppressed innate IL-17A expression by $\gamma\delta$ T cells in vitro and in vivo. Importantly, the increased disease severity observed in the absence of TIR8/SIGIRR is reversible through treatment with an anti-IL-17A neutralization Ab. These data identify a previously unappreciated role for TIR8/SIGIRR in the regulation of innate IL-17A expression by $\gamma\delta$ T cells and as a novel regulator of dermal inflammation in the context of psoriasis.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 mice were purchased from Harlan UK. *Tir8/Sigirr*^{-/-} mice on a C57BL/6 background were provided by A. Mantovani (University of Milan). All mice used were between 6 and 8 wk and were housed under specific pathogen-free conditions at Institute for Molecular Medicine, St. James Hospital (Dublin, Ireland). All animal experiments were performed in compliance with Irish Department of Health regulations (license number B100/4272) and approved by the institutional ethical review board.

Materials

ELISA kits for mouse IL-17A, IL-17F, IL-17A/F, and IL-22 were purchased from eBioscience (Hatfield, U.K.) and were carried out according to manufacturer's instructions. Recombinant mouse IL-1 β and IL-23 were purchased from eBioscience. The following Abs were used for surface

staining CD45 (30-F11), CD3 (145-2C11), $\gamma\delta$ TCR (Ebio GL3), V γ 4 (UC3-10A6) (BioLegend), and CD4 (RM4-5). Intracellular staining was performed for IL-17A (17B7), IL-22 (IL22JOP), IL-17F (eBio18F10), IFN- γ (XMG 1.2), and BrdU (3D4). All Abs were purchased from eBioscience unless otherwise stated. The JNK inhibitor SP600125 and mammalian target of rapamycin (mTOR) inhibitor Temsirolimus were purchased from Sigma-Aldrich.

Aldara-induced model of psoriasiform inflammation and anti-IL-17A treatment

Mice were shaved on their back before hair removal using a depilatory cream. Two days later, imiquimod (5% cream; Aldara MEDA Pharmaceuticals, Dublin, Ireland) or control cream was applied to the back (3.125 mg/day) or ears (1 mg/day) of mice for 1-7 d, depending on experimental requirements. Ear thickness was measured using a thickness gauge (Hitec) before the initiation of the experiment on day 0 and every day thereafter until the termination of the experiment. Cells from skin draining lymph nodes were restimulated with IL-23 (50 ng/ml) for 12 h before further analysis by flow cytometry. Ears from WT and *Tir8/Sigirr*^{-/-} mice were also taken and homogenized in PBS containing a protease inhibitor mixture (Sigma-Aldrich). Protein concentrations were equalized, and homogenate was analyzed for expression of IL-1 β and IL-23 by ELISA. For IL-17A neutralization experiments, mice were treated on days 0 and 2 with 250 μ g/mouse anti-IL-17A Ab (17F3) or mouse IgG1 isotype control (MOPC-21) (BioXCell, West Lebanon, NH), and disease was monitored as described previously (9).

IL-23 intradermal injection model

WT and *Tir8/Sigirr*^{-/-} mice were anesthetized and injected intradermally with either 200 ng IL-23 or PBS in a 20- μ l volume every day using a 30-gauge needle. Ear thickness was measured using a thickness gauge (Hitec) before the initiation of the experiment on day 0 and every day thereafter for a period of 7 d. Cells from psoriatic skin were restimulated with PMA (10 ng/ml) and ionomycin (1 μ g/ml) for 2 h in the presence of brefeldin A (5 μ g/ml) before further analysis by flow cytometry.

Cell preparation

Ears were cut into small pieces and digested for 1 h at 37°C in complete RPMI 1640 medium supplemented with 1 mg/ml collagenase D and 100 mg/ml DNase I before passing through a 40 μ M filter to form a single-cell suspension. Lymph nodes or spleens were processed into single-cell suspensions by dissociation of tissue and passing through a 40 μ M filter.

Flow cytometry

Cells were analyzed for surface and intracellular protein expression using an LSR/Fortessa (BD Biosciences) before further analysis using FlowJo software (Tree Star). For all experiments, initial gating was performed on live lymphocytes from forward versus side scatter plots. Subsequent gating for $\gamma\delta$ T cells and subsets were performed for CD45, $\gamma\delta$ TCR, and V γ 4 positivity as appropriate. Other unconventional cell subsets were gated on CD45-positive and CD3-negative staining. CD4 T cells were gated on CD4-positive cells from the live lymphocyte gate. For intracellular staining, fixation and permeabilization of cells were performed using a Fix/Perm Kit (DakoCytomation), according to the manufacturer's instructions. Appropriate isotype control Abs were used for setting positive gates in intracellular staining experiments.

In vivo footpad model

Mice were injected with 50 ng IL-1 β , IL-23, or a combination of both or with the indicated TLR ligands into each hind footpad in a total volume of 20 μ l as described previously (26). PBS injection was used as a vehicle control. After 4 h, the popliteal lymph nodes were harvested and processed to single-cell suspension before culture in the presence of brefeldin A (5 μ g/ml) for an additional 4 h. Cells were subsequently analyzed for surface and intracellular protein by FACS.

BrdU flow kit (BD Pharmingen) was used according to the manufacturer's instructions for in vivo BrdU labeling of cells. Briefly, mice were injected i.p. with BrdU (1 mg/mouse) 48 and 24 h prior to initiation of the footpad model as described above. Upon isolation, cells were stained for surface protein before fixation and permeabilization with BD Cytotfix/Cytoperm Buffer. Cells were subsequently treated with DNaseI to expose intracellular BrdU and stained with anti-BrdU.

RT-PCR and quantification

Extraction of RNA from whole tissue was performed using an Isolate RNA mini kit (Bioline), and RNA levels were equalized before performing RT-

PCR using a High Capacity cDNA kit (Applied Biosystems), all according to the manufacturer's instructions. Real-time PCR was performed using SensiFAST Probe Hi-ROX kit (Bioline) incorporating predesigned gene expression assays for IL-17A, IL-22, IL-1 β , IL-23, and tir8 (Applied Biosystems). Expression was normalized to 18s rRNA. Samples were assayed on an Applied Biosystems 7900HT Fast Real-Time PCR System.

Histopathological analysis of tissue

Tissue was fixed overnight in 10% formalin before dehydration and embedding into paraffin blocks. Eight-micrometer sections were subsequently cut and stained with H&E. Sections were scored blindly for pathological manifestations of psoriatic inflammation on a scale of 0–4 (0, no observable difference over control; 1, mild; 2, moderate; 3, marked; and 4, severe) for the following parameters: acantosis, desquamation, parakeratosis, and infiltration. Scores for each parameter were combined for an overall histological severity score.

