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The Role of IL-15 in Activating STAT5 and Fine-Tuning IL-17A Production in CD4 T Lymphocytes

Pushpa Pandiyan,*,† Xiang-Ping Yang,‡ Senthil S. Saravanamuthu,§ Lixin Zheng,* Satoru Ishihara,§ John J. O’Shea,‡ and Michael J. Lenardo*

IL-15 is an important IL-2-related cytokine whose role in Th17 cell biology has not been fully elucidated. In this study, we show that exogenous IL-15 decreased IL-17A production in Th17 cultures. Neutralization of IL-15 using an Ab led to increases in IL-17A production in Th17 cultures. Both Il15−/− and Il15r−/− T cell cultures displayed higher frequency of IL-17A producers and higher amounts of IL-17A in the supernatants compared with those of wild-type (WT) cells in vitro. IL-15 down-modulated IL-17A production independently of retinoic acid-related orphan receptor-γt, Foxp3, and IFN-γ expression. Both Th17 cells and APCs produced IL-15, which induced binding of STAT5, an apparent repressor to the Il17 locus in CD4 T cells. Also, in a model of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (EAE), Il15−/− mice displayed exacerbated inflammation—correlating with increased IL-17A production by their CD4+ T cells—compared with WT controls. Exogenous IL-15 administration and IL-17A neutralization reduced the severity of EAE in these mice, helped with manuscript preparation, recorded experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; qPCR, quantitative PCR; rQPCR, quantitative real-time PCR; RA, rheumatoid arthritis; ROR, retinoic acid-related orphan receptor; WT, wild-type.

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; EAE, experimental autoimmune encephalomyelitis; γc, γ-chain; IBD, inflammatory bowel disease; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; qPCR, quantitative PCR; rQPCR, quantitative real-time PCR; RA, rheumatoid arthritis; ROR, retinoic acid-related orphan receptor; WT, wild-type.

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Crohn’s and ulcerative colitis patients, implying that IL-15 promotes the disease (29, 30). However, one study has shown that IL-15 protects against colitis in mice (31). Whether IL-15 controls IBD directly by regulating Th17 cells is unknown. In patients with RA, IL-15 is considered proinflammatory, and blocking of IL-15 using anti–IL-15 mAb (HuMax-IL-15) ameliorated symptoms (29, 30). However, one study has shown that IL-15 suppresses rather than promotes inflammation (34–36). IL-15 produced by astrocytes in MS lesions may promote CD8 T cell functions in the experimental autoimmune encephalomyelitis (EAE) model in mice (37). In contrast, two independent studies have shown that IL-15 mice exhibit heightened EAE, implying that IL-15 suppresses rather than promotes inflammation (38, 39). However, the mechanism by which IL-15 suppresses EAE and its role in Th17 responses has not been investigated.

It has recently been shown that IL-2 and IL-21, members of the same γc cytokine family, perform opposite functions in restraining and promoting Th17 cells, respectively (8, 40). IL-15, known to activate both STAT3 and STAT5 (28), may be inferred either to inhibit or promote Th17 cells. Thus, elucidating the exact mechanism of IL-15 action on Th17 cells is important and may shed new light on the mechanisms of autoimmunity and inflammatory conditions.

In this study, we identify a novel mechanism by which IL-15 mediates inhibition of Th17 differentiation in vitro and immunopathology in the EAE model. We find that both Th17 cells and APCs produce IL-15, which serves to control IL-17A production by activating STAT5 and inducing its binding to Il17a promoter in CD4+ T cells. Thus, our data show the mechanistic details of inhibitory action of IL-15 on IL-17A production in CD4+ T cells, both in vitro and during EAE inflammation in vivo.

Materials and Methods

Mice

C57BL/6 WT or Rag2−/−, Il15−/− mice and the appropriate control mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6, Il15−/− mice and CD45.1 mice in Rag2−/− background were purchased from Taconic Farms (Germantown, NY). All animal experiments were done at the National Institute of Allergy and Infectious Diseases under an approved protocol and in compliance with the guidelines of the National Institute of Allergy and Infectious Diseases Institutional Animal Care and Use Committee.

Reagents and Abs

Purified anti-CD3 (145-2C11), anti-CD28, anti-CD25 (3C7), CD4, CD25, IL-2, IL-4, and IFN-γ Abs were all purchased from BD Biosciences (San Diego, CA). Purified IL-17F, IL-17A, TNF-α, Foxp3, CD45.1, and CD45.2 Abs were purchased from eBioscience (San Diego, CA). PE-conjugated Abs were purchased from eBioscience (San Diego, CA). Fluorescein isothiocyanate (FITC)-labelled anti–IL-15 (20 ng/ml), anti–IL-17A 15 (15 µg/ml) and anti–IL-15 (15 µg/ml). Where indicated, APcs were added at a T cell/APC ratio of 1:1, and T cells were CFSE labeled to assess their proliferation. When indicated, cytokines, anti–IL-15 (20 µg/ml), and anti–IL-2 (10 µg/ml) were added at the beginning of stimulation of cocultures.

