Interleukin-2 Signaling via STAT5 Constrains T Helper 17 Cell Generation

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SUMMARY

Recent work has identified a new subset of effector T cells that produces interleukin (IL)-17 known as T helper 17 (Th17) cells, which is involved in the pathophysiology of inflammatory diseases and is thought to be developmentally related to regulatory T (Treg) cells. Because of its importance for Treg cells, we examined the role of IL-2 in Th17 generation and demonstrate that a previously unrecognized aspect of IL-2 function is to constrain IL-17 production. Genetic deletion or antibody blockade of IL-2 promoted differentiation of the Th17 cell subset. Whereas STAT3 appeared to be a key positive regulator of RORγt and IL-17 expression, absence of IL-2 or disruption of its signaling by deletion of the transcription factor STAT5 resulted in enhanced Th17 cell development. We conclude that in addition to the promotion of activation-induced cell death of lymphocytes and the generation of Treg cells, inhibition of Th17 polarization appears to be an important function of IL-2.

INTRODUCTION

In response to various infectious organisms, the adaptive immune system generates distinct responses, which in part are governed by the pattern of cytokines secreted by effector T cells. Although T helper (Th) cells secrete an array of cytokines, until recently, these cells were divided into two groups on the basis of their cytokine profiles: Th1 cells that secrete IFN-γ and Th2 cells that secrete IL-4, IL-5, and IL-13 (Abbas et al., 1996; Flavell, 1999; Glimcher, 2001; Mosmann and Coffman, 1989; Murphy and Reiner, 2002). Th1 cells are essential for controlling intracellular infectious organisms whereas Th2 cells are important for host defense against helminth infections. These Th cell lineages have also been implicated in the pathogenesis of immune-mediated disease. For instance, Th1 cells have been thought to contribute to pathology in Crohn’s disease (Strober et al., 1998), whereas Th2 cells have been implicated in asthma (Umetsu et al., 2002). In general, though, autoimmune diseases are not easily explained by a dichotomous view of helper T cell differentiation.

The interleukin (IL)-17 family members 17A and 17F have been implicated in host defense against extracellular bacteria; IL-17 receptor-deficient mice are susceptible to bacterial pneumonias and are unable to form abdominal abscesses (Ye et al., 2001). Importantly though, IL-17 has also been found to be overexpressed in a variety of autoimmune and autoinflammatory syndromes, both in mice and in humans (McKenzie et al., 2006). The identification of a helper T cell lineage that selectively produced IL-17 was therefore of considerable interest (Aggarwal et al., 2003; Bettelli et al., 2006; Cua et al., 2003; Harrington et al., 2005; Langrish et al., 2005; Mangan et al., 2006; Murphy et al., 2003; Veldhoen et al., 2006a). Initially, it was thought that IL-23 induced the formation of Th17 effector cells; however, it is now thought that IL-23 maintains and expands this population of cells. The cytokines now thought to be critical for promoting Th17 differentiation are transforming growth factor (TGF)-β1 and IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a). In conjunction with T cell receptor stimulation, these cytokines induce the expression of the orphan nuclear receptor RORγt, which is critical for Th17 differentiation (Ivanov et al., 2006). These findings are also of interest in that TGF-β1 is important for maintenance peripheral regulatory T (Treg) cells (Li et al., 2006; Marie et al., 2006). That is, TGF-β1 alone promotes expression of the transcription factor FoxP3, whereas the combination of IL-6, TGF-β1 suppresses FoxP3 expression and enhances IL-17 production. Thus, the differentiation of Treg and Th17 cells by TGF-β1 appears to be reciprocally related,
through the action of IL-6 (Bettelli et al., 2006; Veldhoen et al., 2006a, 2006b).