In vitro cell stimulation

WT and *Tir8/Sigirr*^{-/-} splenocytes were cultured in the presence of IL-23 (10 ng/ml) and IL-1 β (0.01 ng–10 ng/ml) in the absence of plate bound Ab stimulation for 72 h before analysis of the cell culture supernatants by ELISA. For inhibition of IL-1R1 signaling pathways, cells were

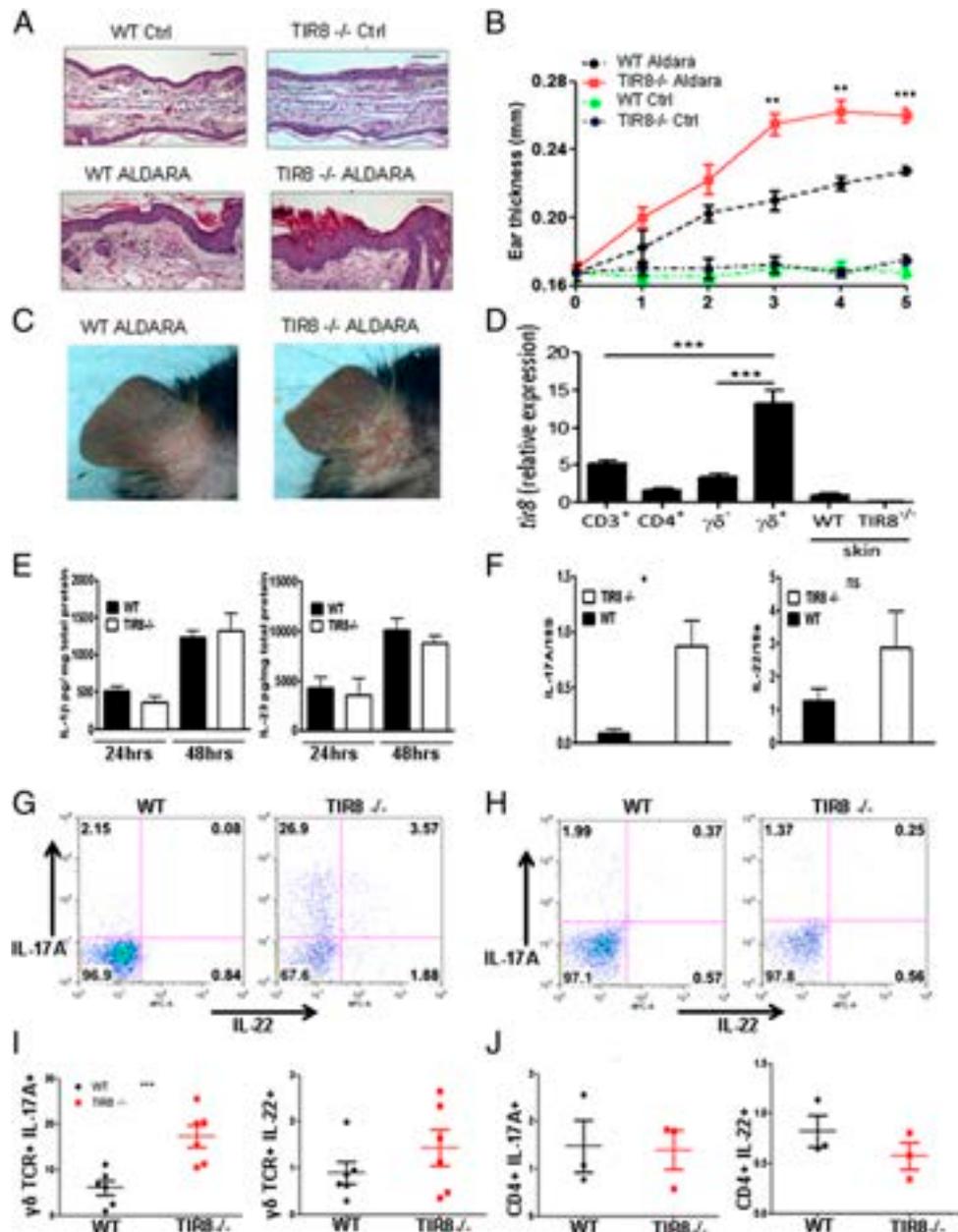
preincubated for 2 h at 37°C at the indicated concentrations prior to washing and stimulation. WT and *Tir8/Sigirr*^{-/-} $\gamma\delta$ T cells were purified (>98%) by FACS sorting using a MoFlo sorter (Beckman Coulter) and cultured in the same manner as above or in combination with rIL-6 (10 ng/ml) and TGF- β (5 ng/ml) or imiquimod (5 μ g/ml) before performing intracellular cytokine staining and analysis of cell culture supernatants by ELISA. Identical experiments were also performed in the presence (1:1 ratio) of APCs (T-depleted splenocytes), which had been prestimulated overnight with imiquimod (5 μ g/ml)

Results

Enhanced severity of Aldara-induced psoriasiform inflammation in *Tir8/Sigirr*^{-/-} mice

Innate $\gamma\delta$ T cells that express IL-17A in response to IL-1R1 stimulation are thought to play a key pathological role in a variety of autoimmune disorders with recent emerging evidence, suggesting a critical role in the progression of psoriasis (10, 27). Interestingly, it has been observed that TIR8/SIGIRR expression is significantly downregulated in the peripheral blood of psoriatic patients; however, despite these observations, the role of TIR8/SIGIRR in the

FIGURE 1. Enhanced severity of Aldara-induced psoriasiform inflammation in *Tir8/Sigirr*^{-/-} mice. **(A)** H&E-stained sections from ears of WT and *Tir8/Sigirr*^{-/-} mice treated with Aldara (1 mg/day) or control cream for 5 d (scale bar, 50 μ m). **(B)** Increased epidermal thickening in *Tir8/Sigirr*^{-/-} mice treated with Aldara. **(C)** Representative macroscopic photographs of ears from WT and *Tir8/Sigirr*^{-/-} mice treated with Aldara. **(D)** Real-time PCR analysis for basal expression of *Tir8* in the indicated cells and tissues. WT unless stated otherwise. **(E)** ELISAs for IL-1 β and IL-23 were performed on tissue lysates of ears from WT and *Tir8/Sigirr*^{-/-} mice treated with Aldara for the indicated times. **(F)** Real-time PCR analysis of IL-17A and IL-22 from psoriatic skin of WT and *TIR8*^{-/-} mice after 5 d of treatment with Aldara. Cells from skin draining lymph nodes of WT and *Tir8/Sigirr*^{-/-} mice were examined by flow cytometry for intracellular IL-17A and IL-22 expression from $\gamma\delta$ T cells (**G, I**) and CD4 T cells (**H, J**). These data are representative of at least three independent experiments with similar results. Statistical significance determined by unpaired Student *t* test; ***p* \leq 0.01, ****p* \leq 0.001.

