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Priming for T helper type 2 differentiation by interleukin 2-mediated induction of IL-4 receptor α chain expression

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Abstract

T-helper type 2 (T_H2) cells are essential for humoral immunity and host defense. Interleukin (IL)-4 drives T_H2 differentiation and IL-2 augments *Il4* chromatin accessibility. Here we demonstrated that IL-2, by inducing STAT5 binding to the *Il4ra* locus, is essential for inducing and maintaining IL-4Rα expression. Although IL-4 induces IL-4Rα expression, T-cell receptor-induced IL-4Rα expression was normal in *Il4*$^{-/-}$ but profoundly diminished in *Il2*$^{-/-}$ cells. Remarkably, forced IL-4Rα expression rescued T_H2 differentiation in *Il2*$^{-/-}$ cells. Moreover, genome-wide mapping by ChIP-Seq reveals broad interaction of STAT5A and STAT5B with genes associated with T_H2 differentiation. These results reveal a previously unappreciated function for IL-2 in 'priming' T cells for T_H2 differentiation and in maintaining expression of *Il4ra* and other genes in T_H2-committed cells.

Introduction

$CD4^+$ T helper cells can differentiate into different functional subsets defined by patterns of cytokine production (T helper type 1 (T_H1), T_H2, T_H-17). Differentiation into these specialized subsets is mediated at least in part by the actions of specific signal transducer and activator of transcription (STAT) proteins (STAT4, STAT6, and STAT3) that control the transition of precursor cells into mature T_H1, T_H2, or T_H-17 cells, respectively 1-5. T_H1 cells are vital for the control of infections by viruses and other intracellular pathogens and are identified by the production of interferon (IFN)-γ, whereas T_H2 cells are important in allergic responses as well as for the clearance of helminths and other parasites and produce interleukin (IL)-4 (http://www.signaling-gateway.org/molecule/query?afcsid=A001262), IL-5, and IL-13[1]. T_H-17 cells produce IL-17A, IL-17F, IL-21, and IL-22, and are important in host defense against certain bacteria and fungi and implicated in autoimmune diseases including Crohn's disease and psoriasis 4,6.

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Previous studies have indicated that T_H2 differentiation is characterized by a STAT protein-dependent initiation phase, a commitment phase dependent on the transcription factor GATA3, and a final stabilization phase in which *Il4* transcription is maintained without further stimulation 3,7-9. IL-4 drives T_H2 differentiation; STAT6 has been considered to be the most important STAT protein for mediating IL-4 signaling 10,11, and STAT5A (http://www.signaling-gateway.org/molecule/query?afcsid=A002234) was reported to augment IL-4 production by altering chromatin accessibility at the *Il4* gene locus in differentiated T_H2 cells 12. However, little is known regarding the initiation phase of T_H2 differentiation. The cellular source of the initial IL-4 production in T_H2 differentiation remains unclear, with $NK1.1^+$ $CD4^+$ T cells, conventional $CD4^+$ memory T cells, eosinophils, mast cells, and basophils as possible contributors 13,14. In order to be able to respond to IL-4, it is clear that cells must express IL-4Rα (http://www.signaling-gateway.org/molecule/query?afcsid=A001263), which is an essential component of both type I and type II IL-4 receptors 15-18. Because resting T cells express little if any IL-4Rα 19, IL-4Rα induction must be another key control point that allows priming of cells for T_H2 differentiation. Unlike the *Il4* gene 3,7-9, relatively little is known about the molecular basis of *Il4ra* regulation.

We previously used DNA arrays to identify genes that are regulated by IL-2 20,21. These genes include those encoding cytokine receptors; IL-2 potently induced IL-2Rα yet repressed IL-7Rα 21. Examination of the array data revealed that IL-2 also induced IL-4Rα expression. We sought to validate this observation and to investigate its potential biological importance.

We now demonstrate that IL-2 potently up-regulates IL-4Rα expression in T cells shortly after T cell receptor (TCR) stimulation, and that IL-2 rather than IL-4, which also is known to be a key regulator of IL-4Rα expression 22,23, is required for TCR-induced IL-4Rα expression. We also show that defective T_H2 differentiation in $Il2^{-/-}$ mice can be rescued by the addition of IL-2, but also by transduction with a retrovirus encoding IL-4Rα, even when no IL-2 is added. These data establish IL-2—dependent IL-4Rα induction as an important TCR-induced priming step for T_H2 differentiation.

Results

IL-2 potently induces IL-4Rα expression

We first confirmed our early DNA array observation that IL-2 could induce IL-4Rα expression 21. We pre-activated mouse splenic T lymphocytes with anti-CD3 and anti-CD28 for 48 h, rested the cells overnight, and cultured them for 4 h with 0, 10, or 100 U/ml of IL-2. IL-2 induced IL-4Rα mRNA expression in a dose-dependent fashion, similar to the induction of expression of the *Pim1* gene, which was previously shown to be IL-2—dependent 24(Fig. 1a). In contrast *Stat5b* (http://www.signaling-gateway.org/molecule/query?afcsid=A002235), which is not an IL-2 target gene, was not induced (Fig. 1a). IL-2 also increased cell surface IL-4Rα expression in a dose-dependent fashion (Fig. 1b); a marked increase in IL-4Rα protein expression was confirmed by immunoblotting (Fig. 1c). Similarly, IL-2 induced IL-4Rα mRNA and cell surface expression in human peripheral blood T cells pre-activated with anti-CD3 and anti-CD28 (Fig. 1d,e). As previously reported 22,23, IL-4 also potently induced IL-4Rα expression (Fig. 1d). *Pim1* was induced by IL-2

but not by IL-4, whereas *Stat5b* mRNA was not induced by either cytokine (Fig. 1d). The increased IL-4Rα expression was functional, as IL-4 induced augmented expression of *Gfi1*, an IL-4—regulated gene 25, in cells that were pre-treated with IL-2 (Fig. 1f).