The development of Treg cells is regulated by IL-2 and IL-2Rα chain (Shevach et al., 2006). Accordingly, 

\[ \text{IL}^2 \rightarrow \text{---} \text{and IL}2\text{a} \rightarrow \text{---} \text{mice develop systemic autoimmune disease characteristic of reduced Treg cell numbers} \]

(Suzuki et al., 1995; Abbas, 2003; Malek, 2003; Refaeli and Abbas, 1998; Refaeli et al., 1999). In view of the apparent connection between Treg and Th17 cells (Bettelli et al., 2006), we were interested in exploring whether IL-2 had a role in Th17 differentiation. In the present study, we report that IL-2 negatively regulates Th17 differentiation and appears to be a mechanism for regulating the balance between Th17 and Treg cells.

**RESULTS**

**IL-2 Inhibits In Vitro Differentiation of Th17 Cells**

We first examined the potential role of IL-2 in Th17 generation by using several complementary approaches. As previously reported, in vitro culture of naive CD4+ T cells with IL-6 and TGF-β1 along with blocking antibodies against IL-4 and interferon (IFN)-γ (denoted henceforth as optimal Th17 conditions) resulted in an increased proportion of cells that selectively produce IL-17 and not IFN-γ compared to cells cultured in medium alone (Figure 1A). We first assessed a role for IL-2 by adding excess exogenous IL-2 to these cultures. This resulted in a dose-dependent decrease in the proportion of

![Figure 1](image-url)
IL-17-producing cells with no change in the proportion of IFN-γ-producing cells (Figure 1A). Activated T cells produce endogenous IL-2, so we next investigated the contribution of this cytokine by adding IL-2 antibodies. Blockade of IL-2 in these cultures increased the proportion of IL-17-producing cells, as determined by intracellular cytokine staining (Figures 1B–1D) and ELISA (data not shown); conversely, addition of exogenous human IL-2 reversed this effect. After 3 days of culture, the addition of human IL-2 to the sample receiving anti-mouse IL-2 resulted in a small increase in cell number that was not statistically significant (0.7 × 10^6 cells/ml ± 0.22 versus 1.01 × 10^6 cells/ml ± 0.25, p = 0.11). Additionally, we observed a decrease in the percentage of FoxP3-positive cells that was inversely proportional to the increased percentage of IL-17-producing cells when IL-2 was blocked (Figure 1D). Thus, these data illustrate the ability of IL-2 to expand Treg cells and simultaneously restrict Th17 development. This is consistent with the idea that Treg and Th17 cells may be reciprocally regulated (Bettelli et al., 2006); in this case, IL-2 is an important factor in fate determination of both lineages. It is notable that TGF-β, an important factor for Th17 polarization, is an inhibitor of IL-2 production; this could be one means by which this cytokine promotes differentiation of this lineage (McKarns et al., 2004).

**IL-2 Deficiency Is Associated with Enhanced IL-17 Production**

To more clearly define a role of IL-2 in Th17 polarization, we took a third approach of culturing Il2−/− CD4+ T cells in vitro under Th17-polarizing conditions in the presence or absence of exogenous IL-2. As shown in Figure 2A, T cells from Il2−/− mice had a marked increase in the percentage of IL-17-producing cells compared to wild-type cells. Addition of IL-2 into polarizing cultures reduced the percentage of IL-17-producing cells in wild-type cells and normalized IL-17 production by Il2−/− cells. These data substantiate the contention that IL-2 limits IL-17 production. The addition of IL-2 to Il2−/− T cells had no significant increase in cell number after 3 days of T cell receptor stimulation (see Figure S1 in the Supplemental Data available online) irrespective of culture condition.

To determine whether IL-2 was an important in vivo regulator of IL-17 homeostasis, we next measured serum concentration of IL-17 in 3-month-old wild-type versus Il2−/− mice. Whereas IL-17 was undetectable in normal mice, Il2−/− mice had significantly elevated concentration of this cytokine in serum (Figure 2B, p = 0.02). The mesenteric lymph nodes from Il2−/− and wild-type mice were analyzed for the presence of IL-17-expressing cells: consistent with the elevated concentration of IL-17 in serum, peripheral lymph node cells from Il2−/− lymph node cells had a greater proportion of IL-17-positive cells, most of which were CD4 positive (Figure 2C). Taking into account the peripheral lymphadenopathy associated with Il2−/− mice, there was a 30-fold difference in absolute numbers of mesenteric Th17 cells compared with wild-type mice (data not shown).