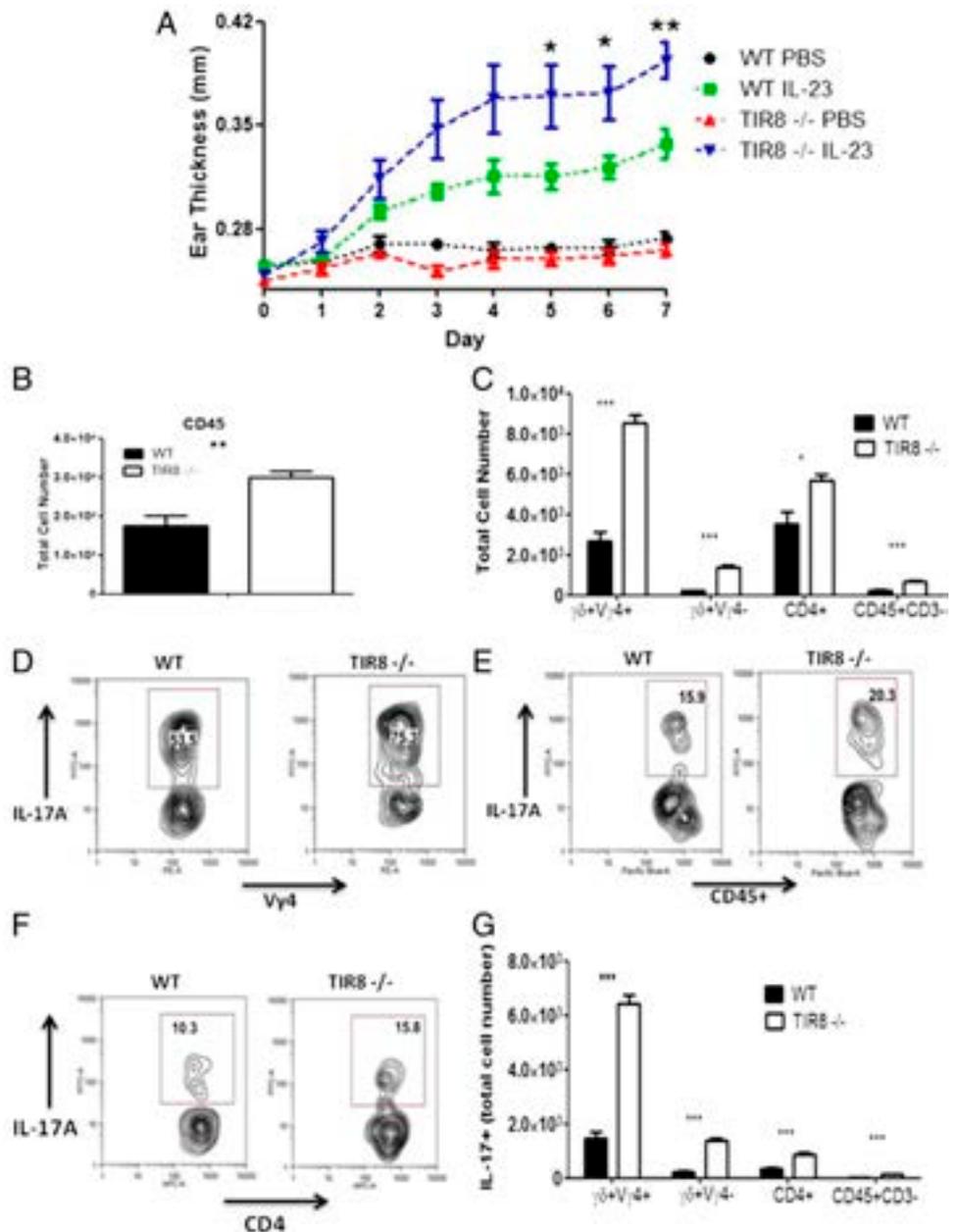


regulation $\gamma\delta$ T cell–driven pathology and more specifically its role in the regulation of dermal inflammation in psoriasis have yet to be addressed (25). To answer these questions, we used a murine model of psoriasiform inflammation where $\gamma\delta$ T cells are known to play a key role in disease progression. This model, which is IL-23 dependent, involves daily topical application of the TLR7 agonist imiquimod formulated in a commercially available cream, Aldara (28). Aldara has been used for some time in the treatment of a variety of dermal malignancies such as actinic keratoses and superficial basal cell carcinomas (29, 30); however, a side effect of such treatment is the exacerbation of psoriatic lesions in some patients, even those with well controlled psoriasis (31, 32). On the basis of this observation, use of Aldara in murine studies as an inducer of psoriasiform inflammation has come to be accepted as a clinically faithful model of psoriasis, particularly as the pathological manifestations induced bear striking similarities to human psoriasis but also because it is responsive to a range of the current frontline psoriasis therapies (9, 10, 28).

Aldara cream applied to the ears of WT control and *Tir8/Sigirr*^{-/-} mice over a period of 5–7 d resulted in an exacerbated inflamma-

tory phenotype in *Tir8/Sigirr*^{-/-} mice. Analysis of H&E-stained sections of treated ears revealed enhanced acantosis, desquamation (scaling and outer layer shedding), and dermal infiltration of nucleated cells in *Tir8/Sigirr*^{-/-} mice (Fig. 1A). Also, measurement of the overall thickness of treated ears revealed significantly enhanced epidermal thickening in *Tir8/Sigirr*^{-/-} mice (Fig. 1B). This enhanced disease phenotype is also clearly visible macroscopically with enhanced desquamation and erythema on the ears of *Tir8/Sigirr*^{-/-} mice (Fig. 1C). To investigate which cell types TIR8/SIGIRR activity may play a role in the context of dermal inflammation, we first examined levels of gene expression on relevant cell subsets and tissues by real-time PCR analysis. Interestingly, basal expression of the *Tir8/Sigirr* gene was found to be significantly higher in $\gamma\delta$ T cells when compared with CD3⁺, CD4⁺, and CD3⁺ $\gamma\delta$ ⁻ cell subsets and murine skin (Fig. 1D). Application of Aldara is thought, in part, to drive psoriasiform inflammation through induction of IL-23 and IL-1 β from dendritic cells and keratinocytes (19, 28). To investigate the possibility that an enhancement of either, or both, of these cytokines in the *Tir8/Sigirr*^{-/-} mouse was

FIGURE 2. IL-23 injection results in significantly enhanced inflammation and IL-17A expression in tissues of *Tir8/Sigirr*^{-/-} mice. (A) Intradermal injection of IL-23 (200 ng) or vehicle control into the ears of WT and *Tir8/Sigirr*^{-/-} mice resulted in enhanced epidermal thickening in *Tir8/Sigirr*^{-/-} mice. Increased numbers of total (B) and specific (C) immune cell subsets infiltrating the skin of *Tir8/Sigirr*^{-/-} mice after rIL-23 treatment for 7 d. Skin-infiltrating immune cell subsets were analyzed by flow cytometry for intracellular IL-17A expression by V γ 4 cells (D), CD4⁺ T cells (E), and CD3⁻CD45⁺ cells (F). (G) IL-17A⁺ cell numbers of skin infiltrating cell subsets from IL-23–treated WT and *Tir8/Sigirr*^{-/-} mice. Statistical significance determined by unpaired Student *t* test; ***p* ≤ 0.01, ****p* ≤ 0.001.



