

Expression and regulation of IL-22 in the IL-17-producing CD4⁺ T lymphocytes

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IL-22 is a novel cytokine in the IL-10 family that functions to promote innate immunity of tissues against infection. Although CD4⁺ helper T lymphocytes (TH) were found as a source of IL-22, the regulation of this cytokine has been poorly understood. Here, we show that IL-22 is expressed at both mRNA and protein levels by a novel subset of TH cells that also makes IL-17. IL-22 and IL-17 were found to be coordinately regulated by TGF β and IL-6 during TH differentiation by real-time PCR as well as ELISA analysis. However, IL-22 does not regulate TH differentiation; exogenous IL-22 or an IL-22 antagonist had no effect on TH differentiation. These data demonstrate a novel cytokine expressed by IL-17-producing T cells, and suggest interaction and synergy of IL-22 and IL-17 signaling pathways in tissue inflammation and autoimmune diseases.

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Introduction

Following activation by antigen-presenting cells, CD4⁺ helper T lymphocytes (TH) differentiate into effector TH cells that are specialized in their cytokine secretion and immunoregulatory function. TH cells have been historically classified into TH1 and TH2 cells [1, 2]. TH1 cells make IFN γ and regulate antigen presentation and cellular immunity against intracellular pathogens. TH2 cells produce IL-4, -5 and -13 to regulate humoral responses and anti-parasite immunity. TH1 and TH2 differentiation is regulated by cytokine environment. IL-12 regulates TH1 differentiation while IL-4 is essential in TH2 differentiation.

Recently, a third subset of TH cells, named as TH17, TH-17 or inflammatory TH (THi), that produce IL-17 was identified to mediate pathogenic inflammatory response [3]. IL-17 regulates chemokines and other proinflammatory gene expression *in vitro* and *in vivo* [4]. IL-17 expression in TH cells was initially found to be regulated by ICOS costimulatory receptor [5]. Subsequently, IL-23, a cytokine

in the IL-12 family, was found to regulate IL-17 expression and expansion of IL-17-expressing T cells [6]. Importantly, impaired IL-17 expression caused by ICOS- or IL-23-deficiency was reported to result in resistance to autoimmune diseases. More recently, IL-6 in the presence of TGF β has been shown by multiple investigators to initiate the differentiation of IL-17-expressing T cells in the absence of IL-23 [7], further supporting a distinct cytokine requirement in generation of IL-17-expressing TH cells.

IL-22 is a recently identified cytokine that belong to the IL-10 family [8, 9]. In contrast to the anti-inflammatory function by IL-10, IL-22 does not inhibit proinflammatory cytokine expression by the innate immune system [8]. Instead, it was found to regulate the expression of β -defensin genes in keratinocytes possibly through activation of STAT3 [10]. Thus, IL-22 may regulate innate immunity and inflammation in the skin and its overexpression has been associated with dermatitis and psoriasis [11].

IL-22 expression was first discovered to be expressed by CD4⁺ T cells [8]. However, it is unknown what subset of TH cells express IL-22, or how it is regulated. Here, we for the first time report that IL-22 is expressed by IL-17-expressing TH cells and is regulated in the same manner as IL-17. Since IL-22 and IL-17 do not have any effect in regulation of TH differentiation, they may jointly regulate tissue inflammation.

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Materials and Methods

T-cell differentiation

OVA-specific CD4T cells isolated from OT-II mice using anti-CD4-microbeads by AutoMACS (Miltenyi Biotec) were cultured with irradiated splenic APC from C57BL/6 mice prepared as described previously [4] in the presence of OVA₃₂₃₋₃₃₉ peptide. For TH1 differentiation, anti-IL-4 (11B11, 10 µg/ml) and IL-12 (2 ng/ml) were added. For TH2 differentiation, anti-IFN γ (XMG1.2, 10 µg/ml) and IL-4 (10 ng/ml) were added. For THi differentiation, anti-IL-4/anti-IFN γ Abs, IL-6 (10 ng/ml), TGF β (5 ng/ml), IL-23 (5 ng/ml) were used. To examine the effect of IL-22 during Th 17 differentiation, recombinant human IL-22 (100 ng/ml) or anti-mIL-22 polyclonal Abs (5 µg/ml, R&D systems) were added into the cell culture.