Quantitative PCR analyses

For quantitative PCR (qPCR) analyses of ROR-α, ROR-γ, Foxp3, and IL-17A mRNA, naïve CD T cells were stimulated in Th17 cultures as described earlier, and RNA was recovered using an RNAeasy Mini Kit (Qiagen). In some experiments, CD4 cells were first separated from APCs using CD90 magnetic beads (Miltenyi Biotech). DNase was added to the columns during RNA purification. cDNA was synthesized from total RNA using Superscript III Reverse Transcriptase plus random primers (Invitrogen) and purified on a MinElute spin column (Qiagen) following treatment with RNase A plus RNase H and extraction with phenol/chloroform. Five nanograms of cDNA was used in one reaction using a QuantiT Fast SYBR Green PCR Kit (Qiagen) in a real-time PCR machine (ABI7900HT; Applied Biosystems). All primers (Invitrogen) for PCR were designed to amplify a coding region within a single exon. The relative amount of cDNA of interest was estimated from its Ct value plotted on a standard curve acquired from the Ct values of a diluted series of genomic DNA. The relative quantification was normalized to the amount of ribosomal 18S RNA, assigning values of “1” or “100” to unstimulated control samples. The primer sequences used were as follows: 15S: 5′-AAGCGGCTCACATCAGAAA-3′, 5′-ATTCCAAATACAGGCGCTCG-3′; β-actin: 5′-GGTTAGGGTCAGGAGCT-3′, 5′-GGTCCACATGAGAGAAGGTG-3′; ROR-α: 5′-TTGCTGCTGGAATCAGGAAAGA-3′, 5′-TGCGAGTTAGTTGAAGTG-3′; ROR-γt: 5′-CAGTCTACATGACAGTG-3′, 5′-ATCTAAGAATGCTGCTGCG-3′, 5′-CAAGGCGTCAGAACCTTCTAG-3′, 5′-GCGTACAGGAGGAGAGGTG-3′; IL-17A: 5′-TCCAGAGGAGTCCCTGACTA-3′, 5′-AGCATCCTCTGACGCTTCA-3′; 5′-AATCCACCTGACACATGGA-3′, 5′-AGCGCTGTTTAATGTGGAGA-3′.

Chromatin immunoprecipitation–qPCR

Chromatin immunoprecipitation (ChiP) assays were performed using Th17 cells stimulated for 4 or 5 d in cultures. In brief, after being fixed in 1% formaldehyde, T cells were lysed for 10 min on ice. Chromatin was sheared by sonication in a Branson 2510 digital sonifier. Lysates equivalent to 107 cells were used for each immunoprecipitation. After being precleared with protein-A agarose beads (Upstate), cell lysates were immunoprecipitated overnight at 4°C with 2 μl STAT5A–STAT5B (PA-STAT5A and PA-STAT5B; R&D Systems). After washing and elution, cross-links were reversed for 4 h at 65°C. Eluted DNA was purified, and samples were analyzed by qPCR with TaqMan qPCR Mastermix (ABI, Roche). Primers and probes. Enrichment of chromatin was analyzed using the 7500 Fast Real-Time PCR instrument (Applied Biosystems). Data were normalized to input values and expressed as “% input.” Primer–probe sets used to detect the enrichment of STAT5 binding sites in Il17a locus were as follows: P2: forward 5′-CACCCTCACAGGACCAAGC-3′, reverse 5′-ATGTGTTGGCAGGTCTCGATC-3′; probe 2: 5′-CACCCACACAGCAGC-3′, 5′-GGATGAGGACGACCGTGGT-3′, reverse 5′-TCTCCACGTTGCTTCTCT-3′; probe 3: 5′-TGGCCAAATGTATGTGAGTA-3′; P4: forward 5′-CTACCGCTTCCCCACAGACA-3′, reverse 5′-GGCATAGTCCGAGGACAGT-3′, reverse 5′-TCTCTCCACGTTGCTTCTCTC-3′; probe 4: 5′-TACCAGACCCGCTTCCCACTT-3′, 5′-AGGAGCCAGACCATGAATC-3′, 5′-AGGAGCCAGACCATGAATC-3′, 5′-AGGAGCCAGACCATGAATC-3′.

Retroviruses transduction

Constitutively active STAT5–RES-GFP pmIGR and control GFP retroviral supernatants (gift from Dr. William E. Paul, National Institute of Allergy and Infectious Diseases) were used for transduction. Naive CD4+ T cells (2 × 106) were plated in one well of a 24-well plate and were stimulated using soluble 1 µg/ml anti-CD3 and 2 µg/ml anti-CD28 under Th0 conditions for 48 h. Retroviruses supernatants (0.5 ml) were added to the cells, replacing 0.5 ml of medium from the cultures, and incubated for 20 min in the incubator. The cells were spun at 2500 rpm for 1.5 h at room temperature with 8 µg/ml polybrene (Sigma). After 24 h, infected cells were
differentiated under Th17 cell conditions for 48 h and stained for intra-cellular cytokines.