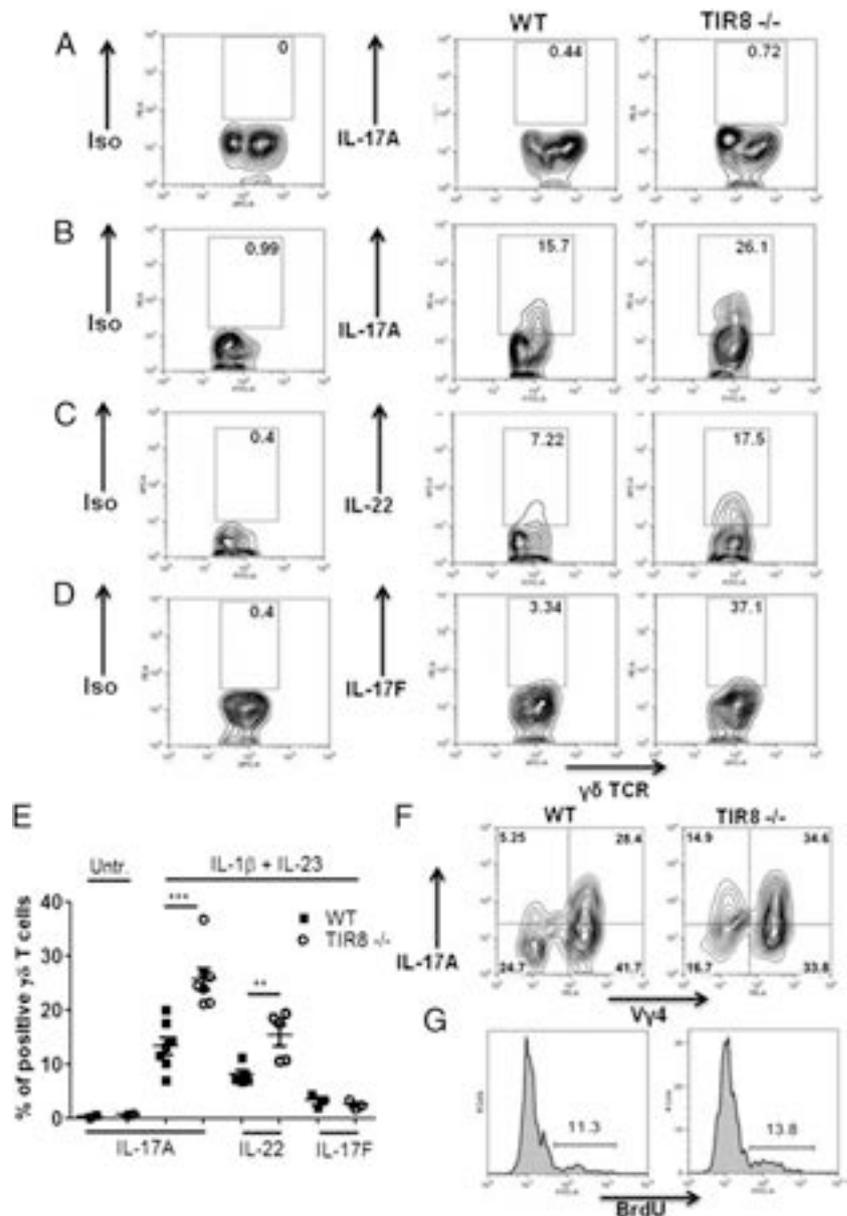
accounting for the disease phenotype observed, skin biopsies from treated mice were homogenized and analyzed for gene and protein expression levels of both IL-1 β and IL-23. Although no significant differences were observed in the levels of expression of IL-1 β or IL-23, we did observe significantly increased levels of IL-17A gene expression in the skin of *Tir8/Sigirr*^{-/-} mice when compared with controls (Fig. 1E, 1F). Furthermore, analysis of $\gamma\delta$ T cells in the skin-draining lymph nodes of Aldara-treated mice revealed significantly enhanced levels of IL-17A, but not IL-22, expression by *Tir8/Sigirr*^{-/-} mice (Fig. 1G, 1I). $\gamma\delta$ T cells, rather than Th17 cells, appear to be the primary source of IL-17A in this setting because limited expression of IL-17A by CD4⁺ T cells was observed (Fig. 1H, 1J). These data demonstrate that in the absence of TIR8/SIGIRR there is increased Aldara-induced psoriasiform dermal inflammation that occurs in association with increased IL-17A expression by $\gamma\delta$ T cells.

Intradermal IL-23 injection results in significantly enhanced dermal inflammation in Tir8/Sigirr^{-/-} mice

Intradermal injection of IL-23 is another widely used model of psoriasiform inflammation, which is robustly comparable to the

Aldara model in terms of the key cellular mechanisms involved such as the induction of an IL-1R1-dependent type 17 response through direct stimulation of T cell subsets (10, 33). Because TIR8/SIGIRR has been characterized as a negative regulator of TLR7 signaling (34, 35), it is possible that the enhanced dermal inflammatory phenotype observed in *Tir8/Sigirr*^{-/-} mice in the Aldara model described above is dependent on this activity. To address this possibility, WT and *Tir8/Sigirr*^{-/-} mice were injected intradermally with rIL-23 or vehicle control daily for up to 7 d. Consistent with previous reports, IL-23 injection induced epidermal thickening in WT mice (Fig. 2A) (33, 36). Similar to the Aldara model, *Tir8/Sigirr*^{-/-} mice display significantly enhanced epidermal thickening compared with WT controls in this model (Fig. 2A). To determine the composition of dermal infiltrating cells and assess any differences in populations between WT and *Tir8/Sigirr*^{-/-} mice, CD45⁺ cells purified from skin tissue were analyzed by flow cytometry. The total numbers of skin-infiltrating cells were significantly increased in the absence of TIR8/SIGIRR activity (Fig. 2B) with the most significant increase observed in the V γ 4 subset of $\gamma\delta$ T cells, which have previously been implicated as important mediators of dermal inflammation in this model

FIGURE 3. Innate IL-1 β and IL-23 drive enhanced IL-17A and IL-22 expression from *Tir8/Sigirr*^{-/-} $\gamma\delta$ T cell subsets in vivo. WT and *Tir8/Sigirr*^{-/-} mice injected in the hind footpad with a combination of IL-1 β (100 ng) and IL-23 (100 ng). After 4 h, popliteal lymph nodes were harvested from treated mice and untreated controls. $\gamma\delta$ T cells were subsequently analyzed by flow cytometry for intracellular expression of the following cytokines. IL-17A from untreated mice (A), IL-17A from treated mice (B), IL-22 from treated mice (C), and IL-17F from treated mice (D). (E) Expression of cytokines by $\gamma\delta$ T cells from untreated and treated mice. Each dot indicates one mouse. (F) Expression of innate IL-17A by the V γ 4 subset of $\gamma\delta$ T cells. (G) Analysis of BrdU incorporation by the V γ 4 subset after stimulation with IL-1 β and IL-23. These data are representative of at least three independent experiments with similar results. Statistical significance determined by unpaired Student *t* test; ***p* \leq 0.01, ****p* \leq 0.001.