Because IL-4 can potently induce IL-4Rα expression, and IL-2 can elevate the production of IL-4 26, it was possible that the induction of IL-4Rα by IL-2 was indirectly mediated by its induction of IL-4. However, although IL-2—induced IL-4Rα expression tended to be somewhat lower in *Il4*$^{-/-}$ mice than in WT mice, suggesting a partial requirement for IL-4, IL-2 potently induced IL-4Rα expression even in the absence of IL-4, indicating existence of a more direct IL-4-independent mechanism (Fig 2a,b). As anticipated, anti-IL-4Rα staining of cells from *Il4ra*$^{-/-}$ mice was similar to that of the isotype control, indicating the specificity of the IL-4Rα antibody (Fig. 2a).

STAT5 mediates IL-2-induced IL-4Rα expression

STAT5A and STAT5B are encoded by head-to-head tandem genes and both proteins are activated by IL-2 as one of its major signaling pathways 27. We therefore evaluated IL-4Rα expression in CD4^{+} T cells from *Stat5b* transgenic mice 28 and found increased IL-4Rα expression (Fig. 3a). We next isolated splenic T cells from *Stat5a*$^{f/f}$*Stat5b*$^{f/f}$ mice 29, transduced them with a retrovirus expressing *Cre* recombinase to delete the *Stat5a* and *Stat5b* loci, cultured the cells in the presence of IL-2 for 16 h, and generated cRNA that was used to screen a limited DNA array (GEArray Q Series mouse Signal Tranduction in Cancer Gene Array). As expected, expression of *Stat5a* and *Stat5b* was decreased, indicative of successful Cre-mediated deletion (Fig. 3b). Expression of *Pim1* was also decreased, whereas expression of cathepsin D (*Ctsd*), which is not known to be regulated by STAT5, was not diminished (Fig. 3b). Consistent with the reported role for STAT5 in chromatin accessibility of the *Il4* locus 12, *Il4* mRNA was slightly diminished, but we observed an even greater defect in *Il4ra* mRNA expression, indicating that IL-4Rα expression is dependent on STAT5 (Fig. 3b). Expression of some genes on the array, such as *Mdm2*, was increased (Fig. 3b and Supplementary Table 1, online).

We next examined the *Il4ra* gene for the presence of TTCN$_3$GAA IFN-γ activated sequence (GAS) motifs that can potentially bind STAT5 30. We found five canonical GAS motifs in the region from -5 kb through the first intron of the mouse *Il4ra* gene (Fig. 3c). GAS1 is 5′ of the *Il4ra* transcription initiation site (TIS), whereas the other GAS motifs are in intron 1 (Fig. 3c). In luciferase reporter assays (please see **Supplementary Methods** for details of plasmids and luciferase reporter assays), only the *Il4ra* promoter constructs containing GAS2 and GAS3 exhibited significant, albeit modest, IL-2—inducible expression (approximately 1.9 fold); duplicating the GAS2 + GAS3 fragment further increased IL-2 inducibility (approximately 2.6 fold) (Fig. 3d). Mutation of GAS3, but not GAS2, eliminated almost all IL-2 inducibility (Fig. 3d); thus, GAS3 contributed markedly to IL-2—induced IL-4Rα reporter activity. Nuclear extracts from IL-2—stimulated human peripheral blood T cells bound to the GAS3 motif probe, forming complex C1, as evaluated by electrophoretic mobility shift assays (Fig. 3e), and this complex was supershifted by antibodies to STAT5A or STAT5B but not by an antibody to STAT3, which is only weakly activated by IL-2 17. A mutant probe (gTCTAAcAA instead of TTCTAAGAA) did not bind any factor. Chromatin

immunoprecipitation (ChIP) analysis confirmed IL-2—induced binding of STAT5A and STAT5B to the region spanning the GAS3 motif, and weaker binding was observed at GAS5 as well (Fig. 3f). As expected, strong STAT5 binding was also observed when we used a known STAT5 binding site from the *Socs3* promoter region 31 as a positive control (Fig. 3f).

As part of a separate project to map the STAT5A and STAT5B binding sites in the human genome, we used pre-activated human $CD4^+$ T cells and ChIP coupled to Solexa sequencing (ChIP-Seq technique; see **Supplementary Methods**) 32. In these cells, IL-2 induced binding of both STAT5A and STAT5B to intron 1 of the *IL4R* gene, with highest tag numbers at the site that corresponds to the mouse *Il4ra* GAS3 region (Fig. 4a,b; see also the alignment of human and mouse sequences in Fig. 4c). A second major binding peak that corresponds to the mouse GAS5 motif was also detected; note that GAS5 in human is a TTCTGGaAA variant of the canonical TTCTGGGAA motif in the mouse (Fig. 4a-c).