**Tissue histology**

For immunocytochemical staining, colon tissues were washed with PBS, fixed with 10% formalin overnight, and suspended in 70% ethanol to prevent overfixation. Paraffin sectioning and H&E staining were performed by Histoserv.

**Intracellular staining of cytokines and STAT proteins and flow cytometry**

For single-cell staining, cells were cultured in Th17 conditions, washed in PBS, and fixed with Cytofix/Cytoperm kit (BD Biosciences). Before fixation, cocultures were restimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h, with brefeldin A (10 μg/ml) added in the last 2 h. For p-STAT3 and p-STAT5 staining, the cells were washed, fixed, and were stained with Phosflow staining kit from BD Biosciences using the manufacturer’s protocol. Data were acquired using Becton Dickinson FACS-Calibur cytometers and were analyzed using FlowJo 9.1 software.

**EAE in mice**

EAE experiments were performed using the myelin oligodendrocyte glycoprotein (MOG)CFA/Purtsessis toxin kit (no. EK0115) from Hooke’s Laboratories. EAE was induced in age- and sex-matched C57BL/6 or Il15−/− mice, at least five per group, according to the Hooke’s Laboratory protocol. EAE was scored as follows: 0, healthy; 1, flaccid tail; 1.5, flaccid tail and impaired righting reflex; 2, impaired righting reflex and hind limb weakness; 2.5, one hind leg paralyzed; 3, both hind legs paralyzed with residual mobility in both legs; 3.5, both hind legs completely paralyzed; 4, both hind legs completely paralyzed and beginning front limb paralysis; 5, moribund or death of the animal after preceding clinical disease.

**IBD induction by Th17 cell transfer in vivo**

CD4.5.1 Rag2−/− mice received 4 × 105 WT or Il15−/− CD4.2 Th17 cells by i.p. injection (in 100 μl PBS). CD4.5.2 CD25−CD44lowCD62Lhigh CD4+ naïve cells were cultured under Th17 polarizing conditions and were used as the source of Th17 cells for the infections. Histology scores were assigned as follows: 0, low level of leukocyte infiltration with infiltration seen in <10% of the section, no structural changes; 2, moderate leukocyte infiltration with infiltration seen in 10–25% of the section, crypt elongation and bowel wall thickening that does not extend beyond the mucosal layer; 3, high level of leukocyte infiltration seen in 25–50%, crypt elongation, infiltration beyond the mucosal layer, thickening of the bowel wall and mild loss of goblet cells; 4, marked degree of leukocyte infiltration seen in >50% of the section, elongated crypts, prominent loss of goblet cells and bowel-wall thickening; and 5, leukocyte infiltration seen in >70% and elongated and distorted crypts, bowel-wall thickening, significant or complete loss of goblet cells, and extensive ulcerations.

**Statistical analyses**

The p values were calculated by Student t test in Microsoft Excel software using unpaired, two-tailed distribution and two-sample equal variance parameters or unpaired t test and Mann–Whitney U test in Prism 4.0 (GraphPad Software).

**Results**

**IL-15 downregulates IL-17A in Th17 cells in vitro**

IL-15 is an activator of both STAT3 and STAT5 transcription factors, which are shown to perform opposing roles in regulating Th17 cells (28, 40). IL-15 may induce STAT3 activation to promote Th17 differentiation. Alternatively, by inducing STAT5, IL-15 may restrict Th17 cells. To test these possibilities, we stimulated CD4+CD44lowCD62LhighCD25− naïve T cells under Th17 polarizing conditions (8), consisting of 1 μg/ml soluble anti-CD3 Ab and APCs with 20 ng/ml IL-6, 2 ng/ml TGF-β, 5 μg/ml anti–IFN-γ, and 5 μg/ml anti–IL-4, in the presence of IL-15. As controls, we also tested other individual γc cytokines including IL-2, IL-21, and IL-7. Whereas IL-2 suppressed and IL-21 enhanced IL-17A production as shown by others (8, 40, 41), IL-7 moderately restrained IL-17A induction (data not shown). When IL-15 was added at the beginning of stimulation under Th17 polarizing conditions, IL-17A produced by CD4 cells was reduced in a dose-dependent manner, as assessed by ELISA and intracellular staining on day 4 after stimulation (Fig. 1A, Supplemental Fig. 1). The unstimulated cells showed no detectable IL-17A (Fig. 1A). However, the survival and proliferation, as assessed by propidium iodide staining and CFSE dilution, were unaffected by IL-15 (Fig. 1B). Because the effect of IL-15 was only moderate, we speculated that the inhibitory effect of endogenous IL-2 in the cultures could mask the effects of IL-15 (10, 40). Therefore, we stimulated the cells under Th17 polarizing conditions with soluble anti-CD3 and anti-CD28 for 5 d in the presence or absence of anti–IL-2 blocking Ab. In some experiments, anti–IL-15 blocking Ab was also used. As expected, blocking of IL-2 dramatically increased the frequency of IL-17A+ cells (Fig. 1C, 1D). In addition, blocking of IL-15 further increased the frequency of IL-17A producers. Also, when we added exogenous IL-15, it strikingly decreased the frequency of IL-17A–expressing cells (Fig. 1C, 1D). Considering the possibility that endogenous IL-15 produced in cultures could also contribute to IL-17A suppression, we used Il15−/− T cells in our Th17 conditions. On day 4 after stimulation, Il15−/− cells displayed increased frequency of IL-17A producers compared with WT T cells, and addition of IL-15 caused the frequency to drop to nearly the same level as for WT cells treated with IL-15 (Fig. 1E). ELISA data showing that IL-17A in the supernatants from Il15−/− cell cultures was increased compared with WT controls on day 2 and day 4 validated the flow cytometry data (Fig. 1F). No detectable levels of IL-17A were found in WT control and Il15−/−Th0 cells (data not shown). Taken together, these results reveal that IL-15 plays an important role in limiting IL-17A production in differentiating Th17 cells.

