IL-17A is a therapeutic target in many autoimmune diseases. Most nonhematopoietic cells express IL-17A receptors and respond to extracellular IL-17A by inducing proinflammatory cytokines. The IL-17A signal transduction triggers two broad, TRAF6- and TRAF5-dependent, intracellular signaling pathways to produce representative cytokines (IL-6) and chemokines (CXCL-1), respectively. Our limited understanding of the cross-talk between these two branches has generated a crucial gap of knowledge, leading to therapeutics indiscriminately blocking IL-17A and global inhibition of its target genes. In previous work, we discovered an elevated expression of 14-3-3 proteins in inflammatory aortic disease, a rare human autoimmune disorder with increased levels of IL-17A. Here we report that 14-3-ζ is essential for IL-17 signaling by differentially regulating the signal-induced IL-6 and CXCL-1. Using genetically manipulated human and mouse cells, and ex vivo and in vivo rat models, we uncovered a function of 14-3-3ζ. As a part of the molecular mechanism, we show that 14-3-3ζ interacts with several TRAF proteins; in particular, its interaction with TRAF5 and TRAF6 is increased in the presence of IL-17A. In contrast to TRAF6, we found TRAF5 to be an endogenous suppressor of IL-17A–induced IL-6 production, an effect countered by 14-3-3ζ. Furthermore, we observed that 14-3-3ζ interaction with TRAF proteins is required for the IL-17A–induced IL-6 levels. Together, our results show that 14-3-3ζ is an essential component of IL-17A signaling and IL-6 production, an effect that is suppressed by TRAF5. To the best of our knowledge, this report of the 14-3-3ζ–TRAF5 axis, which differentially regulates IL-17A–induced IL-6 and CXCL-1 production, is unique.

14-3-3ζ | IL-17A | TRAF

Interleukin-17A (IL-17A) is an inflammatory cytokine that is associated with autoimmune diseases and host-defense (1, 2). Increased IL-17A level in the sera of patients with rheumatoid arthritis, psoriasis, and lupus, is well documented (3–5). The IL-17A blocking therapy has been clinically successful to manage psoriasis, but not other diseases (6–8). Current IL-17A blockers lack specificity and compromise the host-defense mechanisms (9, 10). There is a need to design inhibitors that can target specific outcomes of IL-17A signaling. To achieve such a goal, we need to improve the understanding of IL-17A signal transduction and identify the regulatory mechanisms for each set of genes.

The most common targets of IL-17A signaling include NF-kB–dependent cytokines, chemokines, antimicrobial peptides (AMP), and matrix metalloproteases (11, 12). IL-17A plays a significant role in up-regulating the production of inflammatory cytokines via either transcriptional (gene expression) or post-transcriptional (mRNA stabilization) branches (5, 13). IL-17A signal transduction occurs via IL-17 receptors consisting of the IL-17RA and IL-17RC subunits (14). While IL-17RA is ubiquitously expressed, IL-17RC has limited expression on nonhematopoietic epithelial and mesenchymal cells (15, 16). IL-17A binding to its receptor results in the recruitment of the cytosolic signaling complex consisting of Act1 and several TRAF proteins [see review for further details (17)]. Recruitment of TRAF6 results in the activation of downstream molecules, including TAK1, eventually leading to translocation of NF-kB to the nucleus and transcription of target genes, such as IL6 and IL8 (18). On the other hand, the recruitment of TRAF2 and TRAF5 participates in the stabilization of mRNA transcripts of CXCL1 and CXCL5 (19). The TRAF5/TRAF2-dependent mRNA stabilization pathway is independent of the presence of TRAF6 (19); however, the role of TRAF5/TRAF2 in the transcriptional pathway remains unknown. In addition, IL-17A activates MAP kinases, including ERK, p38, and JNK, that play essential roles in autoimmune diseases (20, 21). Although several regulators of each transcriptional and nontranscriptional branch are known (17), there is a knowledge gap in understanding of cross-talk between the two branches. Identifying regulators that promote one branch over the other will provide critical information to design targeted therapeutics.

To address this, we examined the role of 14-3-3ζ in the IL-17A signaling. 14-3-3ζ is one of the seven members of 14-3-3 family that interacts with 0.6% of the cellular proteome and performs a range of context-dependent functions, including signaling (22–24). The 14-3-3ζ regulates the signal transduction of GM-CSF, IL-3, IL-5, STAT3, Toll-like receptor (TLR)3, and AMPK, among others (25–29). Additionally, it supports the production of several cytokines, such as IL-13, IFN-γ, and IL-17A (30, 31). We previously identified increased 14-3-3 protein levels in the aortic tissues of patients with large-vessel vasculitis (32). Large-vessel vasculitis is a presumed autoimmune disease with increased infiltration of Th1 and Th17 cells in the affected artery, resulting in increased levels of IL-17A and IL-6 (33–35).
present study, we identified a requirement of intracellular 14-3-3ζ in IL-17A signal transduction. We used 14-3-3ζ knockout (KO) murine fibroblasts and human epithelial cells to examine the role of 14-3-3ζ in IL-17A signaling. Our results show that 14-3-3ζ is essential for IL6, IL8, and Defb3 production in IL-17A signaling; however, it has an opposite effect on CXCL1 production. To validate our finding, we engineered 14-3-3ζ global KO Lewis rats using CRISPR/Cas9 and subjected them to IL-17A treatment, a technique successfully used to determine the Act1 role in IL-17A signal transduction (36). Similar to cells, the 14-3-3ζKO rats show a decrease in the IL-17A-stimulated IL-6 production but an increase in CXCL1 level. In the absence of 14-3-3ζ, IL-17A-stimulated ERK phosphorylation (pERK) and p65 translocation are decreased. The 14-3-3ζ interacts with TRAFs via two of its TRAF-binding motifs (TBMs), particularly TRAF5, which bound more strongly in the presence of IL-17A. The TRAF interaction with 14-3-3ζ plays a vital role in determining the effects on IL-6 production. We also uncovered a suppressive role of TRAF5 on the IL-17A-stimulated IL-6 levels. To the best of our knowledge, this report showing a role of 14-3-3ζ and its activity with TRAF5 in regulating IL-17A signal transduction, is unique.