Real-time RT-PCR analysis

T cells differentiated for 5 days were restimulated with plate-bound anti-CD3 Ab (2C11, 5 µg/ml) for 5 h. Total RNA was extracted from these cells with TRIzol reagent (Invitrogen) and the cDNA was generated with an oligo(dT) primer followed by analysis using iCycler PCR with the iQTM SYBR[®] Green Supermix (Bio-Rad) based on expression of β -actin. The following primer pairs were used: β -actin: F-GAC GGC CAG GTC ATC ACT ATT G and R-AGG AAG GCT GGAAA GAG CC; IFN γ : F-GAT GCA TTC ATG AGT ATT GCC AAG T and R-GTG GAC CAC TCG GAT GAG CTC; IL-4: F-AGA TCA CGG CAT TTT GAACG and R-TTT GGCACA TCC ATC TCC G; IL-17: F-CTG GAG GAT AAC ACT GTG AGA GT and R-TGC TGA ATG GCG ACG GAG TTC; IL-22: F-CAT GCA GGA GGT GGT ACC TT and R-CAG ACG CAA GCA TTT CTC AG.

ELISA

T cells (1×10^6 /well), after differentiation for 5 days as described above, were restimulated with plate-bound anti-CD3 Ab (5 µg/ml) in 24-well plates. Twenty-four hours later, culture supernatant was harvested, and the concentration of IFN- γ , IL-4, IL-17 (BD Bioscience) and IL-22 (Antigenix) was measured by ELISA [4], following the manufacturer's direction.

Intracellular cytokine staining

T cells, after differentiation for 5 days, were restimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug (BD Bioscience) for 5 h. These cells were stained with FITC-conjugated anti-V α 2 Abs then permeabilized as per the manufacturer's instruction. Cells were additionally stained with PE-conjugated anti-IL-17 and APC-conjugated anti-IFN γ Abs before FACSCalibur analysis.

Results

Expression of IL-22 in IL-17-producing TH cells

In order to identify genes that are selectively expressed in different subsets of TH cells, we recently compared gene expression profiles of *in vitro* differentiated TH1, TH2 and THi cells. In addition to IL-17 that was highly upregulated by THi cells, we found that IL-22 expression was also selectively upregulated in these cells ($>8\times$ than TH1 cells and $>16\times$ than TH2). This result was further confirmed by real-time RT-PCR analysis. CD4⁺ T cells

isolated from OT-II TcR transgenic mice were activated by Ova peptide and splenic APC and differentiated into TH1 (in the presence of IL-12 and anti-IL-4), TH2 (IL-4 and anti-IFN γ) or THi (IL-6, TGF β , IL-23, anti-IL-4 and anti-IFN γ) cells. While IFN γ gene was highly expressed in TH1 cells and IL-4 was highly expressed in TH2 cells, expression of IL-17 and IL-22 was restricted to THi cells (Figure 1A).

To further characterize the expression of IL-22 in different TH cells, we examined IL-22 protein levels in their supernatants by ELISA following anti-CD3 restimulation. Consistent with the results of real-time RT-PCR analysis, the production of IFN γ , IL-4 and IL-17 was highly restricted to TH1, TH2 and THi, respectively (Figure 1B). The production of IL-22 by TH1 was higher than by TH2; however, this cytokine was produced at greatly increased levels in THi cells ($>2.5\times$ than TH1 cells and $>10\times$ than TH2; Figure 1B). Taken together, these data indicate that IL-22 may be another cytokine uniquely expressed in the THi subset.