STAT5 DNA binding during T_H2 differentiation

We next analyzed STAT5 DNA binding under conditions of T_H2 differentiation in mouse T cells. Because IL-2 regulates both the *Il4* 26 and *Il4ra* (Fig. 3f) loci via STAT5, we extended our ChIP-Seq analysis to study STAT5 binding to these genes at two early time points (8 and 13 h) after initiating T_H2 differentiation, and at a late time point after 2 rounds of T_H2 polarization (see Fig. 5 legend). We applied a motif discovery algorithm to classify predicted STAT5A and STAT5B peaks (see **Supplementary Methods** for details). Read numbers and predicted peak numbers for ChIP-Seq libraries are listed in Supplementary Table 2, online. The motif with the best score and thus lowest error rate at each time point for STAT5A and STAT5B was highly similar to the known GAS motif, with the highest degree of sensitivity tending to occur after two rounds of T_H2 differentiation and in the same general range as that reported in a ChIP-Seq analysis for another sequence-specific transcription factor, NRSF (Table 1)33.

Although some binding of STAT5A and STAT5B was evident at the *Il4ra* GAS3 motif by 8 h, stronger binding for STAT5A and STAT5B was evident at 13 h, as evaluated by tag number, and STAT5A and STAT5B binding was sustained or further enhanced after 2 rounds of T_H2 polarization (Fig. 5a-c). At the GAS1 region, a strong peak was evident in most samples, including the IgG control, indicating that it was non-specific (Fig. 5a-c). We detected little if any STAT5 binding at the other *Il4ra* GAS motifs except for the GAS5 motif (Fig. 5b,c). Binding at the GAS5 region was consistent with binding at this site in human cells (Fig. 4), suggesting that GAS5 may also contribute to *Il4ra* gene regulation, even though we did not observe activity of the GAS5 motif in the context of limited reporter constructs in luciferase assays (Fig. 3d).

After two rounds of T_H2 polarization, when STAT5A and STAT5B ChIP-Seq analysis was performed, peaks were observed at the principal *Il4-Il13-Il5* cluster DNase I hypersensitivity regions. These included HSII (in the *Il4* gene), which was previously identified as being capable of binding STAT5A 12, HSV (between the *Il4* and *Kif3a* genes), CNS1 (between the *Il13* and *Il4* genes), CGRE (in the *Il13* promoter), and the locus control region (LCR) C and B hypersensitivity sites in the *Rad50* gene, with weaker binding at HSIII, LCR A and O

hypersensitivity sites, and within the *Il5* locus; in contrast, at the 8 and 13 h time points, only weak peaks at HSII, HSV, and LCR B and C regions were observed (Fig. 5d-f, Supplementary Fig. 1). Interestingly, after two rounds of T_H2 differentiation, strong peaks were also observed in the *Kif3a*, which is adjacent to the *Il4* gene (Fig. 5f), as well as at the *Maf* and *Gata3* loci (Supplementary Fig. 2a,b). These data are consistent with a potential broad role for STAT5 for many factors associated with T_H2 differentiation. The genes with STAT5A and STAT5B ChIP-Seq peaks at 8 h, 13 h, and after two rounds of T_H2 differentiation are shown in Supplementary Tables 3-8.

To verify that STAT5 binding to GAS3 was induced by IL-2, we used a combination of antibodies to IL-2, IL-2Rα, and IL-2Rβ to block IL-2 signaling in late phase T_H2 cells and examined STAT5B binding by ChIP (Fig. 5g). Treatment with the antibodies lowered STAT5B binding to the *Il4ra* GAS3 region and in the *Socs3* promoter (positive control) but not at the *Il4ra* GAS2 region or to the *Gapdh* gene (negative control) (Fig. 5g). Notably, binding at the *Il4* HSII region was also IL-2—dependent, whereas the weak binding at HSIII was not markedly affected by the antibody treatment (Fig. 5g). Binding of STAT5 proteins to HSV was not anticipated, and this region does not contain a TTCNNNGAA GAS motif, but we identified a TTGNNNTAA motif and used classical ChIP to confirm STAT5A and STAT5B binding (Fig. 5h). Our results collectively indicate that STAT5 proteins bind to the *Il4ra* locus by 8 h after cellular stimulation with IL-2. This binding of STAT5 was maintained or increased following two rounds of T_H2 polarization, suggesting that STAT5 proteins enable and promote expression of IL-4Rα during T_H2 differentiation. STAT5 occupancy at the *Il4* locus and nearby genes was observed primarily later in T_H2 differentiation.

Defective TCR-induced IL-4Rα in *Il2*$^{-/-}$ T cells

Above, we showed that after TCR stimulation, IL-2—dependent IL-4Rα expression was substantially independent of IL-4. However, given that both IL-2 and IL-4 can induce IL-4Rα expression, what then is the relative importance of these cytokines during TCR-induced IL-4Rα expression? As expected, we found similar basal IL-4Rα expression on $CD4^+$ T cells from *Il4*$^{-/-}$ mice and control littermates, and on *Il2*$^{-/-}$ mice and their control littermates (Fig. 6a,b upper panels). However, whereas IL-4Rα expression was similarly induced by TCR stimulation in *Il4*$^{-/-}$ and control T cells, we noted a marked defect in IL-4Rα induction in *Il2*$^{-/-}$ T cells (Fig. 6a,b lower panels). Addition of exogenous IL-2 corrected this defect (Fig. 6b). Thus, although IL-4 can induce IL-4Rα expression, only IL-2 is required for TCR-induced IL-4Rα expression. Consistent with this finding, 4 h after treatment of cells with anti-CD3 and anti-CD28, a time point during which only relatively low levels of IL-2 protein were produced, no increase in IL-4Rα mRNA was observed whereas expression of IL-2Rα mRNA, which is induced directly by TCR stimulation, was markedly increased (Fig. 6c).