Results

14-3-3ζ Is Required for IL-17A-Mediated Gene Induction. To investigate the role of 14-3-3ζ in IL-17A signaling, we took advantage of the human retinal epithelial cells (ARPE-19) and mouse embryonic fibroblasts (MEFs) as in vitro cell models, which respond to exogenous IL-17A in the absence of pretreatment by TNF-α (37). IL-17A treatment resulted in robust induction of IL-6, which was suppressed by BV02, a pan 14-3-3 inhibitor (Fig. L4). This suggested that 14-3-3 proteins play a role in IL-17A signal transduction. To determine the isoform-specific role of the 14-3-3 family of proteins, we generated 14-3-3ζKO ARPE-19 cells using the CRISPR/Cas9 approach (SI Appendix, Fig. S1A). The loss of 14-3-3ζ in ARPE-19 did not affect the cell viability (SI Appendix, Fig. S1B) or the expression of two unrelated 14-3-3 isoforms, ε and γ, measured at RNA and protein levels (Fig. 1B). Importantly, the 14-3-3ζKO cells displayed significant inhibition of IL-17A-induced IL-6 at both mRNA and protein levels (Fig. 1 C and E and SI Appendix, Fig. S1C), suggesting a specific role of the 14-3-3ζ isoform in IL-17A-induced IL-6 expression. We tested additional IL-17A–induced genes (e.g., IL8), which were strongly inhibited in 14-3-3ζKO cells (Fig. 1D).

To validate these results in mouse cells, we examined WT or 14-3-3ζKO MEFs (38). Similar to the human cells, 14-3-3ζKO MEFs exhibited a substantial reduction in IL-17A–induced Il6 and Il8 levels (Fig. 1 F and G). To further solidify the uniquely identified role of 14-3-3ζ in IL-17A–mediated gene induction, we rescued 14-3-3ζ expression in 14-3-3ζKO ARPE-19 cells using lentiviral transfections and tested the effect on the IL-17A–stimulated IL-6 or IL-8 production. As expected, the rescue of 14-3-3ζ expression resulted in increased IL-17A–induced IL6 and IL8 (Fig. 1 H and I). The loss of Il6 or Il8 induction was not due to any change in IL-17 receptor expression on the 14-3-3ζKO cells (SI Appendix, Fig. S1D). To evaluate the specificity of 14-3-3ζ in IL-17A signaling, we studied its role in IFN-γ signaling. The IFN-γ treatment caused increased induction of Il6 in ARPE-19 cells, which was unaffected by the presence of BV02 (SI Appendix, Fig. S2A). Conversely, IFN-γ–induced Il6 gene expression was unchanged in 14-3-3ζKO MEFs (SI Appendix, Fig. S2B). Treatment with IL-17A or BV02 did not affect the 14-3-3ζ levels (SI Appendix, Fig. S2C). Collectively, our results established a role of 14-3-3ζ in IL17A–mediated gene induction.

14-3-3ζ Is Required for IL-17A-Induced NF-κB Activation. To investigate how 14-3-3ζ participates in IL-6 production, we examined IL-17A–stimulated NF-κB activation, a step that is critical for gene induction (1, 18). We coexpressed a reporter plasmid that drives Renilla luciferase by a NF-κB promoter, and a firefly luciferase driven by thymidine kinase promoter in the 14-3-3ζ knockdown (KD) HeLa cells, and measured luciferase activity after the stimulation with IL-17A (39). The ablation of 14-3-3ζ resulted in significant suppression of the IL-17A–induced NF-κB activity in a luciferase assay (lanes 2 and 4, Fig. 2A). The complementation with HA-tagged 14-3-3ζ resulted in a substantial rescue of NF-κB activity in 14-3-3ζKO cells (lanes 4 and 6, Fig. 2A). Encouraged by these results, we focused on a specific stage of NF-κB activation, the nuclear translocation of the p65 subunit of NF-κB, in IL-17A–stimulated cells. IL-17A treatment of ARPE-19 cells resulted in the nuclear translocation of p65, which was inhibited in the 14-3-3ζKO ARPE-19 cells, analyzed by confocal microscopy (Fig. 2B). Similarly, we observed IL-17A–stimulated increase in nuclear p65+ cells in WT MEFs but not in 14-3-3ζKO cells (Fig. 2C). To validate the microscopic results, we performed subcellular fractionation and evaluated the nuclear translocation of p65. Similar results were obtained using subcellular fractionation of ARPE-19 cells and MEFs, confirming that 14-3-3ζ was required for IL-17A–induced p65 nuclear translocation (Fig. 2 D and E and SI Appendix, Fig. S3). Taken together, our results suggest that the role of 14-3-3ζ in IL-17A signaling is upstream of the p65 nuclear translocation event.

