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CD4 effector T cell differentiation is controlled by IL-15 that is expressed and presented *in trans*

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Abstract

T cells are both producers and consumers of cytokines, and autocrine cytokine signaling plays a critical role in T cell immunity. IL-15 is a homeostatic cytokine for T cells that also controls inflammatory immune responses. An autocrine role of T cell-derived IL-15, however, remains unclear. Here we examined IL-15 expression and signaling upon effector T cell differentiation in mice, and, surprisingly, found that CD4 T cells did not express IL-15. CD4 T cells lacked *Il15* gene reporter activity, did not contain IL-15 transcripts, and did not produce IL-15R α , the proprietary IL-15 receptor required for IL-15 *trans*-presentation. Moreover, IL-15 failed to inhibit Th17 cell differentiation and failed to generate Foxp3⁺ Treg cells *in vitro*. IL-2, which utilizes the same IL-2R β / γ c receptor complex, however, successfully did so. Exogenous IL-15 only exerted bioactivity to control T cell differentiation when *trans*-presented by IL-15R α . Consequently, IL-15R α -bound IL-15, but not free IL-15, suppressed Th17 cell differentiation and induced Treg cell generation. Collectively, these results reveal the absence of an IL-15 autocrine loop in CD4 T cells and strongly suggest that IL-15 *trans*-presentation by non-CD4 T cells is the primary mechanism via which IL-15 controls CD4 effector T cell differentiation.

Keywords

common γ -chain; interleukin-15 receptor; Th17 cells; *trans*-presentation; Treg cells

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Disclosures

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1. Introduction

A primary function of CD4 effector T cells is the production of cytokines [1]. Depending on the cytokines they express, CD4 T cells can be classified into different subsets with distinct functions [2]. In a simplified version, IFN γ -producing CD4 T cells are known as Th1 cells, whereas IL-4- and IL-5-producing CD4 T cells are referred to as Th2 cells [3]. CD4 T cells that express the pro-inflammatory cytokine IL-17 have been designated as Th17 cells [4, 5], and they recently came into the limelight because of their role in exacerbating autoimmunity [6]. While T cell-derived cytokines usually act on other T cells or lymphocytes in a paracrine fashion, cytokines also feedback onto CD4 T cells themselves. Thus, T cell immunity is orchestrated by a complex network of both *cis*- and *trans*-acting cytokines [2].

IL-15 is a critical cytokine in T cell immunity [7, 8]. Its importance is illustrated, among others, in IL-15-deficient mice which display severe defects in CD8 effector memory cell generation and T cell effector function [9, 10]. On the other hand, IL-15-deficiency reportedly also exacerbates autoimmune neuroinflammation [11–14], so that IL-15's involvement in inflammatory diseases is evident, but its exact role as a pro- or anti-inflammatory agent remains to be clarified. Also, the cellular source of IL-15 is not yet fully understood, and both T cell-intrinsic and -extrinsic expression of IL-15 has been reported. On one hand, IL-15 is proposed to be expressed by stromal cells, myeloid cells, and medullary thymic epithelial cells, but not by T cells themselves [15, 16]. On the other hand, earlier studies identified IL-15 mRNA transcripts in human T cells [17], so that *ex vivo* isolated human T cells were proposed to survive and proliferate *in vitro* by an autocrine loop of IL-15 expression and signaling [18]. IL-15 gene transcription was also reported in murine T cells [12, 19]. Development of T cell leukemia in IL-15-transgenic mice, for example, was shown to be induced through autocrine IL-15 expression and signaling in CD8 T cells [20]. In the same vein, CD4 T cells from IL-15-deficient mice exhibited dysregulated effector functions, which supported an autocrine role for IL-15 in effector T cell differentiation [12, 17]. Thus, CD4 T cell-derived IL-15 is potentially an important mechanism that controls CD4 T helper function. However, whether this is indeed the case *in vivo* is unclear, and what consequences it might have on effector T cell differentiation remains to be addressed.

Assessing this question is particularly important because IL-15 utilizes the same IL-2R β / γ c cytokine receptor complex as IL-2 for ligand binding and signaling [10]. IL-2 is produced by activated T cells and plays critical and non-redundant roles in many aspects of T cell biology, including Th1/Th2 effector T cell differentiation, Foxp3⁺ T regulatory cell generation, as well as promoting CD8 cytolytic T cell activities [21]. Thus, competing for the same receptor complex with IL-15 could diminish IL-2R β / γ c availability for IL-2 and result in impaired IL-2 signaling and downstream effector function. IL-2 expression is terminated by IL-2 receptor signaling as a negative regulatory feedback mechanism [22]. Because IL-15 also activates IL-2R β / γ c signaling, IL-15 could induce premature termination of IL-2 expression and interfere with IL-2-dependent T cell responses. Under such a scenario, autocrine IL-15 would be detrimental for IL-2 expression. Moreover, IL-2R β / γ c signaling suppresses pro-inflammatory IL-17 production [23], so that IL-2 receptor signals are anti-inflammatory by inhibiting Th17 cell differentiation [24]. Whether autocrine IL-15 also constrains Th17 polarization is an interesting issue, because it could

explain the anti-inflammatory effects of IL-15 [25]. A role for CD4 T cell-derived IL-15 in Th17 cell differentiation had been previously proposed [12]. However, the molecular mechanisms that drive IL-15 expression in CD4 T cells and its potential effects on other helper T cell subsets remain unresolved.