**IL-2 Deficiency Is Associated with Enhanced Th17 Cell Generation In Vivo**

Because of the widespread autoimmune disease in Il2−/− mice and the activated phenotype of CD4+ T cells from these animals, we thought it important to confirm the requirement of IL-2 for suppression of Th17 cells by using a different system. To eliminate the potentially confounding aspect of autoimmune disease, we used mice deficient in IL-2 that express a restricted T cell receptor
repertoire. Thus, B10.A Il2−/− Rag2−/− 5C.C7 mice, which express a transgenic T cell receptor (5C.C7) specific to the nonendogenous antigen pigeon cytochrome c (PCC), were used as a source of naive CD4+ T cells. Culture of the Il2−/− Rag2−/− 5C.C7 T cells under optimal Th17 differentiating conditions also resulted in a large proportion of IL-17-producing cells (Figure 3A, top). Again, the addition of exogenous IL-2 to the cultures substantially reduced Th17 polarization. In contrast, IL-23, anti-IFN-γ, and anti-IL-4 had no effect on polarization irrespective of IL-2, indicative of the fact that this population did not include significant number of memory CD4+ T cells (Veldhoen et al., 2006a). Finally, the addition of IL-2 to Il2−/− Rag2−/− CD4+ T cells stimulated under Th1-polarizing conditions (IL-12 and anti-IL-4) increased the proportion of cells expressing IFN-γ.

To further explore whether IL-2 has the capacity to regulate Th17 generation during a normal in vivo immune response, in the presence of normal numbers of Treg cells, no autoimmunity, and without the use of exogenous cytokines, we used an adoptive transfer approach. Naive B10.A Rag2−/− 5C.C7 TCR transgenic mice with (top) or without (bottom) deletion of the Il2 gene were injected i.v. into congenic (CD45.1) B10.A mice. The next day mice received s.c. injections of PCC peptide together with CFA. The draining lymph nodes were harvested after 7 days. Cells were stimulated for 4 hr with PMA and ionomycin, fixed and stained, and analyzed by flow cytometry. CD44, IL-17, and IFN-γ expression is shown for the gated CD45.2+ cell population. Representative data from four independent experiments are shown.

Figure 3. IL-2 Inhibits IL-17 Production in the Absence of Autoimmune Disease
(A) CD4+ T cells from B10.A Rag2−/− 5C.C7 TCR transgenic specific for pigeon cytochrome c with or without deletion of the Il2 gene were polyclonally stimulated for 3 days either in media alone (Th0); in the presence of IL-12 and anti IL-4 (Th1 conditions); in the presence of IL-23, anti-IFN-γ, and anti-IL-4; or in the presence of optimal Th17 differentiating conditions. The stimulations were performed in the presence or absence of IL-2 and subsequently stained for IFN-γ and IL-17. The numbers indicate the percentage of CD4+ T cells that express either IL-17 (top left quadrants) or IFN-γ (bottom right quadrants) and are representative of data from two independent experiments.
(B) CD4+ T cells from B10.A Rag2−/− 5C.C7 TCR transgenic mice with (top) or without (bottom) deletion of the Il2 gene were injected i.v. into congenic (CD45.1) B10.A mice. The next day mice received s.c. injections of PCC peptide together with CFA. The draining lymph nodes were harvested after 7 days. Cells were stimulated for 4 hr with PMA and ionomycin, fixed and stained, and analyzed by flow cytometry. CD44, IL-17, and IFN-γ expression is shown for the gated CD45.2+ cell population. Representative data from four independent experiments are shown.
reduced. The data shown in Figure 3B are representative of four experiments, which consistently showed a doubling of IL-17 producers. Although the proportions of IL-17 producers is small, it must be borne in mind that this experiment represents in vivo Th17 differentiation in response to vaccination, without addition of exogenous cytokines or blocking antibodies. In contrast with the donor cells, polyclonal nontransgenic host T cells were not activated in this setting. CD44 expression was used to monitor both the lack of responsiveness of the host cells (data not shown) and the activation of the adoptively transferred TCR transgenic T cells (Figure 3B, right). In both settings, the recipient mice were not deficient in IL-2 and it is possible that this diminished the proportion of Th17 cells present in the transgenic T cells. Nonetheless, in vivo, without addition of exogenous cytokines, the presence of IL-2 clearly inhibited the proportion of IL-17-producing T cells. Taken together, these data indicate that IL-2 regulates IL-17 production in vitro and in vivo. Moreover, the overproduction of IL-17 associated with IL-2 deficiency appears not to be secondary to the autoimmune disease but is a primary defect.