14-3-3ζ Is Required for IL-17A-Induced pERK. IL-17A stimulation results in the activation of MAP kinases, which also activate NF-κB (1, 37). To evaluate whether 14-3-3ζ is required for IL-17A–induced pERK, we used WT and 14-3-3ζKO ARPE-19 cells. IL-17A treatment, analyzed at two different time points, caused a robust increase in pERK in the WT, which was significantly suppressed in 14-3-3ζKO cells (Fig. 3A). We further examined this using MEFs, which also showed increased IL-17A–induced pERK in WT cells, but not in the absence of 14-3-3ζ (Fig. 3B). To strengthen our observation, we tested additional doses of IL-17A, which still did not trigger pERK in 14-3-3ζKO ARPE-19 cells (Fig. 3C). To validate the 14-3-3ζ requirement for pERK, we rescued its expression in 14-3-3ζKO HeLa cells by lentiviral transduction. As expected, complementation of 14-3-3ζKO cells with Flag-tagged 14-3-3ζ rescued the IL-17A–induced pERK as compared to empty vector control (Fig. 3D and SI Appendix, Fig. S4). Together, our results indicate that 14-3-3ζ is required for IL-17A–induced pERK in human and mouse cells.

14-3-3ζ Forms Complex with the TRAF Proteins. Our results showed that 14-3-3ζ participates in IL-17A signaling upstream of the activation of NF-κB and ERK, which led us to focus on the TRAF proteins, which are recruited to IL-17RA and are essential for signal transduction (19, 40, 41). We examined whether the TRAF proteins—namely TRAF2, TRAF5, and TRAF6—can interact with 14-3-3ζ. In HEK293T cells, the ectopically expressed Flag-tagged TRAF proteins were successfully pulled down by HA-tagged 14-3-3ζ (Fig. 4A), suggesting that 14-3-3ζ might function at the level of IL-17RA–TRAF complex formation. Next, we determined the interaction at endogenous levels using rat aortic lysates and coimmunoprecipitate (co-IP) 14-3-3ζ–bound proteins. Both TRAF5 and TRAF6 coimmunoprecipitated with 14-3-3ζ (Fig. 4B). To evaluate whether 14-3-3ζ interacts with the TRAF proteins in a signal-dependent manner, we used co-IP of endogenous 14-3-3ζ in unstimulated or IL-17A–stimulated ARPE-19 cells. We observed a robust IL-17A–dependent increase in the interaction of TRAF5, but not TRAF2, with 14-3-3ζ (Fig. 4C). Similarly, we observed increased interaction of TRAF6 with 14-3-3ζ in IL-17A–treated cells (Fig. 4D). We validated these results using confocal microscopy to evaluate the interaction between the endogenous proteins. Using IL-17A–stimulated ARPE-19 cells, we confirmed colocalization of endogenous 14-3-3ζ with TRAF5, and 14-3-3ζ with TRAF6 (Fig. 4E).
To investigate if 14-3-3ζ directly interacts with TRAF5 or TRAF6, we developed a cell-free in vitro interaction assay using the purified proteins from the transiently transfected HEK-293T cells (SI Appendix, Fig. S5). The TRAF5-bound FLAG-beads and 14-3-3ζ-bound HA-beads were washed with 300 mM salt to remove any associated proteins to isolate TRAF5 and 14-3-3ζ proteins in near purity. The washed 14-3-3ζ tagged HA-beads were used to affinity pull-down TRAF5 from the peptide elute of FLAG beads. The HA–14-3-3ζ beads were able to pull-down nearly purified TRAF5, suggesting that the two proteins can directly interact with each other in solution (Fig. 4F). Similarly, TRAF6 and 14-3-3ζ showed the ability to interact directly with each other in the cell-free in vitro interaction system (Fig. 4G). Since 14-3-3ζ is required in IL-17A signal transduction, we next addressed the functional role of 14-3-3ζ–TRAF complex in IL6 induction.