Here, we assessed IL-15 expression and signaling in naïve and effector CD4 T cells. Surprisingly, and in contrast to previous studies [12, 26], *Il15* gene reporter mice analysis and quantitative real-time RT-PCR results failed to provide evidence for IL-15 expression in CD4 T cells. Moreover, CD4 T cells did not express IL-15R α , which is the high-affinity receptor required for IL-15 *trans*-presentation to IL-2R β / γ c expressing target cells [27, 28], and which is required for optimal IL-15 signaling *in cis* [20, 29, 30]. Based on these and other findings, we propose that it is unlikely that IL-15 exerts autocrine effects on CD4 effector T cells. Moreover, we found that recombinant IL-15 alone lacked bioactivity, as it was unable to drive Foxp3⁺ Treg cell generation or suppress Th17 cell differentiation *in vitro*, even as it induced robust STAT5 phosphorylation in activated CD4 T cells. IL-15 complexed with IL-15R α , however, effectively suppressed generation of pro-inflammatory Th17 cells and induced Foxp3⁺ Treg cell differentiation. These results suggested that IL-15 *trans*-presented by IL-15R α is the biologically relevant form of IL-15. Thus, while we exclude an autocrine role of IL-15, this study documents IL-15 as potent regulator of CD4 effector cell differentiation whose bioactivity is controlled and constrained by IL-15R α *trans*-presentation.

2. Materials and Methods

2.1. Animals

C57BL/6 (WT) mice were obtained from the Jackson Laboratory or Charles River. EmGFP/IL-15 reporter mice were provided by Leo Lefrançois [19]. *Il15*^{-/-} and *Il15ra*^{-/-} mice were previously described [9, 31]. IL-2R β ^{Tg} mice were generated by cloning a murine *Il2rb* cDNA under the control of a human CD2 mini-cassette and injection into fertilized B6 oocytes. All animal experiments were approved by the Animal Care and Use Committee (ACUC) of the National Cancer Institute, NIH. Mice were cared for in accordance with NIH guidelines.

2.2. Flow cytometry

Data were analyzed on the FACSCalibur, FACSARIA, or LSRII (BD) using software designed by the Division of Computer Research and Technology at the NIH. Live cells were gated using forward scatter exclusion of dead cells and staining with propidium iodide. *NKT* cells were detected using PBS-57-loaded CD1d tetramer staining. For intracellular cytokine staining, cells were first surface stained, fixed with IC fixation buffer, and then permeabilized using the intracellular permeabilization buffer (eBioscience), according to the manufacturer's instructions. pSTAT5 expression was determined following 30 min of stimulation with IL-2, IL-15 or IL-15/IL15R α and methanol/acetone fixation and intracellular staining as previously described [32]. For Foxp3 transcription factor staining, *in vitro* differentiated cells were fixed and permeabilized using a Foxp3 intracellular staining kit, following the manufacturer's instruction (eBioscience).

2.3. Antibodies

The antibodies with following specificities were used; CD4 (GK1.5, Tonbo), CD8 (53.67, Tonbo), CD11c (HL3, BD), CD11b (M1/70, eBioscience), TCR β (H57-597, BD), NK1.1 (PK136, eBioscience), $\gamma\delta$ TCR (GL3, Biolegend), CD44 (IM7, eBioscience), CD122 (TM β 1, BD), CD62L (MEL-14, eBioscience), IL-15R α (DNT15R α , eBioscience), IL-2R α (3C7, Biolegend), γc (4G3, BD), IL-17 (eBio17B4, eBioscience), IFN γ (XMG1.2, Biolegend), pSTAT5 (clone 47, BD), Foxp3 (MF23, BD). Fluorochrome-conjugated CD1d tetramers loaded with PBS-57 were obtained from the NIH tetramer facility (Emory University, Atlanta, GA).