Coordinated regulation of IL-17 and IL-22 expression during TH differentiation

Recent studies have indicated IL-23 or TGF β and IL-6 as cytokines regulating THi cell differentiation [3, 7]. To substantiate our above findings, we further differentiate OT-II cells under various conditions and assessed the regulation of IL-22 expression in comparison to that of IL-17, IFN γ or IL-4. When OT-II cells were differentiated in the presence of IL-6 or TGF β , there was little IL-22 or IL-17 mRNA expression (Figure 2A). On the other hand, combination of these two cytokines led to greatly increased levels of IL-22 and IL-17 expression and down-regulation of IFN γ and IL-4 expression (Figure 2A). IL-23 also moderately enhanced IL-22 and IL-17 expression. Combination of IL-6, TGF β and IL-23, while inhibiting TH1 and TH2 differentiation by blocking IFN γ and IL-4, led to most optimal mRNA expression of both IL-22 and IL-17 (Figure 2A).

When we analyzed the protein expression of IFN γ , IL-4 and IL-17 from T cells differentiated under the same condition, we observed similar results (Figure 2B). The addition of IL-6 appeared to induce IL-22 substantially, although we did not see any significant increase in its mRNA level (Figure 2B). Moreover, combination of IL-6 and TGF β led to a further increase in IL-22 production, although TGF β alone suppressed the IL-22 production. Further inclusion of IL-23 plus neutralizing Abs against IFN γ and IL-4 led to most optimal production of IL-22 cytokine, consistent with real-time RT-PCR analysis (Figure 2B). All these data indicate coordinated regulation of IL-17 and IL-22 expression, which further supports IL-22 as a novel cytokine