**Activated Th17 cells and APCs produce IL-15 in Th17 cultures**

Next, we sought clearly to delineate the source(s) of IL-15 in Th17 cultures. Although APCs are likely sources of IL-15, we speculated that like IL-2, endogenous IL-15 produced by Th17 cells themselves could be an autocrine negative feedback regulator restricting IL-17 production. This tenet was based on our finding that Th17 cells exposed to anti–IL-15 blocking Ab had increased IL-17A production in the absence of APCs (Fig. 1C, 1D). Although it has been reported previously that activated CD4 cells express IL-15, they are not considered to be an immunologically important source of IL-15 (21, 22, 24). Therefore, we separated CD4 cells from Th0 or Th17 cultures stimulated with soluble anti-CD3 and APCs and measured steady-state IL-15 mRNA. As previously reported, on day 3 after stimulation, we detected IL-15 mRNA in activated Th0 cells (22). However, we observed higher levels of steady-state IL-15 mRNA in Th17 cells (Fig. 2A). Furthermore, quantitative real-time PCR (qRT-PCR) confirmed higher levels of IL-15 mRNA in flow cytometrically sorted naïve cells that were differentiated under Th17 conditions (Fig. 2B). As a positive control, we measured IL-15 mRNA in macrophages that were either left unstimulated or activated with LPS and Pam3CSK4 and found that the activated macrophages expressed significantly higher levels of IL-15 compared with T cells and unstimulated macrophages (24, 42). We validated the results by performing an ELISA to detect IL-15 protein levels in Th17–APC cocultures. We quantified IL-15 protein levels in cocultures where WT or Il15−/− Th17 cells were stimulated with WT APCs. We also stimulated WT Th17 cells with either WT or Il15−/− APCs. We observed obvious reduction of IL-15 expression in the cocultures where either the Th17 cells or APCs were derived from IL-15–deficient mice (Fig. 2C). Thus, both T cells and APCs contribute to IL-15 in Th17 cultures stimulated with anti-CD3 and APCs. To test whether Th17 cells produce IL-15 independently of APCs, we stimulated WT or...
**FIGURE 1.** IL-15 decreases the frequency of IL-17A+ cells and IL-17A production in Th17 cultures. (A) Naive T cells were not stimulated (Unstim.) or were cultured under Th0 or Th17 conditions along with or without 20 ng/ml of recombinant mouse IL-15 for 4 d. IL-17A levels were assessed in culture supernatants by ELISA (left). Intracellular cytokine staining was performed in PMA/ionomycin restimulated cells. Data from flow cytometric analyses show the percentage of IL-17A+ cells (right). (B) Cells were stimulated as in (A). The percentage of viable cells (propidium iodide-negative cells, left) and proliferation (CFSE dilution, right) were assessed by flow cytometry. (C) Naive CD4 cells were stimulated under Th17 skewing conditions using anti-CD3 and anti-CD28, with or without anti–IL-2 blocking Ab (10 μg/ml) for 5 d. Anti–IL-15 Ab and IL-15 were added at 50 μg/ml and 20 ng/ml, respectively. Gates were drawn based on “Unstim.” and “Th0” controls (not shown). (D) Cells were stimulated as in (C). The frequency of IL-17A+ cells on day 4 from three independent experiments is depicted. Data points ±SEM are plotted. (E) WT naive CD4 cells were stimulated with WT APCs, and IL15−/− naive cells were stimulated with IL15−/− APCs. They were stained for IL-17A after 3 d of stimulation (gated on CD4 cells). (F) ELISA quantification of IL-17A in the supernatants of cultures stimulated as in (E) under Th17 conditions. The results are representative of at least three independent experiments.