14-3-3ζ Suppresses the Role of TRAF5 in IL-17A Signaling. The increase in the 14-3-3ζ interaction with TRAF5 and TRAF6 upon IL-17A stimulation led us to question its role in the downstream signaling. Requirement of TRAF6 in IL-17A–stimulated gene induction is well-established, but the role of TRAF5 remains understudied (42). As TRAF6 and 14-3-3ζ have similar effects on IL6 gene induction, we focused our attention to study the 14-3-3ζ–TRAF5 axis in the IL17A–induced gene induction, and used both IL6 and CXCL6 as readouts of IL17A signaling. We used TRAF5KD ARPE-19 cells to examine the IL-6 production upon IL-17A treatment. Surprisingly, the IL-17A–induced IL6 level was significantly increased in the TRAF5KD ARPE-19 cells, compared to nonspecific target shRNA control (NSC) cells (Fig. 5A and B). To rule out the cell-specific effect, we generated TRAF5KD HeLa cells and confirmed TRAF5 KD promotes IL-17A–induced IL6 levels (SI Appendix, Fig. S6). As TRAF5 is known to promote IL-17A–stimulated CXCL1 induction (19), we measured the CXCL1 level in the TRAF5KD cells. As expected, CXCL1 mRNA in TRAF5KD cells was significantly reduced in comparison to NSC cells (Fig. 5C). The opposite effect of TRAF5 on IL6 and CXCL1 gene induction in IL17A signaling suggested that TRAF5 may act as a suppressor of IL-6 production, but not for CXCL1. This intrigued us to evaluate the role of 14-3-3ζ in IL17A–induced CXCL1 level. In contrast to TRAF5, 14-3-3ζ had a suppressive effect on CXCL1 induction (Fig. 5D). The opposite effects of 14-3-3ζ and TRAF5 on IL-17A–stimulated IL6, and CXCL1 gene induction suggested the presence of a novel regulatory axis that controls the signaling output.

To gain mechanistic insight into the TRAF5 role, we measured the pERK levels, which were found to be increased in the TRAF5KD ARPE-19 cells (Fig. 5E). To examine if the increased pERK is responsible for the elevated IL6 induction, we used a well-known MEK inhibitor, U0126, which completely abolished the IL6 induction in TRAF5KD cells. This suggested that pERK is required for increased IL6 levels in IL17A–stimulated TRAF5KD cells (Fig. 5F). To test if the elevated IL6 in TRAF5KD is dependent on 14-3-3ζ, we generated 14-3-3ζKD TRAF5KD ARPE-19 cells that were deficient in both TRAF5 and 14-3-3ζ. The increase in IL17A–stimulated IL6 was completely absent in 14-3-3ζKD TRAF5KD cells (Fig. 5G). Collectively, we conclude that TRAF5 suppresses IL17A–induced IL6 production by suppressing pERK, an effect which is countered by 14-3-3ζ.

14-3-3ζ Interaction with TRAF Is Required for IL-17–Induced IL-6 Production. To understand role of the 14-3-3ζ–TRAF interaction in IL-17A signaling, we questioned the basis of interaction...
between 14-3-3ζ and TRAF. TRAF proteins are known to recognize specific motifs, known as TBMs in their partners (43). We found that 14-3-3ζ protein has two TBMs, 37SNEE and 148AYQE (Fig. 6A). To assess whether the TBMs are required for 14-3-3ζ–TRAF5 interaction, we performed in silico modeling by docking the TRAF domain of TRAF5 (4GJH.pdb) on to 14-3-3ζ dimer (5D2D.pdb) using the ZDOCK server. We restricted the site of interaction to specifically these two TBMs on the 14-3-3ζ. The top 10 structures generated were screened using PRODIGY, and the structure (complex #5) with minimum intermolecular energy in the case of each motif was visualized using Visual Molecular Dynamics (VMD) (Fig. 6B). For both motifs, the 14-3-3ζ–TRAF5 complex predicted comparable free energy and kinetic binding constants (AYQE: ΔG = -17.0 kcal/mol, Kd = 3.5E-13 M, and SNEE: ΔG = -15.6 kcal/mol, Kd = 3.3E-12 M) (SI Appendix, Table S1).

To examine whether these two TBMs are responsible for 14-3-3ζ–TRAF5 binding, we generated site-directed mutants for both putative motifs (SNEE → SNAA, AYQE → AYAA) and tested these mutants for their ability to interact with TRAF5. Importantly, mutation of either of the two motifs (M1 and M2 mutants) resulted in significant loss of interaction with TRAF5 (Fig. 6C).

To further confirm the loss of interaction, we used a proximity ligation assay (PLA), which detects closely located proteins, and established the binding of TRAF5 with WT but not with mutant 14-3-3ζ (Fig. 6D). To evaluate the functional consequence of this interaction, we analyzed pERK by immunoblot. EV, empty vector. All results are representative of at least three experiments, *P < 0.05, **P < 0.005, and ***P < 0.0005.

Fig. 2. 14-3-3ζ is required for IL-17A–mediated NF-κB activation. (A) Dual-luciferase activity was measured in NSC or 14-3-3ζ-KO HeLa cells, transiently coexpressing NF-κB, thymidine kinase (TK), and HA-tagged 14-3-3ζ, were treated with hIL-17A for 20 h. (B) WT or 14-3-3ζ-KO ARPE-19 cells, treated with hIL-17A for 1 h, were immunostained for endogenous p65 and analyzed by confocal microscopy. (Scale bar: 10 μm.) (C) WT or 14-3-3ζ-KO MEFs, treated with mIL-17A for 1 h, were immunostained for endogenous p65 and analyzed by confocal microscopy and the nuclear p65-expressing cells were quantified from at least 100 cells. (D and E) Nuclear fractions from WT and 14-3-3ζ-KO MEFs (D) or ARPE-19 cells (E), treated with IL-17A for 1 h, were analyzed for p65 and HDAC1 by immunoblots (shown in SI Appendix, Fig. S3), which were quantified using imageJ software. The results are representative of at least three experiments, *P < 0.05, **P < 0.005, and ***P < 0.0005.