2.4. *In vitro* T cell differentiation

Naïve CD4 T cells were electronically sorted by gating on CD44^{lo} cells. In case of T helper cell differentiation of *Il15*^{-/-} CD4 T cells, additional parameters such as CD62L⁺, CD25⁻, CD1d tetramer⁻ and CD69⁻ were used to define naïve CD4 T cells. Sorted cells were stimulated with plate-bound anti-CD3/CD28 antibodies and cultured under skewing conditions for 5 days. Th1 condition: recombinant mouse IL-12 (10 ng/ml) and anti-IL-4 antibodies (10 μ g/ml, Biolegend). Th2 condition: anti-IFN γ antibodies (10 μ g/ml), and recombinant mouse IL-4 (20 ng/ml, Peprotech); Th17 conditions: anti-IL-4 (10 μ g/ml) and anti-IFN γ antibodies (10 μ g/ml), recombinant human TGF β (5 ng/ml, Peprotech) and recombinant mouse IL-6 (30 ng/ml, Peprotech). Where indicated, recombinant human IL-15 (25 ng/ml, Peprotech), recombinant human IL-2 (25 ng/ml, Peprotech), or recombinant mouse IL-15/IL-15R α complex (100 ng/ml, eBioscience) was added to the cell cultures. For cytokine production analysis, cultures were stimulated for 3 hours with PMA/ionomycin (Sigma) and Brefeldin A (eBioscience) for 3 hours, followed by intracellular staining and flow cytometric analysis.

2.5. Quantification of soluble IL-15R α proteins

Naïve CD4 T cells were enriched from the LN of B6 mice and stimulated with 1 μ g/ml plate-bound anti-CD3/CD28 antibodies for 5 days under Th1 or Th17 skewing conditions. Culture supernatants were harvested and soluble IL-15R α contents were determined using a mouse IL-15R α DuoSet ELISA kit (R&D Systems).

2.6. Quantitative real time PCR

Total RNA was isolated using RNeasy (Qiagen) or Nucleospin (Machery-Nagel). RNA was reverse transcribed into cDNA by oligo(dT) priming with the QuantiTect reverse transcription kit (Qiagen). Quantitative RT-PCR (qRT-PCR) was performed with the QuantiTect SYBR green detection system (Qiagen) and analyzed on an ABI PRISM 7900HT (Life Technologies) as previously described [33]. The following primers were used for qRT-PCR: IL-15 (5'-AATCCACCTTGACACATGGC-3'; 5'-AGGCTGGTTATCTGCTGACA-3'), ROR γ t (5'-GCTGTCCTGGGCTACCCTACTGA-3'; 5'-TGCGGCGGAAGAAGCCCTTG), RPL13 (5'-CGAGGCATGCTGCCCAACAA-3'; 5'-AGCAGGGACCACCATCCGCT-3') and T-bet (5'-AGCTGCGCGCCTATCCAACG; 5'-TCCTTAGGGCTGGGGCGACG-3').

2.7. Statistical analysis

Statistical differences were calculated using a Student's two-tailed *t*-test. *P* values of less than 0.05 were considered statistically significant. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, NS not significant. All calculations were performed using GraphPad Prism.

3. Results

3.1. Lack of evidence for IL-15 expression in naïve and effector CD4 T cells

To identify potential autocrine effects of IL-15 in CD4 T cells, we utilized emerald GFP (EmGFP)-*Il15* reporter mice to map IL-15 gene expression [19]. While EmGFP reporter proteins were robustly expressed in CD8 LN T cells and other hematopoietic origin cells (Fig. 1A, Supplemental Fig. 1A, B), we failed to detect EmGFP-*Il15* reporter expression in CD4 LN T cells (Fig. 1A). Detailed analysis of CD4 T cell subpopulations further confirmed that EmGFP expression was absent in naïve (CD44^{lo}CD62L⁺) CD4 T cells and not detectable in most of CD44⁺CD62L⁻ effector memory phenotype CD4 T cells (Fig. 1B **top**). In contrast, we found that both naïve (CD44^{lo}IL-2Rβ^{lo}) and memory (CD44⁺IL-2Rβ⁺) CD8 T cells expressed high levels of EmGFP (Fig. 1B **bottom**). These results suggest that CD4 T cells in general, and naïve resting CD4 T cells in particular, are unlikely cellular sources of IL-15.

Next, we wished to examine if activated CD4 T cells induce IL-15 expression, and thus stimulated lymph node (LN) T cells overnight with plate-bound anti-CD3/CD28 antibodies. While TCR stimulation induced expression of the activation marker CD69 on CD4 T cells, we did not observe increase in IL-15 reporter expression (Supplemental Fig. 1C). Activated CD8 T cells also did not upregulate IL-15 reporter expression (Supplemental Fig. 1D), indicating that IL-15 expression in T cells is developmentally set and presumably controlled independently of their activation status. To further address if IL-15 expression is induced in effector T cells, we examined IL-15 reporter activity in activated CD4 T cells that have been polarized *in vitro* under Th1 or Th17 skewing conditions. Strikingly, and in marked contrast to freshly isolated NK cells, IL-15 reporter expression was virtually absent in CD4 effector T cells (Fig. 1C). In agreement with the lack of EmGFP reporter protein expression, we also did not detect discernable amounts of IL-15 mRNA transcript in Th1 and Th17 cells (Fig. 1D). Thus, contrary to previous reports [12, 26], our results indicate that IL-15 expression is not a feature associated with CD4 T cells, and therefore argue against an autocrine role for IL-15 in CD4 T cells.