**IL-15 limits IL-17A and IL-17F production independently of IFN-γ, Foxp3, and ROR-γt**

Because both APCs and Th17 CD4 cells produce IL-17A, we examined the function of autocrine IL-15 in Th17 cells excluding APC effects. Therefore, we stimulated WT or IL15−/− naive CD4 cells with anti-CD3 and anti-CD28 in the absence of APCs under Th17 conditions for 4 d. As a negative control, we also stimulated naive CD4 T cells under neutral conditions (Th0). Intracellular staining of IL-17A revealed that the frequency of IL-17A producers was increased in IL15−/− T cells compared with WT T cells (Fig. 3A). Th0 cells showed no IL-17A production whether or not IL15 gene was intact. Addition of exogenous IL-15 independently caused the frequency of IL-17A producers to drop, especially in IL15−/− Th17 cells (Fig. 3A). Also, ELISA data showed that supernatants from IL15−/− cultures contained increased levels of IL-17A compared with those of WT cells. Moreover, exogenous IL-15 significantly decreased IL-17A (Fig. 3B). We confirmed these findings by comparing WT cells with IL-15Rα (IL15r−/−) cells, showing that the latter had a higher frequency of IL-17A producers under Th17-inducing conditions (Fig. 3C, 3D). As expected, because of the lack of receptor expression in IL15r−/− cells, 20 ng/ml of exogenous IL-15 did not affect the increased production of IL-17A, indicating that the observed suppression was specific (Fig. 3C). Both WT and IL15r−/− cultures polarized toward Th17 lineage and did not deviate to induced regulatory T cell or Th1 lineage, as we found very low frequencies of Foxp3-expressing and IFN-γ-expressing cells, which was comparable in WT and IL15r−/− cells (Supplemental Fig. 2). Although an increase in IL-17F producers was also observed in IL15r−/− CD4 cells, the frequency of IL-22 and TNF-α producers was measurably less compared with WT cells, indicating that certain hallmark cytokines of Th17 cells were not downregulated by IL-15. The activation status was apparently the same in both the cell types, as they were comparable in terms of CD25 expression (Supplemental Fig. 2). GM-CSF was recently found to be produced by Th17 cells, when stimulated with IL-23 in vitro and in vivo (43). Therefore, we tested for GM-CSF production in our WT and IL15r−/− cell cultures (Supplemental
IL-15 limits IL-17A production by activating STAT5

Th17 cells require TGF-β–mediated SMAD signaling and IL-6–mediated STAT3 activation for differentiation (7). We first examined the levels of the activated (phosphorylated) SMAD2 (p-SMAD2) and p-SMAD3 as a measure of TGF-β signaling (11). Upon TGF-β stimulation, Il15r""'"' and WT cells displayed equivalent levels of p-SMAD2 and p-SMAD3 (data not shown). Similarly, p-STAT3 levels were also the same in WT and Il15r""'"' cells at different time points after stimulation in the presence of 100 ng/ml IL-15 or IL-6 (Fig. 5A, upper panel, data not shown). The basal levels of p-STAT3 were high in both of the cell types, possibly due to constitutive IL-6 and IL-21 signaling in these cultures. These data suggest that IL-15 does not affect TGF-β and IL-6 signals during Th17 differentiation.

p-STAT3 binds to multiple DNA recognition sites at the Il17a locus and promotes IL-17A transcription (10). However, its effects are counterregulated by the fact that IL-2 limits IL-17A production through a STAT5-dependent mechanism (10). Activated STAT5 (phosphorylated form of STAT5; p-STAT5) competes with p-STAT3 for the same cognate binding sites and inhibits Il17a transcription and to a lesser extent Il17f transcription. Notably, we found that the p-STAT5 expression was markedly reduced in Il15r""'"' cells compared with the WT cells, indicating that IL-15 promoted inhibitory STAT5 phosphorylation in Th17 cells (Fig. 5A, lower panel). To examine further the effects of IL-15 on Il17f transcription, we stimulated WT or Il15r""'"' CD4 cells under Th17-inducing conditions and determined the binding of STAT5 in Il17af–Il17f locus by ChIP and qRT-PCR. Previous studies using ChIP followed by massive parallel sequencing have mapped STAT5 binding sites to the Il17a–Il17f locus (10). We chose to examine the enrichment of three strong STAT5 binding sites (P2, P3, and P4) in the Il17f locus, as described previously (10). We also designed the primers for two nonbinding sites, NB1 and NB2. We found that the STAT5 binding in the Il17a–Il17f locus was higher in WT cells than in Il15r""'"' cells on day 5 after stimulation (Supplemental Fig. 4). To validate further the effects of IL-15 in WT cells from the same mice, we cultured them alone, with anti-IL-15 blocking Ab or with exogenous IL-15 in Th17-inducing conditions and performed the ChIP-qPCR on day 5. We found that blocking of IL-15 decreased the binding of STAT5, and exogenous IL-15 dramatically increased the binding at all three sites in the il17f locus (Fig. 5B). STAT5 nonbinding sites (NB1, NB2) were detected at very low and equivalent levels. Thus, IL-15 promoted direct binding of STAT5 in the il17af–il17f locus and may have inhibited il17 transcription by displacing STAT3 proteins. To support the hypothesis that IL-15–mediated STAT5 signaling indeed limited IL-17A production, we transduced WT and Il15r""'"' CD4 cells with a retrovirus constitutively expressing active STAT5 and GFP. Il15r""'"' cells in both untransduced and control GFP virus cultures showed increased frequencies of IL-17A""'"' cells compared with their WT counterparts (Fig. 5C). By contrast, transduction with constitutively active STAT5 reduced IL-17A production both in WT and Il15r""'"' cells (Fig. 5C). Because STAT3 upregulates Bcl-2 expression in T cells, we tested for Bcl-2 expression. We found that cells overexpressing STAT5 indeed upregulated Bcl-2 expression, confirming the action of
STAT5 (data not shown). These results show that STAT5 signaling inhibits IL-17A production in differentiating Th17 cells.