IL-2—induced IL-4Rα in T_H2 differentiation

We next investigated whether the amount of IL-4Rα expression correlated with the degree of T_H2 cell differentiation. To this end, we examined IL-4Rα expression in $CD4^+$ T cells from *Il4ra*$^{+/+}$, *Il4ra*$^{+/-}$ and *Il4ra*$^{-/-}$ Balb/c mice and found that T cells from the heterozygous

mice had approximately half the quantity of IL-4Rα expression found in *Il4ra*$^{+/+}$ T cells (Fig. 7a). We then measured intracellular IL-4 expression in cells polarized in T_H2 conditions for 92 h. The number of IL-4—producing cells correlated with the amount of IL-4Rα expression (Fig. 7b). This observation suggests that the extent of T_H2 differentiation depends on the extent of IL-4Rα expression, and that IL-2—mediated regulation of IL-4Rα expression plays an important role in T_H2 differentiation.

Il2$^{-/-}$ CD4$^+$ T cells were previously reported to exhibit defective T_H2 differentiation 26. To investigate whether IL-2—regulated IL-4Rα expression served as a critical control point for T_H2 differentiation, we investigated whether transduction of *Il2*$^{-/-}$ CD4$^+$ T cells with an IL-4Rα retrovirus could restore T_H2 priming in the absence of IL-2. Indeed, whereas a control retrovirus had little effect, retroviral transduction of IL-4Rα resulted in an increase in IL-4—producing cells, even in a setting where no endogenous or exogenous IL-2 was available (Fig. 8a). We confirmed that we were detecting only intracellular IL-4 rather than exogenously added IL-4 by showing that IL-4 was only identified in the permeabilized cells (Fig. 8a). To further analyze this effect, we divided IL-4Rα—transduced *Il2*$^{-/-}$ CD4$^+$ T cells according to low, medium, or high retroviral GFP expression as an indicator of transduction efficiency, which corresponded to the amount of IL-4Rα expression (Fig. 8b). Importantly, IL-4Rα expression directly correlated with the number of IL-4—producing cells (Fig. 8b). As expected, IL-2 stimulation augmented IL-4Rα expression and thus IL-4 production in *Il2*$^{-/-}$ CD4$^+$ T cells, comparable to the amounts observed in wild-type CD4$^+$ T cells (Fig. 8b). When we further subdivided the IL-4Rα—transduced *Il2*$^{-/-}$ T cells according to GFP fluorescence intensity as an indicator of IL-4Rα expression, it became evident that the percent of IL-4—producing cells increased as the intensity of GFP increased (Fig. 8b), further confirming the conclusions of Fig. 7, based on the analysis of *Il4ra*$^{-/-}$, *Il4ra*$^{+/-}$, and *Il4ra*$^{+/+}$ mice. These results reveal that IL-2—induced IL-4Rα expression is vital for T_H2 differentiation and that the requirement for IL-2 could be eliminated by retroviral transduction of IL-4Rα.

Above, we have demonstrated a role for IL-2 in regulating IL-4Rα expression. We hypothesized that other cytokines that activate STAT5 proteins could presumably also contribute to this process. Indeed, like IL-2, IL-15 can also increase IL-4Rα expression in activated T cells, which express IL-15Rα, but not in resting T cells, which do not express IL-15Rα; IL-7 can induce IL-4Rα on both populations of cells (Figure 9). In view of the broad range of cytokines that can activate STAT5 27, including in non-lympho-hematopoietic cells where IL-2 cannot act, we hypothesize that additional cytokines might also act via STAT5 to augment IL-4Rα expression and thus prime cells for T_H2 differentiation and/or responsiveness to IL-4.

Discussion

T_H2 differentiation is known to be driven by IL-4 in a STAT6-dependent fashion 10,11; in addition, IL-2 and STAT5 proteins critically regulate this process 12,26,34. A role for IL-2 in altering chromatin accessibility at the *Il4* gene in a STAT5A-dependent fashion in T_H2 cells was established12. However, ‘priming’ must occur to allow efficient responsiveness to

IL-4. This clearly requires induction of IL-4Rα, which plays a central role in mediating signals by IL-4 and IL-13.

In lymphocytes, the functional IL-4 receptor consists of IL-4Rα plus γ_c (the type I IL-4 receptor), whereas in non-immune cells, the functional IL-4 receptor is IL-4Rα plus IL-13Rα1 (the type II IL-4 receptor); this latter receptor is also the functional receptor for IL-13 15-17. Targeted disruption of the *Il4ra* gene in mice prevents responsiveness to IL-4 and IL-13 and normal T_H2 cell differentiation, with abrogation of the IgE response to parasites, defective allergen sensitization, diminished airway hypersensitivity, and defective mucus secretion 18,35-38.