Opposing Roles of STAT3 and STAT5 in Th17 Differentiation

STAT3 is activated by IL-6 and IL-23, two cytokines that promote IL-17 production by T cells (Harrington et al., 2005; Veldhoen et al., 2006a). We therefore next analyzed the importance of this transcription factor in IL-17 regulation. In comparison to wild-type CD4+ T cells, T cells in which STAT3 amounts were reduced through a conditional Cre-lox targeted deletion revealed marked reduction in the proportion of IL-17-producing cells, indicating a key role in IL-17 regulation (Figure 4A). Conversely, the proportion of Foxp3-expressing T cells was increased when STAT5 was absent, indicative of its role in mediating IL-6 signaling to repress Foxp3 (Yao et al., 2007). The reduction in STAT3 was documented by immunoblotting (Figure 4B). The nuclear orphan receptor RORα was recently identified as a critical transcription factor in the expression of IL-17A by CD4+ T cells (Ivanov et al., 2006). We therefore compared RORα mRNA expression in Stat5-deleted CD4+ T cells compared with littermate controls. In keeping with previous reports (Ivanov et al., 2006), the expression of RORα was elevated under Th17 conditions in littermate controls but not in STAT3-deleted cells (Figure 4C). Moreover, the addition of human IL-2 resulted in a marked reduction in the expression of RORα, suggesting one mechanism of action by which IL-2 might inhibit Th17 differentiation (Figure 4D).

IL-2-Mediated Activation of STAT5 Opposes Th17 Cell Differentiation

IL-2 and other common γ chain (γc)-cytokines are potent activators of STAT5a and STAT5b, transcription factors that have critical in vivo functions in lymphoid development (Beading et al., 1994; Johnston et al., 1996; Yao et al., 2006). Deletion of STAT5a and STAT5b via a transgenic expression of CD4-Cre (Stat5a/bfl/fl; CD4-Cre) with resultant expression in DP thymocytes results in loss of CD8+ T cells in the periphery. The CD4+ T cells that are present overwhelmingly exhibit a memory cell phenotype (Yao et al., 2006). We therefore in vitro polarized Stat5a/bfl/fl; CD4-Cre and wild-type littermate splenic CD4+ T cells as before in the presence of exogenous IL-2 (Figure 5A). Wild-type T cells expressed similar numbers of Th17 cells as documented in previous experiments, whereas, even when STAT5-deficient CD4+ T cells were cultured under conditions that did not normally favor IL-17 production (Th0), a large proportion of T cells selectively produced IL-17 (Figure 5A, bottom left), suggesting that these cells were prepolarized in vivo. In normal naive CD4+ T cells, IL-23 does not induce differentiation into IL-17 producers, but rather acts on memory T cells to expand this population (Mangan et al., 2006; Veldhoen et al., 2006a). Accordingly, IL-23 exposure had minimal effects on IL-17 production in wild-type T cells. However, IL-23 increased IL-17 production in Stat5a/bfl/fl; CD4-Cre CD4+ T cells (Figure 5A, bottom middle), again consistent with the existence of a prepolarized or memory population of Th17 cells in Stat5a/bfl/fl; CD4-Cre mice.