Fig. 3. 14-3-3ζ is required for IL-17A–mediated pERK. (A–C) The WT and 14-3-3ζ-KO ARPE-19 (A and C) treated with hIL-17A, or MEFs treated with mIL-17A (B), for the indicated times and doses, were analyzed for pERK by immunoblot. (D) The 14-3-3ζ (Flag-tagged) restored 14-3-3ζ-KD HeLa cells, treated with IL-17A, were analyzed for pERK by immunoblot. EV, empty vector. All results are representative of at least three experiments, *P < 0.05, **P < 0.005, ***P < 0.0005, and ****P < 0.0001.
interaction, we ectopically expressed the WT or mutant 14-3-3ζ in the 14-3-3ζKO ARPE-19 cells to measure the effect on IL-17A–induced IL6. The ectopic expression of WT, but not mutant 14-3-3ζ, triggered strong IL6 mRNA induction (Fig. 6E), suggesting that 14-3-3ζ–TRAF interaction supports IL-17A–induced IL6 levels. This decrease in the IL6 induction can be interpreted by the loss of 14-3-3ζ’s ability to either promote TRAF6–IL6 branch, or sequester TRAF5 to suppress its inhibition of IL6 induction.

Physiological Role of 14-3-3ζ in IL-17A Signaling. To evaluate the physiological relevance of 14-3-3ζ in IL-17A signaling, we utilized two approaches: Measuring the antifungal activity in a newly designed bioassay, and the gene induction analyses using physiological relevance of 14-3-3ζ measured the effect of 14-3-3ζ KO in IL-17A signaling and the resultant antifungal activity.

First, we studied duodenum tissue from the heterozygous 14-3-3ζKO mice, which allowed us to measure the effect of reduced 14-3-3ζ levels in the IL-17A–stimulated gene induction ex vivo. Similar to the cellular studies, rat IL-17A–stimulated significant increase in Il6 in tissues obtained from WT, but not from heterozygous animals (Fig. 7D). In contrast, tissues from heterozygous 14-3-3ζ animals showed a significant increase in the Cxcl1 levels (Fig. 7E). Second, we performed a time course in 14-3-3ζKO animals to measure sera IL-6 level after the intraperitoneal injection of rat IL-17A, a strategy previously used to determine the critical components of IL-17 signal transduction (36). At the basal level, both WT and KO animals had similar levels of IL-6. Upon IL-17A stimulation, at 12 h WT rats showed significantly higher IL-6 production compared to 14-3-3ζKO rats (Fig. 7F). By 72 h, a smaller yet significant difference between the WT and KO animals was still present. In contrast to IL-6, 14-3-3ζKO animals had slightly higher (but not significant) CXCL-1 at 12 h, which was significantly increased by 72 h (Fig. 7G).

Discussion

In the present study, we identified 14-3-3ζ as a component of IL-17A signal transduction. Using in vitro, ex vivo, and in vivo studies, we measured IL-17A–mediated gene induction in the absence of any additional cytokines, such as TNF-α. First, we studied duodenum tissue from the heterozygous animals, which showed a strong reduction of 14-3-3ζ protein levels (Fig. 7C) and mimic our in vitro KD cell models. This allowed us to measure the effect of reduced 14-3-3ζ levels on the IL-17A–stimulated gene induction ex vivo. Similar to the cellular studies, rat IL-17A–stimulated significant increase in Il6 in tissues obtained from WT, but not from heterozygous animals (Fig. 7D). In contrast, tissues from heterozygous 14-3-3ζ animals showed a significant increase in the Cxcl1 levels (Fig. 7E). Second, we performed a time course in 14-3-3ζKO animals to measure sera IL-6 level after the intraperitoneal injection of rat IL-17A, a strategy previously used to determine the critical components of IL-17 signal transduction (36). At the basal level, both WT and KO animals had similar levels of IL-6. Upon IL-17A stimulation, at 12 h WT rats showed significantly higher IL-6 production compared to 14-3-3ζKO rats (Fig. 7F). By 72 h, a smaller yet significant difference between the WT and KO animals was still present. In contrast to IL-6, 14-3-3ζKO animals had slightly higher (but not significant) CXCL-1 at 12 h, which was significantly increased by 72 h (Fig. 7G). Overall, results from in vivo studies validated our in vitro findings. Together, these results illuminate the critical role of 14-3-3ζ in IL-17A signal transduction, and a point of cross-talk between the TRAF6-dependent gene expression branch and the TRAF2/TRAF5-dependent mRNA stabilization branch of the pathway.
IL-17A signaling must be evaluated in the absence of TNF-α components of TNF signaling and the precise role of TRAF5 in 14-3-3 proteins are known to participate in TNF-α signaling in cellular models (40). We avoided the use of TNF-α stimulation with TNF-α, is a possible mechanism by which 14-3-3 ζ-dependent on the phospho-ERK and 14-3-3, reversed its promotional effect on IL-17A production. Importantly, we also identified a suppressive impact of TRAF5 on the IL-17A-induced IL-6 production, which was dependent on the phospho-ERK and 14-3-3ζ. Our results suggest that increased TRAF5 interaction upon IL-17A stimulation is a possible mechanism by which 14-3-3ζ represses the TRAF5-mediated suppression of IL-6 production. Overall, our results show a functional role of 14-3-3ζ in IL-17A signaling and a regulatory mechanism consisting of a TRAF5-14-3-3ζ axis that controls IL-17A signaling outcomes (Fig. 8).