Despite the important role played by IL-4Rα, little is known of the molecular mechanisms regulating its expression. We herein demonstrated that IL-2 up-regulates expression of the *Il4ra* gene in a STAT5-dependent manner, and thereby promotes augmented IL-4Rα expression and 'priming' cells for responsiveness to IL-4 and T_H2 differentiation after TCR stimulation. As T_H2 differentiation proceeds, STAT5 binding to the *Il4ra* gene is maintained or even further enhanced. In the absence of IL-2, T_H2 differentiation does not progress, but notably can be rescued by retroviral expression of IL-4Rα, underscoring that IL-4Rα induction is a critical IL-2— and STAT5-dependent control step in priming cells for T_H2 differentiation. IL-2 is a TCR-induced cytokine with pleiotropic actions; IL-2 influences T cell proliferation, activation-induced cell death, regulatory T cell development, and B and NK cells 39. In this study, we highlight IL-4Rα upregulation is another critical function of IL-2.

Herein, we also showed that both STAT5A and STAT5B can bind to the *Il4* locus in cells after T_H2 differentiation, consistent with an earlier report focusing on the role of STAT5A 12; our findings differ from this earlier study in indicating a role for STAT5B as well as STAT5A, and moreover we discovered that STAT5 proteins bind to HSV, the LCR, and more broadly within the genes within the T_H2 locus, as well as to the *Maf* and *Gata3* genes. In addition, our data reveal that STAT5 proteins also bind to the *Il4ra* locus relatively early in the T_H2 differentiation process, helping to increase IL-4Rα expression and cellular responsiveness to IL-4.

In summary, we have identified a critical cross-talk among γ_c family cytokines, including IL-2 and others that activate STAT5, that promotes IL-4Rα expression. This induction of IL-4Rα primes cells for responsiveness to IL-4 and thus is a critical early step in the initiation of T_H2 responses. Moreover, our study reveals STAT protein occupancy of T_H2 genes many days after the induction of T_H2 differentiation. Given the very transient nature of STAT protein activation based on *in vitro* assays, sustained STAT occupancy of GAS motifs within chromatin was unanticipated and indicates the importance of STAT proteins in not only the induction but also in the maintenance of a differentiated state.

Methods

Isolation and culture of mouse splenocytes

Stat5a$^{f/f}$*Stat5b*$^{f/f}$ mice 29 and *Stat5b* transgenic mice 40 have been described. *Il2*$^{-/-}$ mice on a *Rag2*$^{-/-}$ 5C.C7 TCR transgenic background (line 110) and controls (5C.C7 *Rag2*$^{-/-}$, line 94) were from Taconic Farms. C57BL/6 *Il4*$^{-/-}$ and *Il4ra*$^{-/-}$ mice were from the Jackson lab. Animal protocols were approved by the NHLBI Animal Care and Use Committee and followed the NIH Guidelines "Using Animals in Intramural Research." Splenic total T cells or CD4^{+} subpopulations were purified from 5-12 week old mice by negative or positive selection using magnetic beads (Miltenyi), cultured in RPMI 1640 medium containing 10 mM Hepes (pH 7.0), 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (complete medium), and activated for 1.5 h at 37°C in dishes pre-coated with anti-CD3 (2 μg/ml in PBS) in complete medium containing 1 μg/ml anti-CD28 (PharMingen). Cells were washed, rested overnight in complete medium, and expanded in complete medium containing 100 U/ml IL-2.

Quantitative RT-PCR

Total RNA was isolated using TRIzol (Invitrogen). First-strand cDNAs were made using the Omniscript reverse transcription kit (Qiagen). Quantitative real time PCR was performed on a 7900H sequence detection system (Applied Biosystems) and expression level of each gene was normalized to *Rpl7*, a ribosomal protein gene. Sequences of the primers and probes are in Supplementary Table 9.

Flow cytometric analyses

Splenocytes or purified CD4^{+} T cells were with stained phycoerythrin (PE)-anti-IL-4Rα (mIL4R-M1), Cy-chrome-anti-CD4 (L3T4), allophycocyanin-anti-CD8 (Ly2), PE-anti-IL-4 (11B11), FITC-anti-IFN-γ (XMG1.2), and isotype-matched control antibodies (PharMingen), and analyzed on a FACSort (Becton Dickinson) using CELLQUEST or FlowJo software.

Immunoblotting

Purified CD4^{+} T cells not stimulated or stimulated with IL-2 were washed in ice-cold PBS, suspended in lysis buffer (50 mM Tris·HCl, pH 7.5 containing 150 mM NaCl, 0.5% Nonidet P-40, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and incubated on ice with occasional shaking for 45 min. Cell lysates were collected by centrifugation at 4°C for 15 min, and 20-60 μg of protein was resolved by 4-12% SDS-PAGE and immunoblotted with antibodies to mouse or human IL-4Rα protein (Santa Cruz Biotechnology).

T_H polarization

Splenic CD4^{+} T cells (approximately 99% pure by flow cytometry) from line 94, line 110, or from Balb/c background *Il4ra*$^{+/+}$, *Il4ra*$^{+/-}$, or *Il4ra*$^{-/-}$ mice, were isolated using a kit (Miltenyi Biotec). T-depleted antigen-presenting cells (APCs) were prepared by incubating spleen cells with anti-Thy1.2 and rabbit complement (Cedarlane Laboratories Limited) at

37°C for 45 min, and irradiated with 30 Gy (3000 rad). $CD4^+$ T cells from lines 94 or 110 were co-cultured with APCs at a 1:5 ratio with 1 μM pigeon cytochrome C peptide; for T_H1 conditions, anti-IL-4 (11B11, 10 μg/ml) plus IL-12 (10 ng/ml) were added; for T_H2 conditions, IL-4 (1000 U/ml), anti—IFN-γ (10 μg/ml)were added, with anti—IL-12 (10 μg/ml) added for retroviral transduction of IL-2—deficient cells but omitted for ChIP-Seq experiments, which used purified $CD4^+$ T cells.