To further explore the idea that Stat5a/bfl/fl; CD4-Cre mice contain a pre-existing population of peripheral lymphocytes that can readily produce IL-17, we activated Stat5a/bfl/fl; CD4-Cre CD4+ T cells with anti-CD3 and anti-CD28 and analyzed their competency to produce IL-17 by ELISA compared to littermate controls. We observed dramatically increased amounts of IL-17 protein in the Stat5a/bfl/fl; CD4-Cre cultures over those produced by cultures of littermate controls at multiple time points (Figure 5B). As further evidence of the tendency of STAT5-deficient T cells to produce IL-17, significantly elevated levels of this protein were detectable in the serum of Stat5a/bfl/fl; CD4-Cre mice, compared to wild-type littermates (p = 0.013) (Figure 5C). In interpreting this experiment, it should be kept in mind that Stat5a/bfl/fl; CD4-Cre mice are modestly lymphopenic compared to wild-type and IL2−/− mice; however, it is clear that absence of STAT5 is associated with overproduction of IL-17 in vivo.

To circumvent the apparent prepolarization of peripheral STAT5-deficient T cells, we next used CD4+ single-positive (SP) thymocytes from Stat5a/bfl/fl; CD4-Cre mice and littermate controls. In contrast to peripheral CD4+ lymphocytes from Stat5a/bfl/fl; CD4-Cre mice, these cells expressed naive cell markers (data not shown). CD4+ SP thymocytes were stimulated as before under Th0 conditions or in the presence of neutralizing antibodies together with IL-23, TGF-β1, and IL-6 or TGF-β1 and IL-6 and IL-2 (Figure 6A). Few wild-type or Stat5a/bfl/fl; CD4-Cre CD4+ SP thymocytes produced IL-17 in response to IL-23, indicative of the absence of memory T cells. Both cell populations produced IL-17 in response to TGF-β1 and IL-6; however, enhanced Th17 differentiation was seen in Stat5a/bfl/fl; CD4-Cre CD4+ SP thymocytes. Furthermore, the proportion of IL-17 producers was significantly reduced by the addition of IL-2 (p = 0.012) in wild-type cells, whereas this response was blocked in Stat5a/bfl/fl; CD4-Cre CD4+ SP thymocytes (Figure 6B).
Thus, STAT5a and STAT5b appear to be important in limiting Th17 differentiation.

As indicated above, by using chromatin immunoprecipitation (ChIP), we have previously shown that STAT3 directly binds to the Il17a promoter and that STAT5 directly binds the Foxp3 promoter (Chen et al., 2006; Yao et al., 2007). We therefore considered the possibility that another mechanism by which STAT5 might attenuate IL-17 production was by direct binding to the Il17a promoter. Initial investigation of the Il17a promoter sequences highlighted several potential STAT binding sites within −2K bp of the Il17a promoter. These sites were found within regions denoted A–D indicated in Figure S2. The binding of STAT5 to these regions was first assessed under conditions that would be expected to maximize its binding (Betti et al., 2006; Yao et al., 2007). Specific STAT5 binding was detected in all regions, although the region denoted D had the most consistent IL-2-inducible binding (Figure S2). Binding of STAT5 to the Foxp3 promoters was used as a control (data not shown; Yao et al., 2007). We next...
used quantitative PCR to more carefully measure the induction of STAT5 binding. Isolated CD4+ T cells were stimulated with anti-CD3 and anti-CD28, washed to remove endogenously produced cytokines, and restimulated with IL-2. In this setting, STAT5 specifically bound to the Il17a gene (Figure 6C). Not surprisingly though, CD4+ T cells activated with CD3 and CD28 alone (which induces endogenous IL-2 production) also had very appreciable amounts of binding of STAT5 to the Il17a gene.

**DISCUSSION**

In this study, we examined the role of IL-2 in Th17 generation and found that this cytokine appears to negatively regulate IL-17 production in vivo and in vitro. Il2−/− mice have widespread autoimmune disease, which has been attributed to impaired Treg cell development and/or function. Overproduction of IL-17 is a common feature of many autoimmune and autoinflammatory disorders, and dysregulation of IL-17 was a previously unappreciated aspect of autoimmunity associated with IL-2 deficiency. The inflammatory effects associated with overproduction of this cytokine help to explain the severe, systemic disease in Il2−/− mice. It will be of interest in the future to determine to what extent the pathology in these mice is IL-17 dependent.