(Fig. 2C) (10). Importantly, as well as increased numbers, expression levels of IL-17A on a per cell basis were also significantly enhanced among *Tir8/Sigirr*^{-/-} V γ 4 cells, with these cells accounting for the vast majority of IL-17A-expressing cells in this setting (Fig. 2D). Expression of IL-17A by V γ 4⁻ γ δ T cell subsets and a small population of CD45⁺ non-T cells were also significantly enhanced in *Tir8/Sigirr*^{-/-} mice (Fig. 2E, 2G). Finally, although increased numbers of CD4⁺ T cells were also observed in inflamed skin, their overall contribution to IL-17A expression when compared with that observed from innate subsets was limited (Fig. 2F, 2G). These data demonstrate that intradermal administration of IL-23 also leads to enhanced psoriasiform inflammation in *Tir8/Sigirr*^{-/-} mice, and this occurs in association with increased skin infiltration of V γ 4 γ δ T cells expressing elevated levels of IL-17A.

IL-1 β and IL-23 induce enhanced innate IL-17A and IL-22 expression from Tir8/Sigirr^{-/-} γ δ T cell subsets in vivo

In support of the role IL-23 plays in the progression of IL-17A-driven dermal inflammation, we were interested to assess the potential role TIR8/SIGIRR plays in regulating complementary IL-1 and IL-23 signaling in the context of innate IL-17A expression by γ δ T cell subsets. To address these questions, we used an in vivo innate stimulation system whereby WT and *Tir8/Sigirr*^{-/-} mice were injected in the hind footpad with a combination of IL-1 β and IL-23 as described previously (26). After 4 h, popliteal lymph node cells were harvested from treated mice and untreated controls and cultured in the presence of brefeldin A, without further ex vivo stimulation, before analysis by flow cytometry. Although no expression of IL-17A was detected in the untreated controls (Fig. 3A, 3E), significantly elevated expression of IL-17A was observed from *Tir8/Sigirr*^{-/-} γ δ T cells from mice treated with IL-1 β and IL-23 (Fig. 3B, 3E). IL-22 was also elevated (Fig. 3C, 3E), but there were no differences in IL-17F expression, which was detected at relatively low levels (Fig. 3D, 3E). The V γ 4 subset once again was the predominant subset expressing elevated IL-17A, but it is noteworthy that another population of V γ 4⁻ γ δ T cells also expressed increased levels of IL-17A (Fig. 3F). This suggests TIR8/SIGIRR acts as a broad regulator of innate γ δ T cell IL-17A expression. Interestingly, both IL-1 β and IL-23 were absolutely required to induce IL-17A expression from γ δ T cells in vivo in this setting because mice injected singly with either IL-1 or IL-23 yielded no discernible cytokine expression. Furthermore, innate expression of IFN- γ was not detected under any stimulus (data not shown). Interestingly *Tir8/Sigirr*^{-/-} γ δ T cells also express enhanced IL-17A in response to in vivo administration of the TLR4 agonist LPS and the TLR9 agonist CpG ODN in this model, suggesting a broader role for TIR8/SIGIRR in the regulation of innate IL-17A expression by γ δ T cells (Supplemental Fig. 1). As TIR8/SIGIRR has previously been reported to suppress Th17 proliferation mediated by IL-1 β , we assessed the possibility that the enhanced IL-17A observed in this assay system was as a consequence of dysregulated expansion of *Tir8/Sigirr*^{-/-} cells (24). However, no detectable differences in γ δ T cell BrdU incorporation over normal homeostatic levels were observed (Fig. 3G). This observation indicates a direct effect of TIR8/SIGIRR activity in regulating innate IL-17A expression without altering cell expansion in vivo.

Enhanced IL-1 β -dependent expression of IL-17A following innate stimulation of Tir8/Sigirr^{-/-} splenocytes is dependent on mTOR

TIR8/SIGIRR has previously been reported to inhibit IL-1R1 signaling (20). On the basis of our findings from the in vivo footpad model, we hypothesized that enhanced innate IL-17A

expression by γ δ T cells in *Tir8/Sigirr*^{-/-} mice occurred as a consequence of enhanced IL-1R1-dependent signaling. To address this possibility, we carried out an innate stimulation of WT and *Tir8/Sigirr*^{-/-} splenocytes in vitro with increasing levels of IL-1 β in the presence of IL-23. These conditions resulted in significantly elevated expression of IL-17A, IL-22, and IL-17A/F heterodimer in an IL-1 β -dependent manner after 72 h (Fig. 4A-C). Levels of expression of IL-17F were not altered (Fig. 4D). To examine the molecular mechanisms at play in the dysregulated expression of these cytokines in the absence of TIR8/SIGIRR activity, we stimulated splenocytes in the presence of inhibitors of two established IL-1R1-dependent signaling pathways mediated by JNK and mTOR. Although no effects on IL-17A expression were observed in the presence of the JNK-specific inhibitor SP600125, inhibition of mTOR-dependent signaling with Temsirolimus resulted in a significantly decreased innate IL-17A expression by both WT and *Tir8/Sigirr*^{-/-} cells, with a complete loss of the enhanced IL-17A expression in the absence of TIR8/SIGIRR (Fig. 4E). These data indicate a role for TIR8/SIGIRR in the modulation of mTOR-mediated IL-1R signaling in γ δ T cells.