IL-4Rα retroviral construct and intracellular staining

Mouse IL-4Rα cDNA was PCR-amplified using high-fidelity PCR kit (Invitrogen) and cloned into the pGFP-RV *Bgl*II site to yield pGFP-RV-mIL-4Rα and sequenced. Retroviruses were packaged as described in Supplementary Table 1. For retroviral transduction, 1×10^6 purified $CD4^+$ T cells from line 94 or 110 mice were activated for 24 h under T_H2 conditions with 1 μM pigeon cytochrome C peptide and 5×10^6 irradiated T-depleted spleen cells. Supernatant was replaced with a virus-containing supernatant containing 8 μg/ml polybrene (Sigma), IL-4, anti—IFN-γ, and anti—IL-12. Plates were centrifuged at 1,000 × *g* for 45 min at room temperature. Retroviral transduction was repeated 24 h later, fresh medium containing the same cytokines was added, and 72 h later, cells were restimulated with 25 ng/ml PMA and 1 μg/ml ionomycin for 6 h and treated with BD GolgiPlug (BD Bioscience) for 4 h. For staining, cells were incubated with permeabilization buffer (BD Biosience) and antibodies for 40 min. Data were analyzed with CELLQUEST and FLOWJO software (Becton Dickinson). Percent cytokine-producing cells was obtained by gating on live $CD4^+$ T cells with forward-versus-side scatter profiles and GFP^+ staining.

Short read data

The short read data for Fig. 5 and Supplementary Figs. 1 and 2 have GEO accession number GSE12346.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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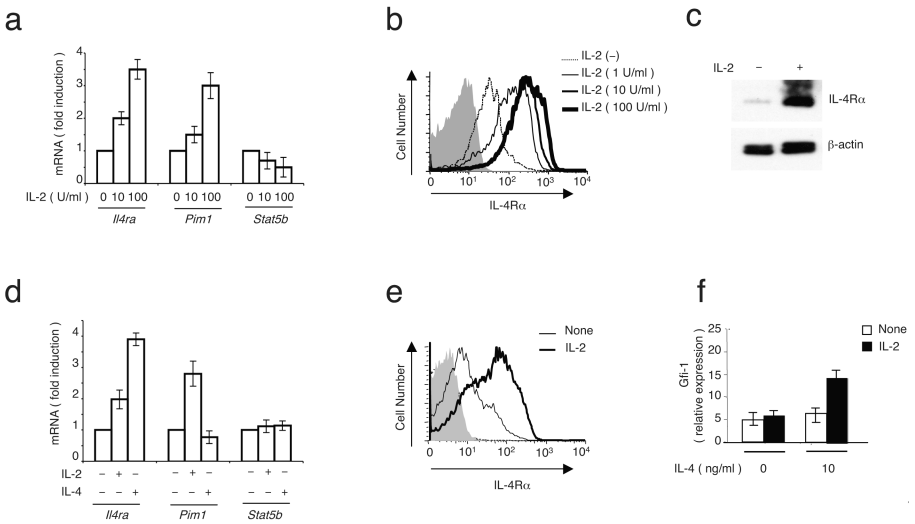

Figure 1.

IL-2 potently induces IL-4Rα expression. (**a—c**) Mouse splenic T lymphocytes were pre-activated by anti-CD3 and anti-CD28 for 48 h, rested overnight, and then 0, 10, or 100 U/ml of IL-2 was added for 4 h. (**a**) Real time PCR was used to measure expression of the indicated mRNA transcripts. (**b,c**) Flow cytometry (**b**) and immunoblotting (**c**) were used to measure cell surface (**b**) and total (**c**) IL-4Rα expression. (**d**) Increased *IL4R* mRNA expression in human peripheral blood T cells pre-activated with anti-CD3 and anti-CD28 and then stimulated with IL-2 or IL-4 for 4 h. (**e**) Increased IL-4Rα protein expression in human T cells pre-activated with anti-CD3 and anti-CD28 and then treated with IL-2 for 16 h. (**f**) Purified splenic $CD4^+$ T cells were pre-activated with anti-CD3 and anti-CD28 for 72 h, washed and incubated without or with 10 U/ml IL-2 for 16 h, then washed twice with PBS, rested 18 h, and cultured without or with 10 ng/ml IL-4 for 4 h. Gfi1 mRNA was measured by RT-PCR. For each panel, 3-5 independent experiments were performed.

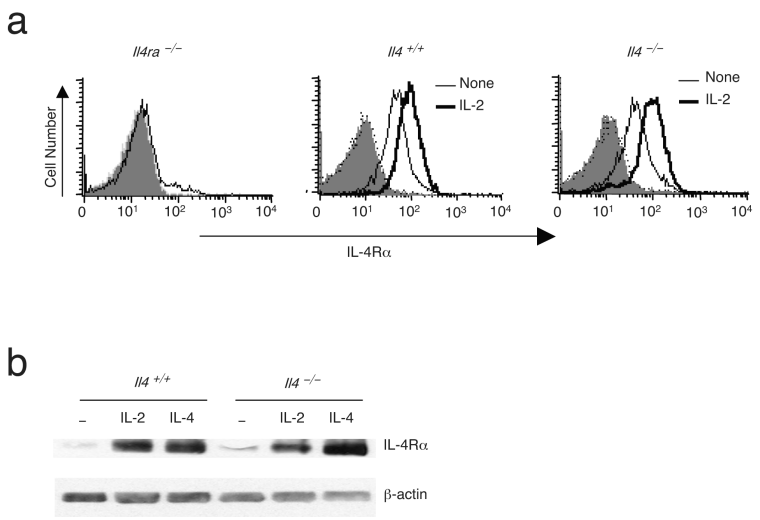

Figure 2.