Because of the systemic abnormalities associated with Il2−/− deficiency, we considered the possibility that the overproduction of IL-17 might be a secondary manifestation of disease and potentially not directly due to IL-2
itself. However, we also noted enhanced IL-17 production in TCR transgenic, IL-2-deficient mice that did not have autoimmune disease. Moreover, by using adoptive transfer experiments, we observed increased propensity of IL-2-deficient T cells to become IL-17 producers during a normal vaccination response. Thus, even in the context of normal numbers of Treg cells and without addition of exogenous cytokines or cytokine antibodies, the capacity of T cells to produce IL-2 regulated IL-17 production. This is not to say that in the context of autoimmunity other mechanisms might also contribute to IL-17 dysregulation; however, we believe that the present data argue that IL-2 is a relevant physiologic regulator of Th17 differentiation independent of autoimmune disease. Our data suggest a model in which IL-2, acting via STAT5, attenuates overproduction of IL-17, which in addition to its positive effects on Treg cells provides another mechanism to constrain immune-mediated damage to the host.

Recently, TGF-β1 has been implicated in the peripheral induction of both Treg and Th17 cells (Bettelli et al., 2006; Veldhoen et al., 2006a, 2006b). Whereas TGF-β1 is evidently important for both lineages in vitro, this cytokine...
clearly does not selectively direct lineage commitment; rather, the additional stimulus from proinflammatory cytokines produced by dendritic cells including IL-6 and IL-23 are needed. These cytokines activate STAT3 to direct and maintain Th17 differentiation. In this regard, it is notable that reduction in STAT3 levels resulted in impaired Th17 differentiation. Previous studies from our laboratory have documented direct binding of STAT3 to the Il17a promoter, suggesting a direct role in transcriptional regulation.

In contrast to IL-6 and IL-23, IL-2 provides a counterregulatory mechanism that promotes Treg differentiation and FoxP3 expression while concomitantly antagonizing Th17 polarization. In this regard, it is worth noting that Treg cells reportedly stimulate Th17 differentiation in vitro via TGF-β/1 production (Veldhoen et al., 2006a) but can also inhibit IL-2 production (Thornton and Shevach, 1998), which could also promote Th17 polarization. IL-2 preferentially activates STAT5, and in contrast to STAT3, STAT5 appears to negatively regulate IL-17 production. Like Il2−/− mice, Stat5−/− mice have widespread autoimmune disease (Antov et al., 2003; Snow et al., 2003). This has been attributed to impaired Treg cells, but STAT5-deficient CD4+ T cells also exhibit dysregulation of IL-17, which may be another contributor to the pathology in these mice. While the overproduction of IL-17 secondarily might also be related to loss of Treg cells, autoimmunity, or the memory phenotype of the CD4+ T cells, we found that naive STAT5-deficient CD4+ SP thymocytes also had increased propensity to become IL-17 producers, and IL-2-dependent inhibition of Th17 generation was blocked. Furthermore, STAT5 was found to bind the Il17a promoter, suggesting that in contrast to STAT3, it could serve as a repressor. This is of interest in that other cytokines, which activate other STAT family transcription factors, can also inhibit IL-17 production. Specifically, IL-4 (which primarily activates STAT6) and IFN-γ and IL-27 (which activate STAT1) all inhibit IL-17 production (Batten et al., 2006; Harrington et al., 2005; Stumhofer et al., 2006). We have also observed binding of STAT1 and STAT6 to the Il17a promoter (data not shown). This raises the intriguing possibility that different STAT family members can recruit different coactivators or regulators of chromatin modification. It will therefore be important to determine precisely how different STAT family members might positively and negatively IL17 transcription. Alternatively, although STAT family transcription factors have the potential to serve as direct repressors of Il17a, they may also have indirect effects. For instance, STATs regulate Socs3, which in turn also regulates Th17 cell differentiation (Chen et al., 2006). Additionally, the actions of STAT3 and 5 may occur via RORγt. Thus, STATs may have both direct and indirect modes of regulating IL-17 production.