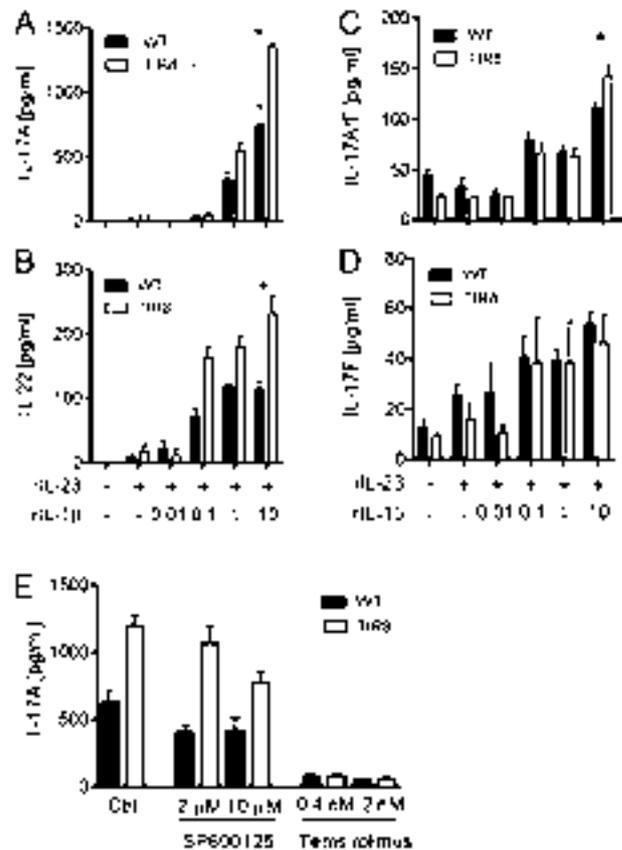


FIGURE 4. Enhanced IL-1 β -dependent expression of IL-17A following innate stimulation of *Tir8/Sigirr*^{-/-} splenocytes is mTOR dependent. WT and *Tir8/Sigirr*^{-/-} splenocytes were innately stimulated with IL-23 (10 ng/ml) and increasing concentrations of IL-1 β for 72 h. Cell culture supernatants were analyzed by ELISA for levels of IL-17A (A), IL-22 (B), IL-17A/F (C), and IL-17F (D). (E) WT and *Tir8/Sigirr*^{-/-} splenocytes were pretreated for 2 h with the indicated doses of SP600125 or Temsirolimus before washing and culturing in the presence of IL-1 β and IL-23 for an additional 72 h. Cell culture supernatants were subsequently analyzed for levels of IL-17A. These data are representative of at least two independent experiments with similar results. Statistical significance determined by unpaired Student *t* test; **p* \leq 0.05, ***p* \leq 0.01, ****p* \leq 0.001.

Purified $\gamma\delta$ T cells express enhanced IL-17A and IL-22 in response to IL-1 β and IL-23 in vitro

$\gamma\delta$ T cells have been previously demonstrated as capable of expressing IL-17A directly in response to IL-1 β and IL-23 stimulation in vitro (27). Similar to the splenocyte experiments described above, purified $\gamma\delta$ T cells from *Tir8/Sigirr*^{-/-} mice expressed enhanced IL-17A when stimulated with IL-1 β and IL-23 (Fig. 5A, 5B). We also extended this analysis to examine other cytokines known to induce type 17 responses in CD4⁺ T cells. Purified WT and *Tir8/Sigirr*^{-/-} $\gamma\delta$ T cells were also stimulated with different combinations of IL-1 β , IL-23, IL-6, and TGF- β or with the TLR7 agonist imiquimod. However, only IL-1 β and IL-23 together were found to induce innate IL-17A expression even in the presence of TLR stimulated APCs (Fig. 5D, 5E). These data confirm the dependency of innate IL-17A from $\gamma\delta$ T cells on IL-1 β and IL-23 and further confirms the ability of TIR8/SIGIRR to regulate this signaling axis.

*Enhanced severity of psoriasiform inflammation in *Tir8/Sigirr*^{-/-} mice is dependent on IL-17A*

Because we have identified a novel mechanism through which TIR8/SIGIRR regulates innate IL-17A expression, we next sought to determine whether the observed exacerbation of psoriasiform dermatitis in *Tir8/Sigirr*^{-/-} mice was mediated by increased IL-17A expression. To achieve this, we used an IL-17A neutralization

Ab in the Aldara disease model. Similar to our earlier observations, *Tir8/Sigirr*^{-/-} mice treated with isotype control Ab displayed enhanced disease characterized by enhanced acantosis, desquamation, and infiltration of nucleated cells into the dermal layers when compared with isotype control Ab-treated WT mice. Strikingly, treatment of *Tir8/Sigirr*^{-/-} mice with an anti-IL-17A neutralizing Ab reduced disease severity to levels comparable with those observed in WT mice (Fig. 6A, 6B). This reduction in disease severity was evident from histological scoring performed on H&E-stained tissue sections from diseased skin and also through analysis of epidermal skin thickening in treated mice (Fig. 6C, 6D). Taken together, these data confirm that the enhanced dermal inflammation observed in *Tir8/Sigirr*-deficient mice is mediated by dysregulated expression of IL-17A.

Discussion

Psoriasis is a common autoimmune disease of the skin which continues to pose a significant socioeconomic burden. Despite major advances in our understanding of the specific mechanisms that contribute toward disease pathogenesis significant questions remain to be answered (1). In recent times it has become evident that innate cell subsets provide a major source of IL-17A expression in settings of autoimmunity and host defense, particularly during the early stages of the response (37). A number of studies in murine models of psoriasiform inflammation have defined a role for innate cells