3.2. CD4 T cells are refractory to IL-15 stimulation

To directly assess if there would be cell-intrinsic effects of CD4 T cell-derived IL-15, we stimulated naïve CD4 T cells from WT and IL-15-deficient mice (*Il15*^{-/-}) under Th17 skewing conditions and examined their IL-17 production. IL-2 signaling downregulates IL-17 expression [23], and IL-15 reportedly acts the same way on Th17 cells [12, 26]. If there would be autocrine effects of IL-15, we expected to see increased IL-17 expression in *Il15*^{-/-} CD4 T cells. However, this was not the case, and we did not find any significant differences in IL-17 production between WT and *Il15*^{-/-} Th17 cells (Fig. 2A). Additionally,

we did not find difference in IL-17 production between *in vitro* differentiated WT and *Il15ra*^{-/-} CD4 T cells (Supplemental Fig. 2A).

Because the absence of IL-15 did not alter Th17 cell generation, next, we wished to examine if the presence of IL-15 would affect IL-17 expression. To this end, we stimulated naïve WT CD4 T cells with plate-bound anti-CD3/CD28 antibodies under Th17 skewing conditions in the presence or absence of recombinant IL-15. As previously reported, IL-2 potently inhibited Th17 cell generation (Fig. 2B)[23]. IL-15, however, did not affect IL-17 production under the same conditions (Fig. 2B). Inducing Th17 cell differentiation by stimulating with irradiated antigen presenting cells (APCs) also did not potentiate any effects of IL-15, so that IL-17 production remained unaffected by addition of IL-15 or the expression of IL-15Rα on APCs (Supplemental Fig. 2B). These results revealed that suppression of IL-17 production is a cytokine-specific effect that is downstream of IL-2, but not of IL-15 signaling. Collectively, these findings indicated that *in vitro* Th17 differentiation is refractory to recombinant IL-15 stimulation.

3.3 IL-2Rβ overexpression promotes IL-15 signaling in CD4 T cells

IL-15 signaling requires IL-2Rβ expression, but CD4 T cells express very low levels of IL-2Rβ relative to other lymphocyte populations, such as CD8 T cells, NK cells, and α NKT cells (Supplemental Fig. 3). Thus, we asked if insufficient IL-2Rβ expression was the reason that IL-15 failed to suppress Th17 cell differentiation. To address this possibility, we generated transgenic mice expressing a murine IL-2Rβ cDNA under the control of human CD2 promoter/enhancer elements (IL-2Rβ^{Tg}). CD4 T cells from IL-2Rβ^{Tg} mice expressed high levels of surface IL-2Rβ (Fig. 3A). Transgenic IL-2Rβ proteins were functional because they conferred dramatically increased IL-2 and IL-15 responsiveness to IL-2Rβ^{Tg} CD4 T cells as demonstrated by the marked increase of STAT5 phosphorylation upon IL-2 and IL-15 signaling (Fig. 3B, 3C). Altogether, these results indicate that increased IL-2Rβ expression was sufficient to induce IL-15 responsiveness on CD4 T cells. Next, to determine if increased IL-2Rβ expression enables IL-15 to inhibit Th17 differentiation, we stimulated IL-2Rβ^{Tg} CD4 T cells under Th17 skewing conditions in the presence of IL-2 or IL-15. While IL-2 stimulation substantially inhibited IL-17 production, surprisingly, IL-15 stimulation failed to suppress IL-17 expression (Fig. 3D). These results indicated that IL-15's inability to suppress IL-17 production is not because of limited quantities of IL-2Rβ proteins on CD4 T cells.

3.4. IL-15 *trans*-presented by IL-15Rα suppresses IL-17 production in CD4 T cells

IL-15 differs from other γ c cytokines as it utilizes a mechanism known as *trans*-presentation for ligand binding and signaling [34]. IL-15 binds to IL-15Rα with high-affinity [35, 36], and IL-15 that is *trans*-presented by IL-15Rα exerts enhanced bioactivity compared to IL-15 alone [37, 38]. To examine if IL-15 *trans*-presentation is required to suppress IL-17 expression, we assessed surface IL-15Rα expression on Th17 cells. IL-15Rα was robustly expressed on cultured CD11c⁺ dendritic cells (DCs), and surprisingly also on Th1-skewed CD4 T cells (Fig. 4A). However, IL-15Rα was conspicuously absent on Th17 cells (Fig. 4A). ELISA for soluble IL-15Rα proteins also showed that Th17 cells did not secrete IL-15Rα, whereas Th1 cells expressed large amounts of soluble IL-15Rα (Fig. 4B).