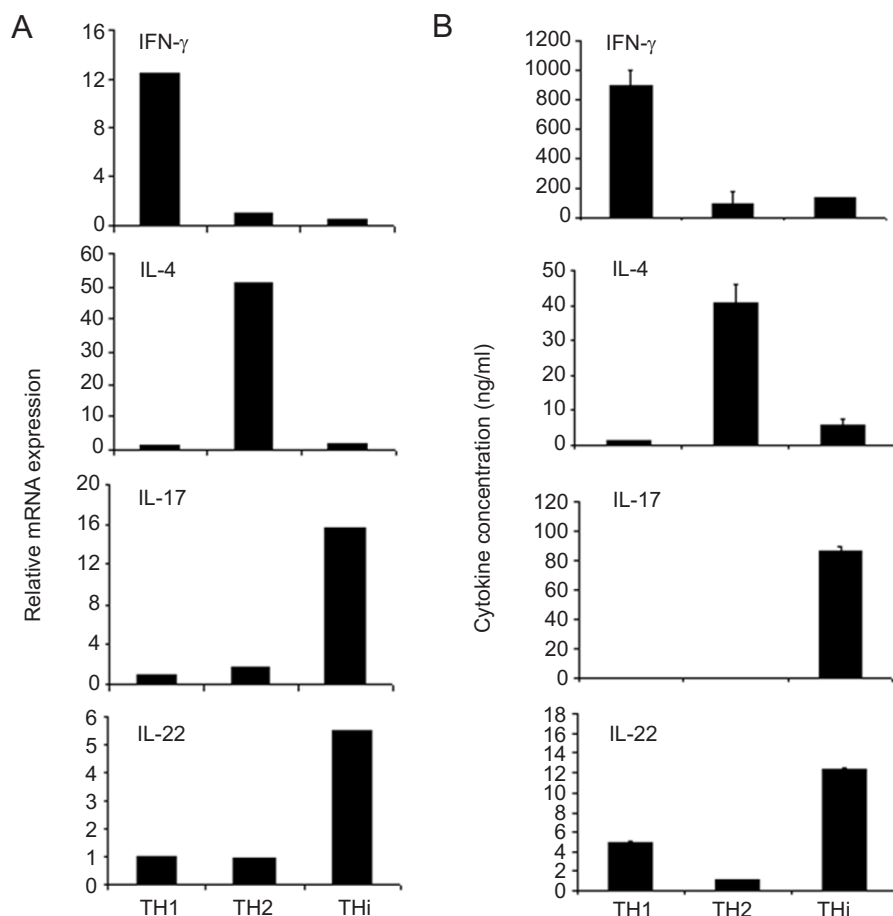


Figure 1 Preferential expression of IL-22 in THi cells. **(A, B)** Naïve CD4⁺ T cells isolated from OT-II mice were differentiated into TH1, TH2 or THi cells. **(A)** Five days later, cells were restimulated with plate-bound anti-CD3 Ab and analyzed for cytokine mRNA levels by real-time RT-PCR. Results are normalized to β -actin. Data are representative of two independent experiments. **(B)** Five days later, T cells were restimulated with plate-bound anti-CD3 Ab for 24 h and the expression of IFN- γ , IL-4, IL-17 and IL-22 in the supernatant was measured by ELISA. Bars are mean \pm SD.

expressed in THi cells.

IL-22 does not regulate TH differentiation

While IFN γ regulates TH1 differentiation and IL-4 mediates TH2 differentiation, it is unclear whether there is a cytokine produced by THi cells to regulate their own differentiation. We thus tested the ability of IL-22 in THi differentiation. Addition of exogenous IL-22 to the above OT-II differentiation system did not augment THi differentiation under suboptimal or optimal conditions; IL-17 and IFN γ expression was not affected by an intracellular cytokine staining assay (Figure 3). We also utilized an antibody against IL-22 in the culture to block the effect of IL-22 produced by T cells. This antibody did not affect THi differentiation either (Figure 3). Thus, IL-22 does not regulate T-cell differentiation.

Discussion

Upon activation, naïve TH cells differentiate into different subsets of effector T cells that are specialized in their cytokine expression and immune function. A novel lineage of TH cells that produce IL-17 was recently identified and strongly implicated in host responses to infection and in autoimmune diseases. Here, we report an additional cytokine product expressed in these cells – IL-22.

In a gene-chip analysis of TH1, TH2 and THi cells, we found IL-22 mRNA was significantly upregulated in THi cells compared with the other two subsets. The idea that IL-22 is a THi cytokine was further supported by a real-time RT-PCR and ELISA analysis of IL-22 expression in these subsets (Figure 1). Furthermore, in analysis of TH differentiation conditions that result in IL-22 expression,