In summary, IL-2 and STAT5a/b, which were known to be key regulators of Treg cells, also serve to constrain Th17 polarization. It is notable that a major cytokine, which promotes clonal expansion, curtails the generation of a highly inflammatory T cell subset. We envision the role of IL-2 as another means of limiting inflammation that might occur during immune responses. Homeostasis of both lineages, Treg and Th17 cells, by IL-2 can ensure that immune responses do not inflict damage to host tissues. Understanding the precise mechanisms by which cytokines and STAT family transcription factors regulate these lineages will certainly be of continuing interest.
analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Events were collected and analyzed with FlowJo software (Tree Star Inc, Ashland, OR). Cytokine production in cell culture supernatants was analyzed by enzyme-linked immunosorbent assay (ELISA) with mouse IL-17 Quantikine assay kits (R&D Systems) and mouse IL-27 Quantikine assay kits (R&D Systems) according to the manufacturer’s instructions.

**Determination of STAT3 Deletion**
STAT3 protein concentration in T lymphoblasts after treatment with IL-6 was determined by western blotting of whole-cell lysates with anti- phospho-STAT3 and anti-pan STAT3 polyclonal antibodies (Cell Signal Technology). Total protein was determined by reprobing membranes with actin monoclonal antibody (Chemicon International).

Preparation of cell lysates and immunoblotting were as described previously [Astoul et al., 2003].

**Determination of ROR-γ mRNA Expression**
cDNA was made from lysates of two million cells with the RNaseasy kit (Qiagen, MD). Quantitative PCR was performed with an ABI PRISM 7700 sequence detection system with site-specific primers and probes (Applied Biosystems, Foster City, CA).

**Chromatin Immunoprecipitation**
ChIP was performed as previously described [Chen et al., 2006]. To summarize, unless indicated otherwise, CD4+ Th cells were polyclonally activated for 3 days followed by resting in cytokine-free media for 24 hr followed by the addition of IL-2 (100 IU/ml) for 1 hr. DNA-bound transcription factors were subsequently crosslinked by infusing complete medium containing 1% formaldehyde for 10 min followed by sonication of the cell lysate. After preclearing with protein A agarose beads (Upstate, Charlottesville, VA), cell lysates from five million cells were immunoprecipitated with anti-STAT5 antibody (R&D Systems) or normal rabbit serum (Upstate USA, Chicago, IL) overnight at 4°C. After washing and elution, crosslinks were reversibly at 65°C for 4 hr. The eluted DNA was purified and samples analyzed by either conventional PCR or quantitative-PCR with β-actin promoter site-specific primers. Primers used are as follows. Region A, forward GCC TTT GTG ATT GTT TCT TGC, reverse TGT GAG TCT CTT GAC AGT CTC; Region B, forward ACC TCA TGC TGC ATG GAC GAG GTC AGT GAC GCT, reverse CTG AAA GAG AGT GAG GGC ACT; Region C, forward CAA TCA GAG GTG TGT GTG AGC, reverse GTC TGA CAT GCA GCA GGA TGA GGT; Region D, forward GCT CAG CAG TGA GTA CTG, reverse ACA TAC CAC AAC ATT GGC TTC. In the case of quantitative PCR, ABI PRISM 7700 sequence detection system (Applied Biosystems) was used with the following primers and probe: forward primer, GGA ATG GCT CAG CAG TTA AG; reverse primer, TGG TTT CTG GGA ATT GAA CTC A; probe, TAC TGA CTG CTC TGC CAG AGG. The Ct value for each sample was normalized to corresponding input value and expressed as fold induction relative to the normal rabbit serum control, which was arbitrary set as 1.0.

**Supplemental Data**
Two Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/26/3/371/DC1/.

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