Importantly, even as Th1 cells produced both membrane and soluble IL-15R α , they did not *trans*-present IL-15 as illustrated by the lack of anti-IL-15 surface staining of *in vitro* differentiated Th1 cells (Fig. 4C). The same was the case for Th17 cells (Fig. 4C). Collectively, these results documented that Th17 cells neither express nor secrete IL-15R α , and thus exclude Th17 cells as a source for IL-15 *trans*-presentation.

To further examine the importance of IL-15 *trans*-presentation, we stimulated CD4 T cells with recombinant IL-15 alone or with IL-15 complexed to IL-15R α under Th17-skewing conditions. In stark contrast to IL-15 alone which had no effect, we found that IL-15 *trans*-presented by IL-15R α induced a marked reduction of IL-17 expression in CD4 T cells (Fig. 4D). Thus, these results formally establish that IL-15 can suppress IL-17 expression in CD4 effector T cells, and that IL-15 bioactivity requires *trans*-presentation by IL-15R α to suppress Th17 cell differentiation. Collectively, these data unveil distinct downstream effects of free versus IL-15R α -bound IL-15 in controlling CD4 effector T cell function.

3.5. IL-15 *trans*-presented by IL-15R α promotes Foxp3⁺ Treg cells generation *in vitro*

To further determine the importance of IL-15R α in establishing IL-15 bioactivity, we examined IL-2R β / γ c signaling upon IL-2, IL-15, and IL-15/IL-15R α stimulation. To this end, we activated CD4 LN T cells overnight with plate-bound anti-CD3/CD28 antibodies, rested them in fresh media for 16 hours, and then examined cytokine signaling by assessing STAT5 phosphorylation. Activated CD4 T cells expressed large amounts of surface IL-2R β and γ c (Fig. 5A), and showed robust STAT5 phosphorylation upon cytokine signaling (Fig. 5B). Notably, IL-2 and IL-15 did not differ in their potency to induce pSTAT5, suggesting that these cytokines are equally effective to trigger proximal IL-2R β / γ c signaling. Interestingly, complexing IL-15 to IL-15R α did not further increase pSTAT5 contents relative to IL-15 alone.

To demonstrate that IL-15 can be equipotent to IL-2 when complexed with IL-15R α , we set up *in vitro* Foxp3⁺ Treg cell differentiation cultures. IL-2 receptor signaling is a critical driver of Treg cell generation [39], but curiously only IL-2, and not IL-15, can drive generation of mature Foxp3⁺CD25⁺ Treg cells *in vitro* [40, 41]. Because Treg cells do not produce IL-15 (Supplemental Fig. 4), so far, a role for IL-15 in Treg cell function or generation has been largely ignored. Considering the role of IL-15R α to confer IL-15 bioactivity, however, we wished to know if IL-15 *trans*-presented by IL-15R α could drive Foxp3⁺ Treg cell differentiation. Thus, we activated naïve CD4 T cells with plate-bound anti-CD3/CD28 antibodies and TGF β to induce Foxp3 expression [42], and further stimulated them with different cytokines to assess Foxp3⁺CD25⁺ Treg cell generation [43]. As previously reported [41], addition of IL-2 induced generation of Foxp3⁺CD25⁺ Treg cells *in vitro*, but IL-15 alone failed to do so (Fig. 5C). IL-15 complexed with IL-15R α , however, potently induced Foxp3⁺CD25⁺ Treg cell generation (Fig. 5C), reaffirming that IL-15 *trans*-presentation is required to convert IL-15 into a biologically active form that drives CD4 T cell effector differentiation.

4. Discussion

Effector CD4 T cells express a vast array of different cytokines to control and coordinate cellular and humoral immune responses. Here we show that IL-15 is not one of such CD4 T cell produced cytokines. Despite playing a critical role in inflammation, autoimmune diseases, and tissue immunity [7, 44], IL-15 expression was conspicuously absent in CD4 T cells. Moreover, we did not find evidence for IL-15 gene reporter activity in terminally differentiated effector CD4 T cells. CD4 T cells also did not express IL-15R α , the unique receptor for IL-15 that is necessary for intracellular transport and secretion of IL-15 [45, 46]. Collectively, these results argue against an autocrine role of IL-15 in CD4 effector T cells, and they suggest that immune dysregulation of IL-15- or IL-15R α -deficient CD4 effector T cells would be mediated by T cell-extrinsic effects of IL-15-deficiency.