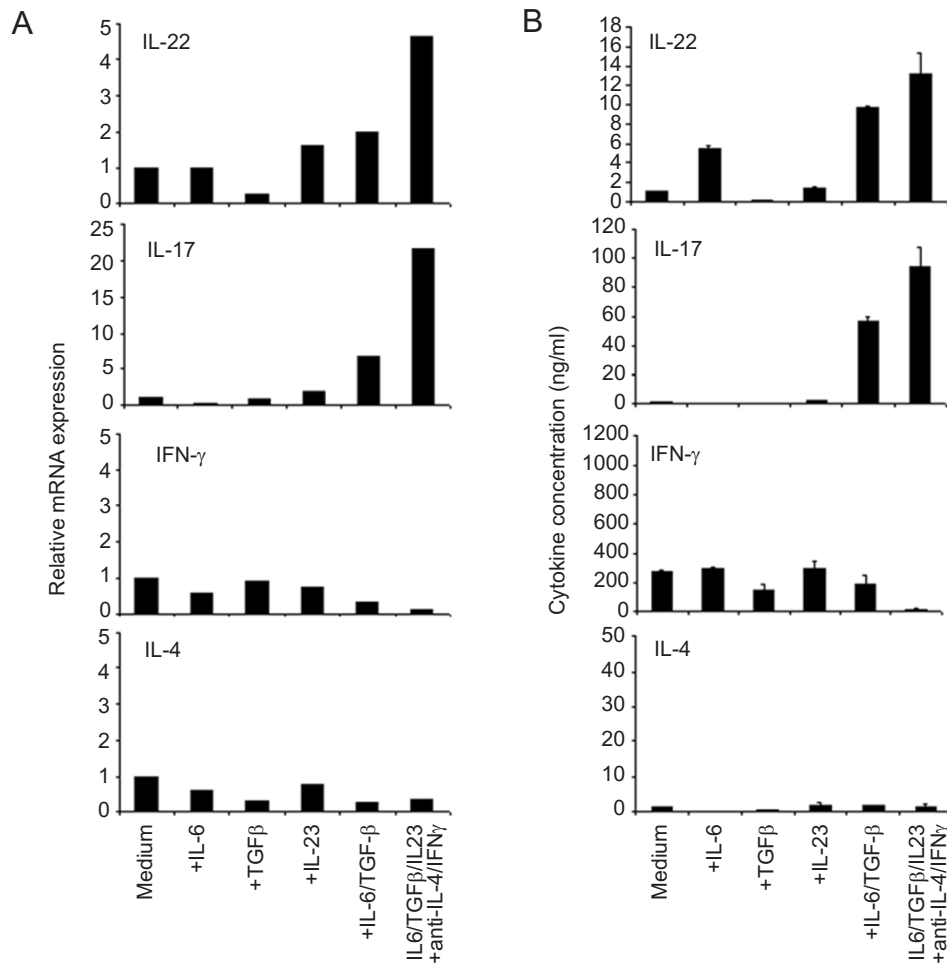


Figure 2 Coordinated regulation of IL-22 and IL-17 in TH differentiation. **(A, B)** CD4T cells isolated from OT-II mice were activated in the presence of indicated cytokines or cytokine blockers. **(A)** Five days later, cells were restimulated with plate-bound anti-CD3 Ab and analyzed for cytokine mRNA levels by real-time RT-PCR. Results are normalized to β-actin. Expression level in medium alone was set to 1. **(B)** Five days later, T cells were restimulated with plate-bound anti-CD3 Ab for 24 h and the concentration of IFN-γ, IL-4, IL-17 and IL-22 in the supernatant was measured by ELISA. Bars are mean ± SD.

we found that the mRNA and protein expression of IL-17 and IL-22 were coordinately regulated (Figure 2). All these data indicate that IL-22 is expressed by IL-17-producing THi cells. However, at this stage, we do not know whether these two cytokines are expressed by the same or different cells at a single-cell level. The intracellular protein analysis of these cytokines is needed in the future. In addition, their relationship with IL-17F, another cytokine expressed in the THi cells [3, 7] needs to be addressed.

In our TH cell differentiation experiments, we did not find any significant effect by exogenous IL-22 or inhibiting IL-22 on subsequent cytokine expression by the effector cells (Figure 3). This further supports the previous conclusion that immune cells may not be the targets of IL-22.

Similarly, IL-17 also mainly functions to promote tissue inflammation *in vitro* and *in vivo* [4]. With this in mind, IL-17 and IL-22 may have synergistic function on their common target tissues or cells. IL-17 strongly induced proinflammatory cytokine expression in mouse fibroblasts [4]. However, we did not observe any synergistic effect by IL-22 in these cells (data not shown). Epithelial cells may be a common target cell type of both cytokines [4, 10]. It has been reported that IL-22 in plasma of psoriatic patients is higher than that of healthy individuals [11]. This cytokine induces keratinocytes to produce anti-microbial peptides and S100 proteins, chemokines that recruit neutrophils. On the other hand, the association of microbial infection and chronic inflammation has been suggested in autoim-