IL-2-induced IL-4Rα expression is independent of IL-4. (**a,b**) Splenic $CD4^+$ T cells from *Il4*$^{-/-}$ mice were pre-activated with anti-CD3 and anti-CD28 for 48 h, washed, and then 0 or 100 U/ml of IL-2 was added. IL-4Rα expression was measured by flow cytometry (**a**) or immunoblotting (**b**). Shown are results representative of five **(a)** or three **(b)** independent experiments.

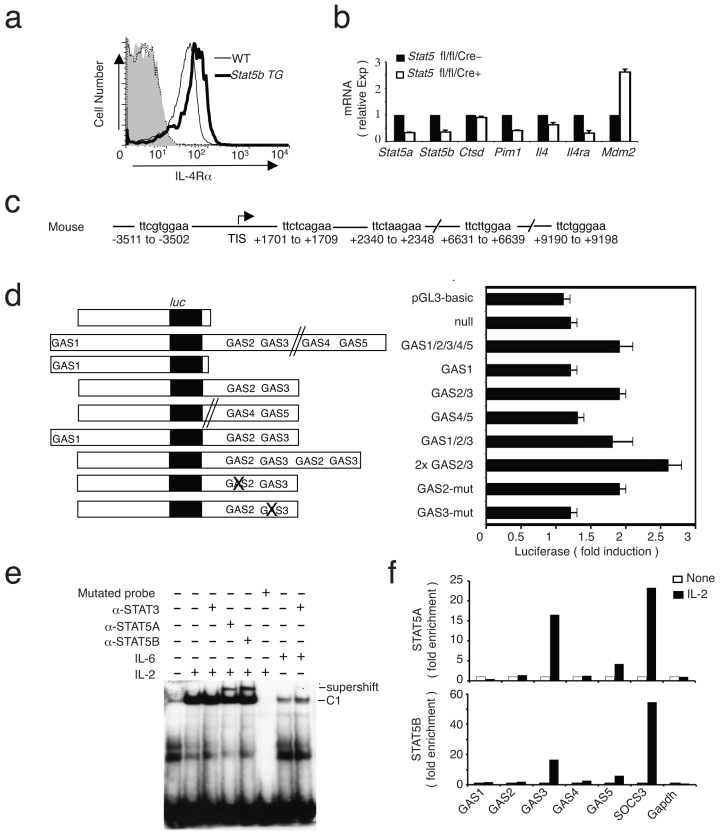

Figure 3.

STAT5-dependent regulation of IL-4Rα expression. (**a**) IL-4Rα expression on splenic CD4$^+$ T cells from wild-type and *Stat5b* transgenic mice 40, as evaluated by flow cytometry. The experiment shown is representative of two independent experiments with 2 to 3 mice in each group. (**b**) Expression of indicated transcripts, after Cre recombinase-mediated deletion of the LoxP-flanked *Stat5a*/*Stat5b* locus 29 in splenic T cells that then were cultured with IL-2. See Supplementary Table 1, online for the entire list of genes. Three independent experiments were performed. (**c**) Schematic of five TTCN$_3$GAA potential GAS motifs in the mouse *Il4ra* gene 5′ regulatory region and first intron. GAS1 is approximately 3.5 kb (mouse) or 1.5 kb (human) 5′ of the *Il4ra* transcription initiation site (TIS), whereas GAS2, GAS3, GAS4, and GAS5 are in the first intron. (**d**) Indicated PCR-generated constructs (left; *luc*, luciferase) were transfected into YT cells not treated or treated with 100 U/ml of IL-2 and cell lysates were analyzed for luciferase activity (right). Three independent experiments were performed. (**e**) EMSA 41 using an *Il4ra* probe spanning GAS3 and nuclear extracts 41 from human peripheral T cells. Cells were untreated or treated with IL-2 or IL-6. For supershifting assays, each antiserum was pre-incubated with nuclear extracts before adding labeled probe. In lane 6, a probe mutated at GAS3 was the control. The experiment shown is representative of three independent experiments. (**f**) ChIP assays 41 of STAT5A and STAT5B binding using CD4$^+$ splenic T cells from Balb/c mice pre-activated with anti-CD3 and anti-CD28 for 3 days, rested overnight, not treated or treated with 100 U/ml IL-2 for 4-5 h at 37 °C, followed by cross-linking with formaldehyde. Nuclear lysates

were immunoprecipitated at 4°C overnight with anti-STAT5A, anti-STAT5B (R&D Systems) or an isotype control antibody to allow normalization of the fold induction by IL-2. After deproteination and cross-link reversal, selected DNA sequences were assessed by real-time PCR. Primers spanning the *Socs3* STAT binding site were used as a positive control and *Gapdh* as a negative control. See Supplementary Table 10 for sequences or primers used in ChIP experiments. The experiment shown is representative of three independent experiments.