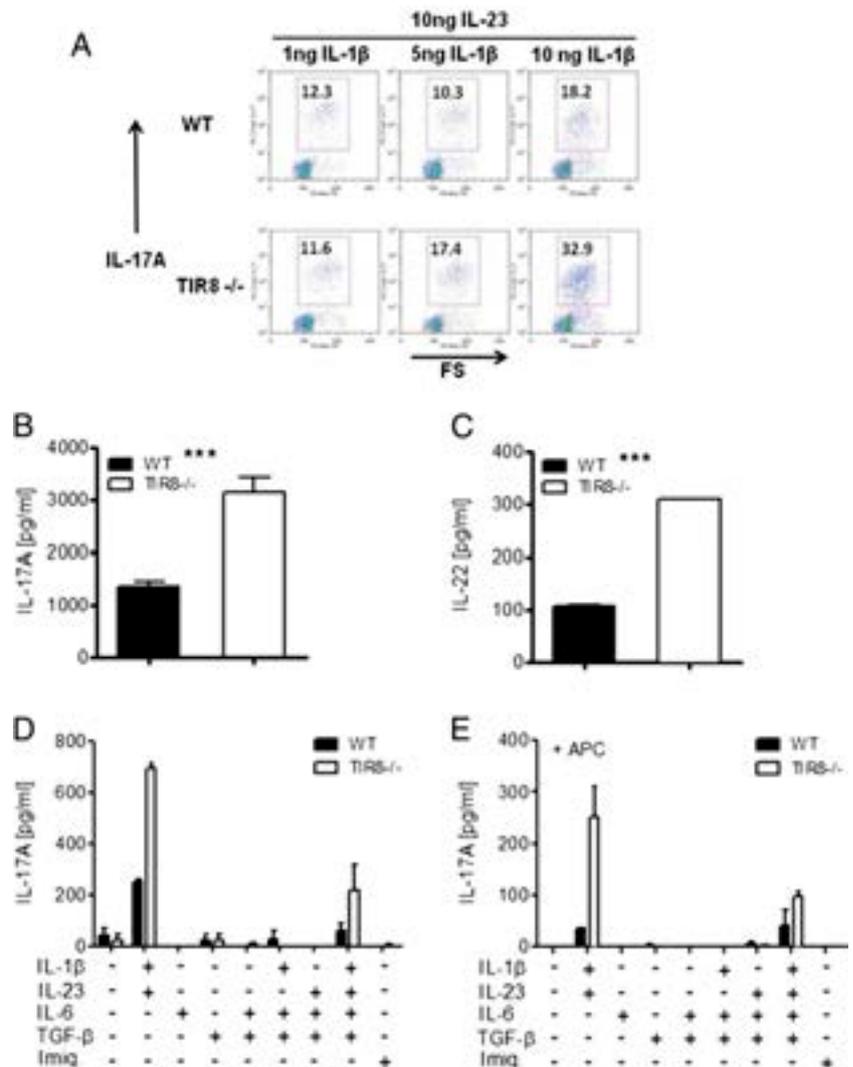
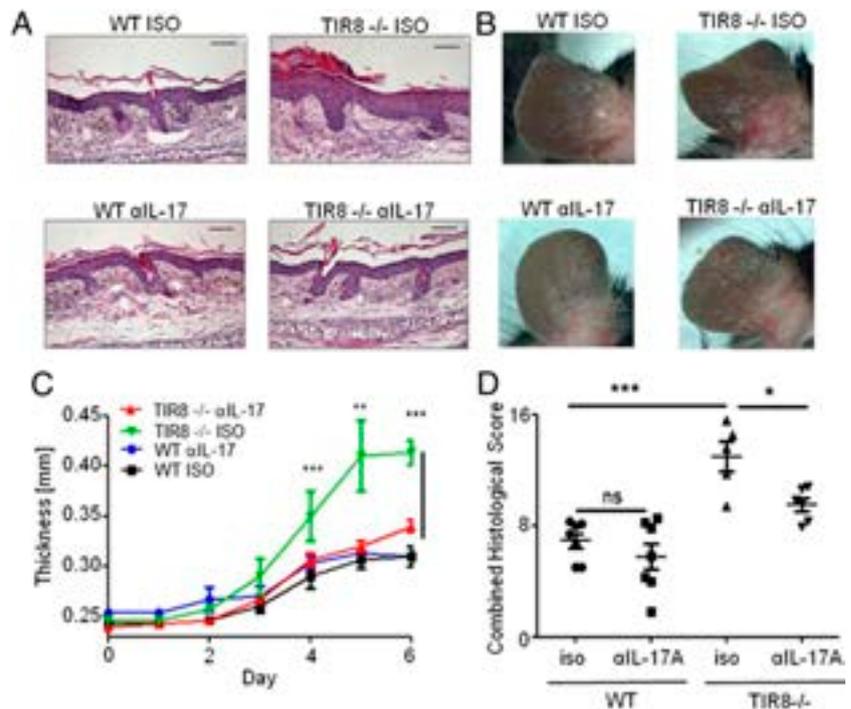


FIGURE 5. FACS purified $\gamma\delta$ T cells express enhanced IL-17A and IL-22 in response to IL-1 β and IL-23 treatment. (A) FACS purified WT and *Tir8/Sigirr*^{-/-} $\gamma\delta$ T cells were innately stimulated with IL-23 (10 ng/ml) and increasing concentrations of IL-1 β for 72 h before analysis of IL-17A expression by intracellular staining. Cell culture supernatants were also analyzed for expression of IL-17A (B) and IL-22 (C) after 72 h. Auto-MACS-purified $\gamma\delta$ T cells were cultured in the absence (D) or presence (E) of TLR-activated APCs along with the indicated combinations of cytokines for 72 h before analysis of the cell culture supernatants for IL-17A.

FIGURE 6. Enhanced Aldara-induced psoriasiform inflammation in *Tir8/Sigirr*^{-/-} mice is reversed by neutralization of IL-17A. **(A)** Representative H&E-stained skin sections of WT and *Tir8/Sigirr*^{-/-} mice treated with Aldara and either isotype control or anti-IL-17A neutralization Ab, illustrating the reversal of increased epidermal thickening and inflammatory cell infiltration with anti-IL-17A treatment (scale bar, 50 μ m). **(B)** Representative macroscopic pictures of ears from treated mice. **(C)** Increased ear thickness from *Tir8/Sigirr*^{-/-} mice treated with Aldara is reversed by treatment with anti-IL-17A. **(D)** Combined histological scoring of the H&E-stained sections capturing scoring for acantosis, parakeratosis, desquamation, and infiltration. These data are representative of at least three independent experiments with similar results. Statistical significance determined by unpaired Student *t* test; **p* \leq 0.05, ***p* \leq 0.01, ****p* \leq 0.001.



governed by the IL-23/IL-17 signaling axis as central mediators of disease (9, 10, 33, 36). However, specific pathways that regulate the responses of these innate subsets remain to be identified. This study uncovers a novel role for the receptor TIR8/SIGIRR as a regulator of innate IL-17A-driven dermal inflammation in psoriasis, which further underscores its importance as signaling mediator with therapeutic potential for interventions focusing on IL-1R1 and its interplay with the IL-23/IL-17 signaling axis.

The role of IL-1R1 signaling has received renewed focus in the context of psoriasiform inflammation as a consequence of observations implicating the IL-23/IL-17 axis as a central mediator of disease (19). Interestingly mice deficient in IL-1R1 antagonist, an endogenous suppressor of IL-1R1 signaling, develop spontaneous autoimmune inflammation in the skin (38). This phenotype is strikingly similar to common manifestations of human psoriasis, with marked acantosis, hyperkeratosis, and a nucleated cell infiltrate. Furthermore these mice also display severe aortic inflammation, a serious cardiovascular complication observed in some chronic psoriasis sufferers (39). These observations highlight the importance of examining IL-1R1-mediated inflammation in psoriasis and further highlight the potential importance of TIR8/SIGIRR as a critical signaling mediator in this context. TIR8/SIGIRR has been described as a negative regulator of inflammation in settings of fungal infection and murine autoimmunity (24, 40). However, these studies have not addressed the role of TIR8/SIGIRR in the regulation of innate IL-17A and how this may impact the progression of autoimmune pathology. We have demonstrated that *Tir8/Sigirr*^{-/-} mice develop enhanced inflammation, displaying a number of the histological characteristics of human psoriasis upon topical application of Aldara containing the TLR7 agonist imiquimod. *Tir8/Sigirr*^{-/-} mice display enhanced acantosis, desquamation, infiltration of nucleated cells, and also ear thickening compared with WT mice. This model has been described as IL-23 and IL-1 dependent, with innate $\gamma\delta$ T cells, expressing IL-17A, established as critical mediators of disease pathogenesis. Protein expression levels of IL-1 β and IL-23 in the skin while upregulated in response to Aldara treatment were unaltered between WT and *Tir8/Sigirr*^{-/-} mice. This lack of difference in protein expression levels is suggestive of a less

prominent role for dysregulated stromal cell or myeloid cell involvement in the progression of dermal inflammation in the absence of TIR8/SIGIRR. Furthermore the overall higher expression of TIR8/SIGIRR on $\gamma\delta$ T cells compared with CD4 T cells and skin underscores the importance TIR8/SIGIRR plays in the regulation of inflammation driven by these cells in such settings of dermal inflammation.