Lack of IL-15 expression results in multiple defects in innate and adaptive immunity, including the loss of NK and α NKT cells as well as impaired survival and function of CD8 effector memory cells [9]. Generation and homeostasis of CD4 T cells remain intact in the absence of IL-15 [21]. However, recent studies reported the unexpected finding that IL-15 signaling can play a role in generation and function of CD4 effector T cells, such as in differentiation of IL-17-producing Th17 cells, imposing Th1 lineage fate, and immune suppression by Foxp3⁺ T regulatory cells [12, 26, 47]. Specifically, IL-15- and IL-15R α -deficient CD4 T cells were found to be significantly impaired in generating IL-17-producing cells under Th17 polarizing conditions, and stimulation with exogenous IL-15 potently suppressed Th17 cell differentiation *in vitro* [12]. Consequently, it has been proposed that IL-15 is expressed by CD4 T cells themselves to be complexed with IL-15R α , and thus to inhibit their differentiation into Th17 cells in an autocrine fashion [12]. These results are reminiscent of Th17 cell suppression by IL-2 [23], which shares with IL-15 the same IL-2R β / γ c cytokine receptor complex for signaling. Therefore, Th17 suppression by IL-15 would be a logical corollary, considering that IL-2 and IL-15 bind to the same cytokine receptors and utilize a common signaling pathway in target cells [48–50].

Curiously, the same study that initially reported an IL-15 effect on Th17 cells also found that IL-15-mediated Th17 suppression was critically dependent on the anatomical location of Th17 cells [12]. As demonstrated in an *in vivo* EAE model with IL-15-deficient mice, only activated CD4 T cells in the draining LN, but not in the spleen, were receptive to IL-15-mediated suppression of IL-17 expression [12]. These results suggested that factors other than CD4 T cell-intrinsic IL-15 would be required and contribute to the inhibitory effect of IL-15 on Th17 differentiation. In agreement, recent analysis of IL-15 transgenic mice (IL-15^{Tg}) underscored the Th17 suppressive function of IL-15 being dependent on the local tissue environment [51]. The IL-15 transgene is driven by a universal promoter/enhancer [52], so that in IL-15^{Tg} mice, all CD4 effector T cells are converted into IL-15 producers. However, decreased frequencies of Th17 cells were only observed among CD4 T cells in the small intestine lamina propria (LP), and not in the spleen. These results indicated that IL-15 from the tissue environment can mediate suppression of Th17 cells, but that autocrine CD4 T cell-intrinsic IL-15 was not sufficient to do so [13]. We propose that a likely mechanism behind this observation would be the requirement for IL-15R α , which binds to and complexes with IL-15 for *trans*-presentation to IL-2R β / γ c on target cells.

IL-15 *trans*-presentation by IL-15R α is the major mechanism of IL-15 signaling *in vivo* [53], and multiple studies have demonstrated that IL-15R α expression is dispensable on target cells but required on IL-15-producing neighboring cells for effective IL-15 signaling *in trans*. Notably, IL-15R α is mostly expressed in cells of non-lymphoid tissues, such as lung, liver, muscle and endothelial cells [35, 54, 55]. Increased IL-15R α availability in the gut would explain the selective decrease of IL-15^{Tg} Th17 cells in the small intestine LP, and the failure to suppress Th17 differentiation in the spleen [51]. Along these lines, IL-15/IL-15R α signaling in the gut would also contribute to the differentiation and maintenance of Foxp3⁺ Treg cells, thus creating an overall immune suppressive environment that promotes intestinal Treg cell homeostasis but suppresses pro-inflammatory Th17 cell differentiation. In fact, loss of intestinal IL-15 signaling results in downregulation of Foxp3 expression and triggers inflammatory bowel disease that was associated with enhanced ROR γ t expression and accumulation of Th17 cells [26]. Thus, intestinal IL-15 plays a dual role for Foxp3⁺ Treg and Th17 cells to maintain immune quiescence in the gut.

Here we wish to emphasize that we found Th17 CD4 effector T cells being absent for IL-15R α expression. IL-15R α is produced both as a conventional membrane-bound form and a secreted form, whereby the soluble IL-15R α is thought to be generated by proteolytic cleavage of the membrane form [56]. Notably, complexing IL-15 with soluble IL-15R α conferred bioactivity to IL-15 that altered CD4 effector cell generation. Thus, the lack of IL-15R α expression on CD4 T cells explains the inability of free IL-15 to suppress Th17 cell differentiation and the failure to induce Foxp3⁺ Treg cell generation *in vitro* when using purified naïve CD4 T cells for stimulation. IL-15 *trans*-presentation by IL-15R α has been considered a vital mechanism for efficient IL-15 signaling because IL-15- and IL-15R α -deficient mice display largely overlapping phenotypes [9, 31]. The pre-association of IL-15 with IL-15R α , and the *trans*-presentation of IL-15 in context of IL-15R α could be important for multiple reasons, such as to prevent degradation and excretion of circulating IL-15 or concentrating IL-15 to the cell surface for localized and higher density signaling [53]. Without physiological data that can clarify the differences between free IL-15 and IL-15R α -complexed IL-15 binding to IL-2R β / γ c, however, it remains difficult to provide a rationale why IL-15/IL-15R α complexes would be superior in signaling compared to unbound IL-15. Because both free and IL-15R α -bound IL-15 induced robust STAT5 phosphorylation in CD4 T cells, we propose that it is not quantitative, but possibly kinetic differences that determine IL-15 bioactivity to control effector T cell differentiation.