Because IL-17A is a weak stimulator of NF-κB activity, costimulation with TNF-α is often used as a strategy to activate the IL-17A-mediated proinflammatory response in several in vitro cellular models (40). We avoided the use of TNF-α for our studies because 14-3-3ζ proteins are known to participate in TNF-α-mediated NF-κB activity and mRNA stabilization (28, 29, 45, 46). Furthermore, TRAF proteins, including TRAF5, are critical components of TNF signaling and the precise role of TRAF5 in IL-17A signaling must be evaluated in the absence of TNF-α. Therefore, we preferred cells (ARPE-19 and MEFs) that can produce small yet measurable outcomes of IL-17A stimuli in the absence of a costimulus. Similarly, we utilized IL-17A alone for the ex vivo and the in vivo experiments, which resulted in a maximum of fourfold cytokine induction. However, we cannot rule out the contribution of costimuli, e.g., TNF-α, produced by myeloid cells, in these physiological models.

Like other cytokines signaling, IL-17A binding to the receptor triggers a series of phosphorylation (Act1, MAPK, TPL2 kinase, IKK, Syk, TBK) and ubiquitination (TRAF6, HuR, USP25, A20)-based molecular events that play essential roles in the regulation of both de novo gene transcription and mRNA stabilization-related outcomes (18, 47–49). Several regulators of the TRAF6-IL6 branch include TRAF3, TRAF4, and IKxB (inhibitor of NF-κB-ζ) that affect the transcription of IL-17A target genes (17). Similarly, specific regulators of the TRAF5-CXCL1 branch include SF2, endoribonucleases MCPiP1 (Regnase-1), and Ard5a, which are responsible for regulating the mRNA stability of IL-17A target genes (19, 50, 51). It is known that TRAF6 plays no role in the TRAF5-CXCL1 branch; however, the role of TRAF5 on the TRAF6-dependent IL6 induction remains unknown (52). Because several growth factors can amplify the IL-17A signaling, it is reasonable to assume the presence of strict control by which both branches cross-talk and influence each other to regulate specific output. Our exciting results show that loss of TRAF5 promotes the TRAF6-IL6 branch, suggesting that TRAF5 is an endogenous suppressor of IL-17A-induced IL6 induction, which requires 14-3-3ζ and MEK activity. It is important to note that TRAF5 as a negative regulator of TLR signaling and suppressor of IL-6 production in B lymphocytes has been previously reported (53). Similar to our observation in TRAF5KD cells, TRAF5 KO animals also show increased NF-κB activation and pERK (53, 54). Additional work is needed to examine if TRAF5 is a regulator that diverts the IL-17A–ACT1 signal in favor of the mRNA stabilization branch (55). In contrast to TRAF5, the presence of 14-3-3ζ, is required for de novo transcription of IL6 but not for CXCL1. The
is a mechanism by which 14-3-3 function: 1) IL-17A-induced increased interaction with TRAF5 and brake on IL-6 production. In either case, 14-3-3ζ or 2) the 14-3-3ζ function, which results in the increased IL-6 production; or 3) cause mutation of the TRAF-binding motif can also disrupt 14-3-3ζ plays a critical role in IL-17A
interact with TRAF5, TRAF2, and TRAF6, and this interaction
TRAF6 interacts with 14-3-3ζ and TRAF5 on IL-17A
stimulated IL-6 and CXCL-1 production suggest the presence of counter
mechanisms to regulate the IL-17A signaling. Whether a pathway-specific role of
14-3-3ζ exists to modulate the two TRAF branches is an intriguing point and will require 14-3-3ζ mutants that separate these functions. Our structural modeling studies may help identify such mutants in the future. In addition, several 14-3-3 proteins are known to stabilize the phospho-proteins and are critical regulators of MAPK (62). Our result shows that 14-3-3ζ activity in IL-17A signal transduction is upstream of pERK; therefore, it will be interesting to examine 14-3-3ζ’s role in the regulating MAPK activity to promote IL-17A signaling.
Overall, our results show 14-3-3ζ is an essential component of the IL-17A signaling pathway that promotes IL-6 production and antifungal activity via activating NF-κB. Importantly, we discovered, as a part of the molecular mechanism, 14-3-3ζ–TRAF5 is a regulatory axis in determining the functional outcome of the IL-17A–TRAF5 branch. Since this axis regulates IL-6 and CXCL-1 production, its role in several immune diseases, including rheumatoid arthritis, lupus, host-defense, and so forth, is implied. Our goal is to achieve in-depth insight into IL-17A signaling and uncover regulators that can help fine tune the outcomes of signal transduction. We expect that further insight of the 14-3-3ζ–TRAF5 axis will make the basis of future therapeutics to promote desired gene outcomes of IL-17A signaling.