Examination of the skin draining lymph nodes revealed significantly enhanced expression of IL-17A by $\gamma\delta$ T cells from *Tir8/Sigirr*^{-/-} mice. IL-22, another type 17 cytokine important for driving inflammation in the Aldara model (41), was unaltered between WT and *Tir8/Sigirr*^{-/-} mice. Both IL-17A and IL-22 expression were virtually undetectable from CD4⁺ T cells, which is in line with previous reports implicating $\gamma\delta$ T cells as the primary source of IL-17A in this setting (9). The low expression of IL-22 in both WT and *Tir8/Sigirr*^{-/-} mice is somewhat surprising in particular as it is clear from our in vivo and in vitro stimulation experiments that TIR8/SIGIRR activity can also inhibit IL-1R1 driven IL-22 expression. However, the levels of IL-22 detected in vitro are in the order of 5- to 10-fold less than the levels of IL-17A detected, which may explain our failure to detect any such difference in the Aldara model. It is also noteworthy that non-T cells have previously been implicated as important sources of IL-22 in this model (9, 41). Imiquimod stimulation of TLR7 on myeloid cells is thought to drive IL-23 expression in the Aldara-induced model of psoriasiform inflammation (10, 28). TIR8/SIGIRR has also been described as a negative regulator of TLR7 signaling (34, 35) and as such, the observed enhancement of inflammation in *Tir8/Sigirr*^{-/-} mice, could be as a consequence of this activity. Therefore, we also used an established model of intradermal injection of IL-23 to assess the impact of TIR8/SIGIRR activity on dermal inflammation. In this model, *Tir8/Sigirr*^{-/-} mice also displayed an enhanced disease phenotype compared with their WT counterparts confirming that TIR8/SIGIRR activity more broadly regulates dermal inflammation independently of its role as an inhibitor of TLR7 signaling. The IL-23 model is also primarily dependent on the direct stimulation of proinflammatory T cell subsets strongly indicating that TIR8/SIGIRR activity plays a prominent

regulatory role on these cells in the context of psoriasiform inflammation. In line with previous reports, we observed the $\gamma\delta$ subset of $\gamma\delta$ T cells as the primary source of innate IL-17A expression. Notably, these cells are also present in the inflamed skin in significantly elevated numbers. TIR8/SIGIRR has previously been described as a negative regulator of IL-1-driven proliferation of Th17 cells, which could account for this observed expansion of infiltrating cells (24). These elevated cell numbers could also be as a consequence of a feed-forward mechanism in what are remarkably inflamed tissues, which is facilitating of further uncontrolled infiltration of proinflammatory cells. Interestingly, we have also demonstrated that TIR8/SIGIRR can directly negatively regulate innate IL-1-driven IL-17A expression in vivo without altering expansion. As well as $\gamma\delta$ T cells we have also identified CD4⁺ T cells and a population of CD45⁺CD3⁻ non-T cells as sources of elevated IL-17A in the inflamed skin of *Tir8/Sigirr*^{-/-} mice. These cells have also been identified in a number of similar studies as sources of IL-17A in this setting (9). However their relative contribution toward disease progression especially given their low numbers warrants further investigation. These data confirm dysregulated activity in dermal infiltrating hematopoietic cells in the absence of TIR8/SIGIRR activity and identify a novel role for TIR8/SIGIRR as a suppressive signaling mediator of the innate arm of the IL-23/IL-17 pathway. We have also demonstrated enhanced expression of IL-17A, IL-22 and IL-17A/F but not IL-17F from *Tir8/Sigirr*^{-/-} splenocytes in vitro and in vivo in an IL-1 dependant manner. This enhanced expression appears to be dependent on dysregulated mTOR activity, in agreement with a previous report describing the role TIR8/SIGIRR plays in the regulation of IL-1R1 mediated mTOR activity (24). Despite the unaltered expression of IL-23 and IL-1 β in the inflamed skin of WT and *Tir8/Sigirr*^{-/-} mice, the enhanced sensitivity of the *Tir8/Sigirr*^{-/-} cells to IL-1R1 stimulation coupled with the established role aberrant IL-1R1 signaling plays in the progression of dermal inflammation provides a potential mechanistic explanation for the enhanced disease observed in these mice.

IL-17A expression is established as a key cytokine driving inflammation in humans and murine models of psoriasis (7). *Tir8/Sigirr*^{-/-} mice display an enhanced psoriatic disease phenotype associated with enhanced IL-17A expression from $\gamma\delta$ T cells in both the skin draining lymph nodes and tissue-infiltrating immune cells. Treatment of *Tir8/Sigirr*^{-/-} mice with an IL-17A neutralization Ab reverses the enhanced disease to levels observed in WT mice. These observations in *Tir8/Sigirr*^{-/-} mice are broadly in line with observations in the clinic where neutralization of elevated IL-17A in human patients results in amelioration of disease symptoms (12). Interestingly in this study, WT mice treated with anti-IL-17A displayed a mild but not significant reduction in disease severity after histological examination (Fig. 5D). This lack of efficacy in WT mice is potentially explained by compensatory mechanisms associated with other proinflammatory IL-17 family members such as IL-17C, which is significantly upregulated in psoriatic plaques and utilizes IL-17A receptor for its signaling activities (42). Also pathways independent of IL-17A and IL-17 receptor A have recently been identified as playing an important role in the progression of Aldara- induced disease in WT mice (43). Because anti-IL-17A is demonstrating considerable promise in clinical trials among psoriasis patients, and decreased TIR8/SIGIRR expression/activity has previously been reported in PBMCs of psoriatic patients, these data identify an important mechanism that may have direct relevance in human disease. These observations also highlight the requirement for greater understanding of the molecular and cellular mechanisms at play in such settings.

Taken together, to our knowledge, this study has for the first time identified TIR8/SIGIRR as a central regulator of innate type 17 responses with particular significance in the context of psoriasiform inflammation where its role as a critical mediator of IL-1R1 signaling identifies it as a potential target for future therapeutic interventions.

Disclosures

The authors have no financial conflicts of interest.

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