**IL-15 regulates the severity of EAE and limits IL-17A production in CD4 cells in mice**

IL-17A plays an important role in the development of EAE in mice (44). Therefore, we used the EAE model to examine the effect of IL-15 on Th17 cells in a disease setting and to validate the regulatory role of IL-15 (38). We induced EAE in WT and Il15^−/− mice using MOG (35–55) peptide and pertussis toxin. In line with the previous studies, we found that Il15^−/− mice exhibited earlier onset of EAE with higher disease scores compared with WT mice (38, 39) (Fig. 6A). This correlated with increased serum IL-17A in Il15^−/− mice (data not shown). However, we found that serum

**FIGURE 3.** Genetic deficiency of IL-15 or IL-15R increases IL-17A production in Th17 cultures. (A) Flow cytometric histograms of IL-17A expression of naive WT (upper panel) or Il15^−/− (lower panel) CD4 cells stimulated using anti-CD3 and anti-CD28 under nonpolarizing conditions (Th0) or Th17 conditions for 96 h with or without 20 ng/ml of IL-15. (B) ELISA quantification of IL-17A in supernatants from cells that were stimulated as in (A) under Th17 conditions (white bars) in the absence or presence of IL-15 (black bars). (C) Flow cytometric histograms of IL-17A expression of naive WT (upper panels) or Il15r^−/− (lower panels) CD4 cells stimulated under Th0 or Th17 conditions as in (A). (D) ELISA quantification of IL-17A in supernatants from WT (white bars) or Il15r^−/− (black bars) Th17 cells that were stimulated as in (C) at different days after stimulation.

**FIGURE 4.** IL-15 limits IL-17A expression independently of ROR-γt expression. (A) WT or Il15r^−/− cells were stimulated as in Fig. 3C. RNA was isolated at indicated times of stimulation to assess the il17a, il17f, rorα, or rorγt RNA levels (indicated by y-axes). These data are representation of two independent experiments with triplicate samples. (B) WT or Il15r^−/− cells stimulated for 48 h as in (A) were stained with isotype control Ab (Stain control) or anti-ROR-γt Ab (ROR-γt). Shown is the fraction of cells in the indicated gate. Gates were drawn based on “Unstained” controls (not shown). These data represent three independent experiments.
GM-CSF levels were comparable in WT and \textit{Il15} \textsuperscript{2/2} mice induced with EAE, showing that GM-CSF did not exacerbate EAE in \textit{Il15} \textsuperscript{2/2} mice (data not shown). On day 9 after EAE induction, we isolated CD4 single-cell suspensions from the spleen as well as axillary and inguinal lymph nodes to assess cytokine production. Flow cytometry analyses revealed that CD4 \textsuperscript{+} T cells in lymph nodes of \textit{Il15} \textsuperscript{2/2} mice contained a small but clearly increased population of IL-17A–producing T cells compared with WT mice (Fig. 6B, x-axis), 6C). A slightly higher frequency of IL-17A+ cells was seen in splenic CD4 cells in \textit{Il15} \textsuperscript{2/2} mice compared with WT mice, but the differences were not significant because of low cell numbers (Fig. 6C). Notably, the increased frequency of IL-17A+ cells in \textit{Il15} \textsuperscript{2/2} mice correlated with a decreased frequency of IFN-\gamma+ CD4 cells (Fig. 6B, y-axis). Thus, the removal of IL-15 augmented the Th17 response in vivo, and that was associated with a higher susceptibility to EAE. Although the direct effect of IL-15 on Th17 cells was not investigated, it has been previously shown that injection of IL-15 can suppress the inflammation in EAE-induced mice (39). Therefore, we examined the IL-17A production in CD4 cells upon administration of IL-15 in \textit{Il15} \textsuperscript{2/2} mice after the induction of EAE. Similar to previous findings, we also found that the administration of two doses of 200 ng IL-15 in \textit{Il15} \textsuperscript{2/2} mice suppressed EAE inflammation (data not shown) (39). More importantly, we found that the suppression of EAE by IL-15 correlated with lower frequencies of IL-17A–producing CD4 \textsuperscript{+} T cells in the EAE-induced mice (Fig. 6D).