As a corollary, we further conjecture that distinct downstream effects of IL-15 and IL-2, which utilize the same IL-2R β / γ c complex for signaling but induce quite different cellular outcomes, could also be the results from differences in their signaling kinetics. IL-2R β / γ c signaling induces CD25 (IL-2R α) expression on the same cells, which potentiates and increases IL-2 binding and signaling. In contrast, IL-15-induced IL-2R β / γ c signaling does not affect expression of IL-15R α on IL-15-*trans*-presenting cells, so that IL-15 signaling does not induce further amplification of IL-2R β / γ c signaling. Thus, it would be not qualitative but kinetic differences of IL-2 and IL-15 signaling that would cause different effects of IL-2R signaling. If such was the case, we can speculate that Foxp3⁺ Treg cells which were generated by IL-2 signaling could differ in their phenotype or effector function from those generated by IL-15 signaling. Because intestinal Foxp3⁺ Treg cells mostly rely

on IL-15 [26], assessing the gene signature and epigenetic changes of IL-15-induced Treg cells versus IL-2-induced Treg cells would be an important aspect in tissue-specific Foxp3⁺ Treg cell biology that remains to be addressed.

Mechanistically, the difference in free versus IL-15R α -*trans*-presented IL-15 signaling might reside in the distinct signaling kinetics of these two forms of IL-15. Earlier studies demonstrated that IL-15R α -associated IL-15 induced prolonged and persistent downstream signaling, whereas signaling by IL-15 alone was equally strong as IL-15R α -complexed IL-15 but rapidly declined [30, 57]. In addition to higher binding affinity toward the receptor [35, 36], internalization and recycling of the IL-15/IL-15R α complex is proposed to be another key mechanism that prolongs IL-15 signaling in responder cells and can explain the difference between free and *trans*-presented IL-15 [58]. Whether such difference in signaling kinetics is indeed the molecular basis for distinct bioactivity between free IL-15 and IL-15R α -complexed IL-15 is an attractive idea that will be pursued in future studies.

In conclusion, here we demonstrated a role for IL-15 in CD4 effector cells, specifically during pro-inflammatory Th17 cell differentiation, and we revealed an absolute requirement for IL-15R α and for IL-15 *trans*-presentation in this process. Because we found IL-15 being not produced by CD4 T cells, we also exclude both autocrine and paracrine effects of IL-15 in CD4 cells. IL-15 shares and co-utilizes the IL-2R β / γ c cytokine receptor complex with IL-2, so that the inability of CD4 T cells to produce IL-15 could be a developmentally set mechanism to ensure effective IL-2 signaling during effector cell generation and differentiation.

Conclusions

Free IL-15 induced STAT5 phosphorylation but failed to control effector cell differentiation of CD4 T cells. IL-15-bound to soluble IL-15R α , on the other hand, was effective for both activities. Thus, the current study provides new mechanistic insights into the biological function of IL-15 in CD4 effector cell generation, and puts forward IL-15 *trans*-presentation as a critical mechanism to control the pro- versus anti-inflammatory effects of IL-15.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Unlike most other γc cytokines, IL-15 is not produced by activated CD4 T cells
- Free IL-15 phosphorylates STAT5 but does not affect CD4 T cell differentiation
- IL-15R α -bound IL-15, but not free IL-15, suppresses Th17 cell generation
- IL-15 *trans*-presentation is required to control CD4 effector T cell differentiation

FIGURE 1. IL-15 gene reporter expression in T cells

(A) EmGFP/IL-15 reporter expression in CD4 and CD8 LN T cells. Data are representative of 4 independent experiments.

(B) EmGFP/IL-15 reporter activity was determined in naïve (CD44^{lo}CD62L⁺) and memory (CD44⁺CD62L⁻) CD4 LN T cells (top) and naïve (CD44^{lo}IL-2Rβ^{lo}) and memory (CD44⁺IL-2Rβ⁺) CD8 LN T cells (bottom). Data are representative of 4 independent experiments.

(C) IL-15 reporter activity in Th1 and Th17 skewed CD4 T cells and fresh NK cells. Naïve CD4 T cells from WT and EmGFP/IL-15 reporter mice were differentiated under Th1 or

Th17 skewing conditions for 5 days then analyzed by flow cytometry. Freshly isolated LN NK cells were used as a positive control. Data are representative of 3 independent experiments.

(D) IL-15, ROR γ t and T-bet mRNA levels in Th17 differentiated cells. Naïve CD4 T cells were stimulated under Th1 and Th17 conditions for 5 days. IL-15, ROR γ t and T-bet mRNA levels relative to RPL13 were determined by qRT-PCR. Data are representative of 2–7 independent experiments.