Materials and Methods

Cell Culture and Reagents. The HEK-293T, HeLa, and MEFs were maintained in DMEM containing 10% FBS, penicillin, and streptomycin. The human ARPE-19 cells were maintained in DMEM-Ham’s F-12 media containing 10% FBS, penicillin, and streptomycin. All cell lines used in this study were maintained in the authors’ laboratory at the University of Toledo. Unless stated otherwise, IL-17A stimulation in ARPE and MEFs was performed at 50 ng/mL of
the tail DNA isolated from the indicated rat strains (+/+, +/-). An immunoblot of 14-3-3ζ from the tail biopsy of the indicated strains is shown. (D and E) The duodenum of WT or heterozygous 14-3-3ζ; Lewis rats were either un treated or treated with rat (r) IL-17A for 20 h, and the iL (D) and Cxcl1 (E) mRNA levels were analyzed by qRT-PCR. (F and G) The WT and 14-3-3ζKO rats (n = 8) were injected with rIL-17A intraperitoneally for the indicated times, and cytokine (IL-6 and Cxcl1) levels in the sera were analyzed by ELISA. The results are representative of at least three experiments, *p < 0.05, **p < 0.005, ***p < 0.0005, and ****p < 0.00001.

**Fig. 7.** Functional significance of 14-3-3ζ participation in IL-17A signaling. (A) Culture supernatants from either untreated or IL-17A–treated WT or 14-3-3ζKO MEFs were analyzed for the killing of C. albicans. (B) WT or 14-3-3ζKO MEFs, treated with rIL-17A, were analyzed for Defb3 by qRT-PCR. (C) The schematic design of 14-3-3ζ; KO Lewis rat generation is shown; the exon 3 of rat 14-3-3ζ was targeted by the gRNAs, as indicated. A representative genotyping result is shown from the tail DNA isolated from the indicated rat strains (“+”, “+/-”, and “-“). An immunoblot of 14-3-3ζ from the tail biopsy of the indicated strains is shown. (D and E) The duodenum of WT or heterozygous 14-3-3ζ; Lewis rats were either untreated or treated with rat (r) IL-17A for 20 h, and the IL (D) and Cxcl1 (E) mRNA levels were analyzed by qRT-PCR. (F and G) The WT and 14-3-3ζKO rats (n = 8) were injected with rIL-17A intraperitoneally for the indicated times, and cytokine (IL-6 and Cxcl1) levels in the sera were analyzed by ELISA. The results are representative of at least three experiments, *p < 0.05, **p < 0.005, ***p < 0.0005, and ****p < 0.00001.

Cell Lysis and IP. Immunoblot analyses were performed using previously described procedures (64, 65). Briefly, the cells were lysed in 50 mM Tris buffer, pH 7.4 containing 150 mM of NaCl, 0.1% Triton X-100, 1 mM sodium orthovanadate, 10 mM of sodium fluoride, 10 mM of β-glycerophosphate, 5 mM sodium pyrophosphate, protease and phosphatase inhibitors (Roche). For IP, either 2 μg of antibody followed by Protein G Sepharose or 10 μL of tagged beads were added to the cell lysate and incubated overnight at 4°C. Total protein extracts or pull-down beads were analyzed by SDS/PAGE, followed by immunoblot.

**ELISA.** ELISA kits for rat IL-6, rat Cxcl1, human IL-6 (R&D Systems) were used as per the recommended protocols. Conditioned media from same number of cells or equal volume of animal sera was used for comparison between the WT and KO set of samples.

**In Vitro Protein-Protein Interaction Assay.** HEK293T cells transiently expressing either HA-tagged 14-3-3ζ or Flag-tagged TRAF5 were lysed in EPPS buffer containing protease inhibitors by five freeze-thaw cycles and lysates were immunoprecipitated separately with HA or Flag beads overnight at 4°C. Beads were washed thrice with EPPS buffer containing 300 mM NaCl. The TRAF5 elute, from beads using Flag peptide, was incubated with washed HA beads bound to 14-3-3ζ or HA beads alone, for 2 h at room temperature. Beads were washed twice with EPPS buffer and once with RIPA buffer before performing final elution for bound proteins by boiling in 2× SDS sample loading buffer.

**RNA Isolation and qRT-PCR Analyses.** Total RNA was isolated using Trizol (Invitrogen), cDNA was prepared using ImProm-II Reverse Transcription Kit (Promega), and the cDNA was analyzed using Radiant SYBR Green PCR mix (Alkali Scientific) in Roche LightCycler 96 instrument and analyzed with the LightCycler 480 Software, v1.5. The expression levels of the mRNAs were normalized to 18S rRNA. For the qRT-PCR analyses of the respective genes, the following primers were used:

- **IL6-** GTAGCCGCCGCCACAGAGACATGTCCCTTTCGAGGCGTG
- **Cxcl1-** AACCCAGACTCATAGGCACCAGTTGGATTGGTCACTGTTCAGC, YWHAZ-ACCAGATGAGCAGGTTTGGATTCACTGATAAAGAAGCAGTGGT, YWHAG- CCTGACATGGAAGCTGAA/GAGCTACGGGCTACAACAA

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**Fig. 8.** A proposed model for the identified 14-3-3ζ–TRAF5 axis in regulating IL-17A signaling pathways. IL-17A binding triggers a receptor complex (IL-17A–ACT1–TRAF) activation that transduces the signal to generate transcriptional output (IL-6/IL-8/CXCL-1). Our results show that 14-3-3ζ is a TRAF-binding component that is needed for IL-17A-stimulated pERK, NF-κB activation, and IL-6/IL-8 production, but not for CXCL-1. Importantly, we uncovered a role of TRAF5 in suppressing the IL-17A-stimulated IL-6 production. The IL-17A stimulation increases the 14-3-3ζ–TRAF5 interaction that potentially inhibits the TRAF5 function and supports the IL-6 production. Overall, our model suggests that IL-17A stimulation increases 14-3-3ζ interaction to sequester TRAF5, which results in increased IL-6 levels. These results have broad implications covering IL-17A’s role in antifungal immunity to regulation of inflammation.