Finally, to confirm that increased IL-17A contributed to more severe EAE in \textit{Il15} \textsuperscript{2/2} mice, we blocked IL-17A in vivo by administering anti–IL-17A blocking Ab on days 2, 4, 7 and 9 after EAE induction. We observed that the anti–IL-17–treated \textit{Il15} \textsuperscript{2/2} mice presented with significantly reduced EAE inflammation scores, comparable to the WT levels (Fig. 6E). This finding confirms that the increased IL-17A indeed contributed to more severe inflammation scores in \textit{Il15} \textsuperscript{2/2} mice. Taken together, these results show that IL-15 controls EAE by restraining IL-17A production in CD4 \textsuperscript{+} T cells.

**Discussion**

In this study, we have shown that IL-15 induces the activation of STAT5 and restricts Th17 differentiation in vitro and in vivo. Our results from in vitro experiments demonstrate that IL-15 by itself modulated IL-17A production consistently, but only moderately, in normal TH17 cultures. This was due to the presence of IL-2 produced by TH17 cells, wherein IL-2 could independently control IL-17A production. When we removed the effect of IL-2 by using anti–IL-2 Ab, the ability of IL-15 to suppress IL-17A production was much more evident in vitro. In contrast, blocking of IL-15 or genetic deficiency of either IL-15 or IL-15R all increased the frequency of IL-17A \textsuperscript{+} cells in TH17 differentiation conditions. We
found only moderate increases in the IL-17A⁺ population in cells deficient in IL-15 signaling compared with WT cells. Nevertheless, these data together with the observations of increased levels of IL-17 mRNA, and supernatant IL-17A protein, from the cultured II15⁻/⁻ or II15r⁻/⁻ cells support our conclusion that IL-15 plays an important role in control of Th17 differentiation. The data in this report indicate that IL-15 may function in conjunction with IL-2 to limit Th17 cells. We previously found that IL-2 restrains IL-17A only during the early induction period and may play a less prominent role at later time points of differentiation or in the maintenance of Th17 cells (45). Hence, IL-15 may play a more prominent role in restricting Th17 cells late in the immune response or when CD4 cells produce non-optimal levels of IL-2. The evidence suggests that IL-2 is a stronger negative regulator of Th17 cells, whereas IL-15 may be involved in fine-tuning Th17 inflammatory responses at late phase of the immune response. This tenet is based on the fact that whereas II2⁻/⁻ and Stat5⁻/⁻ mice exhibit widespread autoimmunity that associates with increased Th17 differentiation, II15⁻/⁻ mice do not show spontaneous autoimmune symptoms. It would be interesting to analyze II2⁻/⁻ and II15⁻/⁻ double knockout mice. It is noteworthy that in vivo, the major source of IL-2 is CD4 cells, whereas IL-15 is produced by a variety of cell types including activated CD4 T cells, monocytes, skeletal muscle cells, and keratinocytes (21–24). Thus, IL-2 provides only an autocrine regulatory loop, whereas IL-15 may facilitate broader paracrine regulation by a host of nonlymphoid cells to limit Th17 differentiation in certain anatomical niches. Moreover, Th17 cells themselves also produce higher levels of IL-15 compared with cells that are activated under Th0 conditions. Although there are a few reports showing activated CD4 cells produce IL-15 (7, 24), the general belief is that APCs and not T cells are the predominant source of IL-15 in an adaptive immune response. In this study, we show that CD4 cells, when activated under Th17 conditions, produced biologically significant levels of IL-15, which limits IL-17A production. IL-15 produced by APCs also limits IL-17A production in a paracrine manner in cultures (data not shown). Thus, IL-15 takes on a new role in directly limiting Th17 functions. At the molecular level, IL-15 inhibits II17 expression independently of ROR-γt. Although IL-15 can activate STAT3, we found that STAT3 phosphorylation was comparable in WT and II15r⁻/⁻ Th17 cells (28). However, IL-15 induced STAT5 phosphorylation in WT cells, and this is impaired in II15r⁻/⁻ cells (28). Notably, we found that excess of IL-15 (100 ng/ml) can cause some STAT5 phosphorylation also in II15r⁻/⁻ cells (Fig. 5A, lower panel). We speculate that this could be due to the effect of IL-15 bypassing the α-receptor at excess concentrations (27). Importantly, IL-15 clearly induced STAT5 phosphorylation, which
is required for the inhibitory effect on Th17 differentiation. This is illustrated by the results from retroviral transduction of active STAT5 in Th17 cells. Our ChIP data also indicate that IL-15 has a direct effect on iIl17a expression by enhancing the binding of inhibitory STAT5 at different sites in the iIl17a locus. Thus, IL-15 downregulated iIl17a expression by inducing STAT5 binding and likely displacing STAT3 proteins at the iIl17a locus (10). However, to gain insights into the mechanistic details of STAT5-mediated iIl17a repression, further investigation is needed.