FIGURE 2. No cell intrinsic role for IL-15 in suppressing IL-17 production in CD4 T cells
(A) IL-17 production from IL-1- deficient CD4 T cells. Naïve CD4 T cells (CD4⁺, TCR β ⁺, CD1d tetramer⁻, CD69⁻ (and/or CD62L⁺), CD25⁻, CD44^{lo}) were sorted from lymph nodes of WT and *Il15*^{-/-} mice. Cells were skewed under Th17 conditions. IL-17 production was determined on day 5 of stimulation. Data are representative of 5 independent experiments with 10 WT and 8 *Il15*^{-/-} mice.
(B) Exogenous IL-15 does not inhibit IL-17 production by CD4 T cells. Electronically sorted naïve (CD44^{lo}) CD4 T cells from WT mice were skewed under Th17 culture conditions for 5 days. Cultures were additionally supplemented with IL-2 (25 ng/ml) or IL-15 (25 ng/ml). IL-17 and IFN γ protein production were determined by flow cytometric analysis following restimulation with PMA/ionomycin in the presence of Brefeldin A. Data are representative of 4 experiments with a total of 8 mice.

FIGURE 3. IL-2Rβ expression does not limit IL-15 responsiveness under Th17 skewing conditions

(A) IL-2Rβ and γC expression on naïve CD4 LN T cells from WT and IL-2Rβ^{Tg} mice. Data are representative of 2 independent experiments with 4 WT and 5 IL-2Rβ^{Tg} mice.

(B) LN cells from WT and IL-2Rβ^{Tg} mice were stimulated with IL-2 (1 μg/ml) or IL-15 (100 ng/ml) and stained for phosphor-STAT5. Data are representative of 4 independent experiments.

(C) LN cells from WT and IL-2Rβ^{Tg} mice were stimulated with IL-2 or IL-15 and assessed for intracellular pSTAT5 content. Results show summary from 4 independent experiments.

(D) Electronically sorted naïve (CD44^{lo}) CD4 T cells from IL-2R β ^{Tg} mice were skewed under Th17 cultured conditions for 5 days. Cultures were additionally supplemented with IL-2 (25 ng/ml) or IL-15 (25 ng/ml). IL-17 and IFN γ protein production were determined by flow cytometric analysis following restimulation with PMA/ionomycin in the presence of Brefeldin A. Data are representative of 2 independent experiments with a total of 4 mice.

FIGURE 4. Trans-presented IL-15 inhibits IL-17 production by CD4 T cells

(A) Electronically sorted naïve (CD44^{lo}) CD4 T cells from WT mice were stimulated under Th1 or Th17 skewing conditions for 5 days. Surface IL-15R α expression was determined by flow cytometry. Overnight medium-incubated splenic CD11c⁺ DCs were used as a positive staining control. Data are representative of 3 independent experiments with 5 mice.

(B) Naïve WT CD4 T cells were enriched by magnetic selection and skewed under Th1 or Th17 conditions for 5 days. Culture supernatants were harvested, and soluble IL-15R α production was determined by ELISA.

(C) Naïve WT CD4 T cells were enriched by magnetic selection and skewed under Th1 or Th17 conditions for 5 days. Surface-bound IL-15 was determined on skewed CD4 T cells and freshly isolated CD11c⁺ DCs by flow cytometry.

(D) Electronically sorted naïve (CD44^{lo}) CD4 T cells from WT mice were stimulated under Th17 skewing conditions for 5 days. Cultures were additionally supplemented with IL-15

(25 ng/ml) or IL-15/IL-15R α complex (100 ng/ml). IL-17 and IFN γ protein production were determined by flow cytometric analysis following restimulation with PMA/ionomycin in the presence of Brefeldin A. Data are representative of 4 experiments with a total of 8 mice.

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FIGURE 5. IL-15R α /IL-15 complex drives Foxp3⁺ Treg cell differentiation *in vitro*

(A) WT CD4 T cells were activated overnight with plate-bound anti-CD3/CD28 antibodies, rested for 16 hours, and then assessed for surface cytokine receptor expression. Results are representative of 2 independent experiments in triplicate cultures.

(B) WT CD4 T cells were activated overnight with plate-bound anti-CD3/CD28 antibodies, rested for 16 hours, and then stimulated with IL-2 (100 ng/ml), IL-15 (100 ng/ml), or IL-15/IL-15R α (200 ng/ml) for 30 min. Intracellular pSTAT5 contents were determined by flow cytometry and quantified as mean fluorescence intensities (MFI). Results are representative of 2 independent experiments in triplicates.

(C) Highly purified naive CD4 T cells were activated in the presence of 5 ng/ml TGF β for 5 days in the presence or absence of the indicated cytokines: IL-2 (25 ng/ml), IL-15 (25 ng/ml) or IL-15/IL-15R α (100 ng/ml). Viable cells were enumerated by trypan blue exclusion, and intracellular Foxp3 and surface CD25 expression were determined by flow cytometry. Results are representative of 2 independent experiments in triplicates.