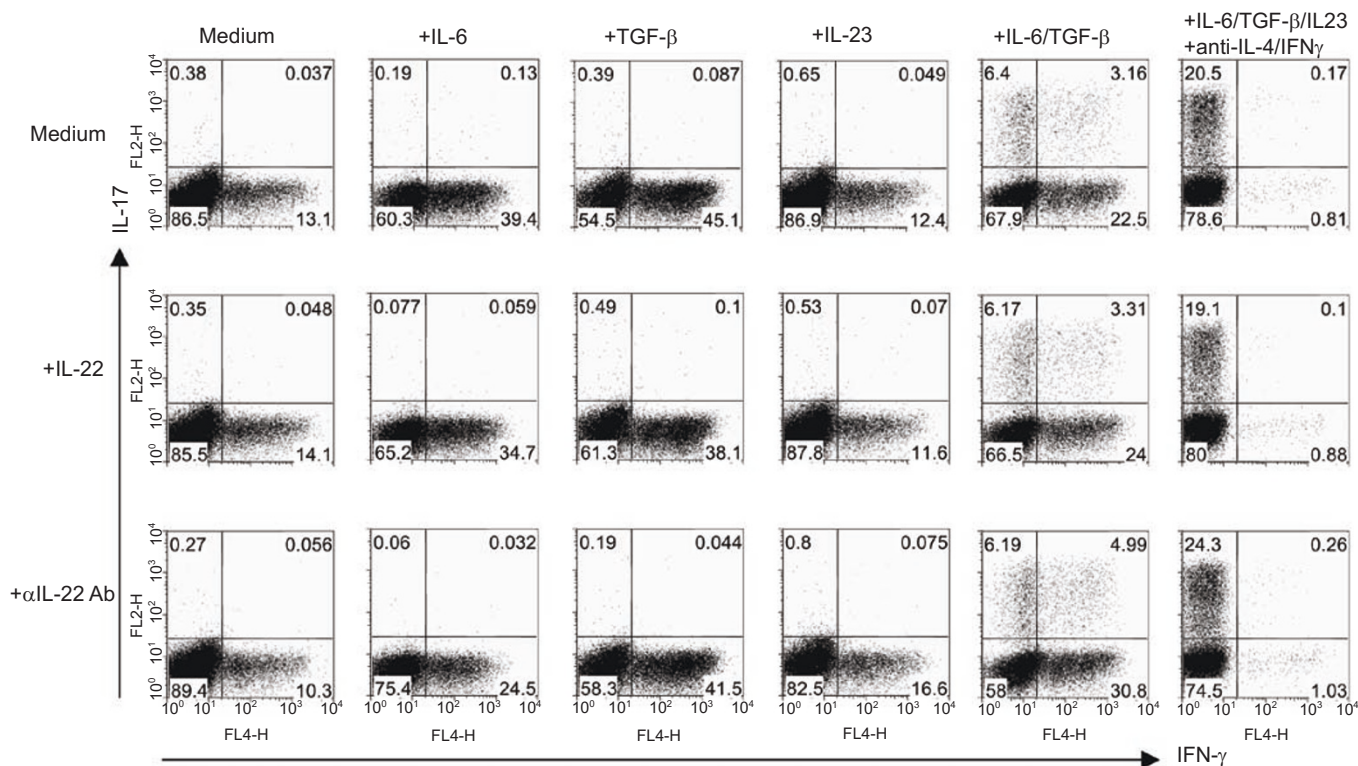


Figure 3 IL-22 does not regulate the generation of THi *in vitro*. CD4⁺ T cells isolated from OT-II mice were activated in the presence of indicated cytokines or cytokine blockers. IL-22 or anti-IL-22 polyclonal Abs were added into indicated samples. Five days later, cells were restimulated with PMA and ionomycin for 5 h and analyzed for intracellular IL-17 and IFN γ expression on gated CD4⁺ V α 2⁺ OT-II cells.

mune disease models including psoriasis [12]. Based on our findings in the present study, we propose that these two cytokines secreted from THi cells synergize to promote autoimmunity and tissue inflammation such as psoriasis. IL-22 and IL-17 may be crucial mediators of this inflammatory disorder in different stages of pathogenesis and may have effects on different target cells.

IL-17-expressing TH cells have recently emerged as an attractive target for immunotherapy against autoimmune diseases. While IL-17 itself has potent inflammatory function, our current study has added another layer of complexity on these cells. One may need to consider the effect of IL-22 in THi-mediated diseases. Understanding the function and regulation of IL-22 would certainly be beneficial in the future in treatment of inflammatory diseases such as dermatitis and psoriasis in which IL-22 was found overexpressed.

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