Although IL-15 promotes inflammation in patients with RA, the direct effect of IL-15 in inducing Th17 cells and in promoting inflammation in vivo is unclear (13, 32). In RA, IL-15 promotes synovial fluid T cell activation by upregulating the expression of CD69 and CD154 and promoting the survival and activation of APCs, synovial neutrophils, NK cells, fibroblasts, and vascular endothelial cells (46–48). Although Th17 cells are present in the synovial compartments in RA, IFN-γ+ Th1 rather than Th17 cells predominate in the synovial joints of RA patients (49, 50). Thus, IL-15 could worsen the disease probably by promoting the proliferation of memory T cells (Th1 cells) or by a completely different mechanism, such as promoting RANTES5 in myeloid cells independently of its effects on T cells in synovial fluid (51). Recent studies clearly show that IL-15 signals differently in myeloid immune cells and lymphocytes, which may also account for different functional outcomes. The details of these different signaling mechanisms will provide insights into biology and therapeutic applications of IL-15 (51, 52). Anti–IL-15 Ab (HuMax-IL-15) ameliorates disease activity in RA patients, but it was associated with decreased release of IFN-γ (Th1) and CD69 expression by synovial mononuclear cells and was likely independent of Th17 cells (32, 33). However, Halvorsen et al. (50) showed that addition of exogenous IL-15 resulted in increased IL-17A as well as IFN-γ, IL-6, IL-10, IL-22, and TNF-α in CD4SRO+ memory synovial T cell culture supernatants. This study showing the effect of IL-15 in promoting Th17 cells is in apparent contradiction with our study. However, they have not clarified whether IL-15 increased the survival and proliferation of differentiated memory T cells, which might have contributed to the overall increases in cytokines in the supernatants, or if it directly induced IL-17A in naive CD4 cells (15). We support the hypothesis that similar to IL-2, IL-15 likely increases the proliferation and cytokine production of differentiated T cells in general. Also, the differences between human and mice may also be responsible for the differences (53). On the basis of our current findings, it seems unlikely that the effect is due to direct induction of IL-17A in naive cells, but we have not examined synovial T cells to address this question directly. Another study showed that mast cells and necessarily not the T cells are the main source of IL-17 in the inflamed joint in the RA condition (54). Therefore, distinguishing between the effects of IL-15 on Th17 cells and mast cells from the synovial tissue is important and needs further investigation. Whether IL-15 can also promote Th17 differentiation in certain contexts, depending on the cell types and their STAT3/STAT5 ratio and their binding to iIl17a locus, remains a possibility to be investigated.

Consistent with the hypothesis that IL-15 limits Th17 cells, IL15−/− mice showed heightened susceptibility to EAE, which is also in agreement with previous studies (38, 39). Whereas those studies did not address the mechanism of IL-15–mediated suppression of EAE, we now show that IL-15 limits Th17 cells in EAE-induced mice. Also in our EAE experiments, we found that IL-15 mediated regulation of Th17 cells only in the draining lymph nodes of the hind limbs and forelimbs but not in the spleen. The site-specific functions of IL-15 are interesting and need to be investigated in further detail. Importantly, we found that IL-15 decreased EAE susceptibility not by reducing IFN-γ+ CD4 cells. Actually, IL-15 enhanced the frequency of IFN-γ+ CD4 cells while reducing IL-17A+ CD4 cells. Therefore, altered IL-17A and not IFN-γ increased the susceptibility of IL15−/− mice to EAE. Our experiments block IL-17A in IL15−/− mice, which showed less severe EAE, further confirm the role of increased IL-17A in their exacerbated EAE. Taken together, our results strongly support the notion that IL-15 is not only involved in promoting the development of certain innate cells and adaptive memory cells but also controls inflammation involving Th17 responses. These findings shed new light on the increase in the serum IL-15 in MS and IBD patients, which could be indicative of a compensatory mechanism to ameliorate the Th17-mediated mediated. Clinical trials using anti–IL-15 Ab in inflammatory diseases other than RA are not encouraging, implying that IL-15 may not be responsible for the proinflammatory effects in those conditions (47, 55). Our data suggest a cautionary note that targeting and blocking of IL-15 in suppressing Th17 responses and the associated autoimmune diseases.

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Disclosures

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References


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