**A proposed model for the identified 14-3-3ζ–TRAF5 axis in regulat

**Anti-Candida effect**

**Regulation of Inflammation**

\[ \text{Intracellular energy constants of each simulated complex (67, 68). The}

**Antifungal Assay.** *C. albicans* cultures were grown to an ODmax of 0.08 prior to adding conditioned media of MEFs or 14-3-3ζKO MEFs that were treated with IL-17A for 48 h. Aliquots of 45 μL of fungal cells were treated with 5 μL conditioned media for 1 h at 37 °C. After treatment, samples were serially diluted and plated for enumeration. The percent survival was determined by dividing the CFU of treated samples by those of untreated samples times 100. Assays were performed in triplicate and *P* values were calculated from all three experiments using ANOVA with Tukey’s post hoc test. MEF pellets were used for RNA analysis.

**14-3-3ζ/Δ (YwhazΔ) Lewis Rat Generation.** KO rats were prepared as described previously (69). In brief, two single-guide RNAs (sgRNAs) were designed to have at least 3 bp of mismatch between the target site and any other site in the rat genome. Ribonucleoprotein (RNP) complexes were formed by incubating the 30 ng/mL gRNA#1 (5′-CACGACACCCUGACGAGU-3′) or 30 ng/mL gRNA#2 (5′-GCUUUCUGCCUAGGACA-3′) with 50 ng/mL Cas9 protein (St. Jude Protein Production Core) at the room temperature for 10 min. The RNP were combined in a 1:1 ratio and placed on ice until rat yogetes microinjections at the Transgenic Animal Model Facility at the University of Michigan. Rat yogetes for microinjection were obtained by mating superovulated Lewis rat females with males of the same strain (LEW/ Cr, strain code; Charles River Laboratory) and used for microinjection. As described previously (70). Animals were housed in American Association for Accreditation of Laboratory Animal Care-accredited animal care facility at the University of Michigan or the University of Toledo under the Institutional Animal Care and Use Committee-approved protocols. One-hundred sixty rat yogetes were microinjected; 349 survived injection and were transferred to pseudopregnant Sprague-Dawley females (Crl:CD(SD), strain code 001; Charles River Laboratory). A total of 20 G0 rat pups were obtained, of which 9 were found to be homozygous null, and 11 were heterozygous for the Ywhaz gene. All heterozygous G0 pups were mated with WT Lewis rats (Charles River Laboratory) to obtain germ-line transmission of gene KO. Ear or tail piece was used for DNA extraction and genotype of each generation of animals was determined by PCR. Three sets of Ywhaz2 primers (TCTTTTCTCTGAACTGTTGTTGG, ATATAAGCTAACCAGAACCACAA, CTACACTTGGAGAAGCGATGG, GATCACACTTTGTAGTATCCTC, CCTCCATGCTACATGCACATG) were used to identify the 170-, 549-, and 1,026-bp product for the WT gene. The heterozygous animals were maintained for breeding and to obtain KO animals. The generation 5b animals were utilized for this study.

All animals were housed in a standard animal care facility with controlled temperature and unlimited access to food and water. Age- and sex-matched rats were used in the study. For in vivo experiments, animals were injected intraperitoneally with 500 ng of rat IL-17A, and blood was collected via saphenous vein at 0, 4, 12, and 72 h. For the ex vivo experiments, 12-wk-old WT and heterozygous male animals were killed to collect duodenum under aseptic environment. Ingest from duodenum was removed and cleaned with sterile PBS containing antibiotics (71). Tissue was cut into 5- to 8-mm size and incubated with 10% FBS and antibiotic-containing DMEM for few hours before adding rat IL-17A at 50 ng/mL followed by incubation for 20 h.

**Statistical Analysis.** All experiments were performed at least thrice unless stated otherwise. Depending upon the number of sets for comparison, either unpaired Student’s *t* test or ordinary one-way ANOVA was used.

**Data Availability.** All study data are included in the article and SI Appendix.

**ACKNOWLEDGMENTS.** We thank Dr. Lopez for sharing the 14-3-3ζKO mouse embryonic fibroblasts; Dr. Bina Joe, Dr. Cheng Xi, Dr. Thom Saunders, and Ms. Blair Bell, who helped us in generating the animal model; and several present and former laboratory members in shaping the project, and in particular Ms. Anna Glanz, who provided technical assistance. We acknowledge funding from American Heart Association Grant 15SDG25008025 (to R.C.), NIH/NIDCR R01DE026889-01 (to H.C.), 1R01DE27343-01A1 (to H.C.), and the University of Toledo College of Medicine and Life Sciences startup funds (R.C.).
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