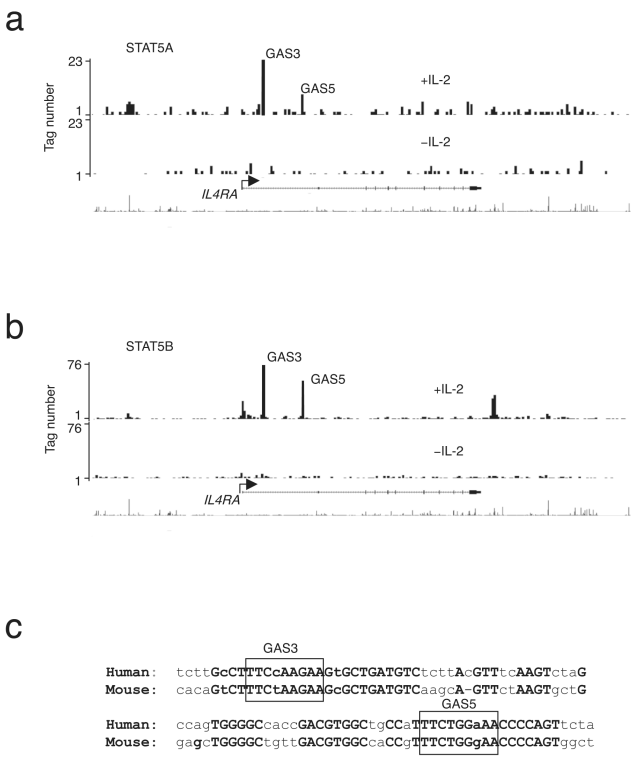

Figure 4.

Analysis of STAT5 binding sites in the human *IL4R* gene. **(a,b)** Human T cells were pre-activated, not stimulated or stimulated with IL-2, and then ChIP-Seq analysis was performed using antibodies for STAT5A and STAT5B. Distribution of STAT5A **(a)** and STAT5B **(b)** protein binding locations are shown as custom tracks on the UCSC genome browser. Samples were from cells stimulated or not stimulated with IL-2 as indicated. The direction of transcription is indicated by the arrow. **(c)** Sequence comparison between human and mouse in the GAS3 and GAS5 regions; the GAS motifs are boxed and conserved residues shown in upper case. The experiments shown are representative of three independent experiments.

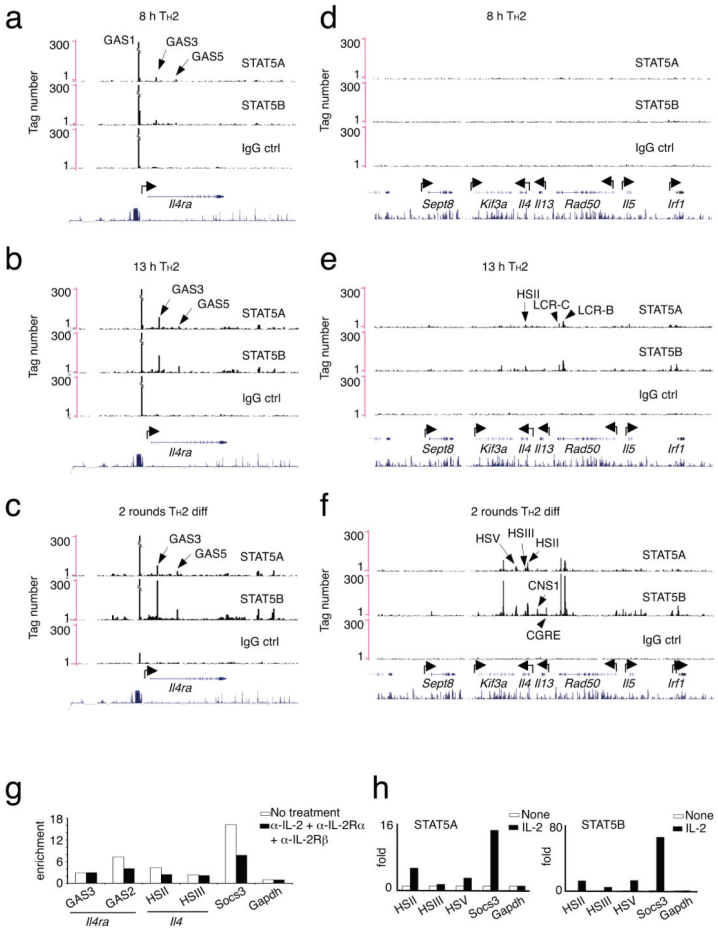

Figure 5.

Analysis of STAT5 binding to the *Il4ra* and *Il4* loci. **(a-f)** ChIP-Seq analysis was performed to analyze STAT5 binding at the *Il4ra* (**a-c)** and *Il4-Il13-Il5* **(d-f)** loci in $CD4^+$ T cells cultured under T_H2 conditions (anti-CD3 + anti-CD28 + 10 ng/ml IL-4 + 10 ug/ml anti—IFN-γ) for the indicated amounts of time. Cells subjected to two rounds of T_H2 differentiation refers to cells cultured under T_H2 conditions for 3 days, expanded with IL-4 and anti—IFN-γ for 2 days, washed, re-cultured under T_H2 conditions for another 3 days and then analyzed without further cytokine stimulation. These cells were not exposed to exogenous IL-2. Unique sequence reads were first adjusted to center them on the corresponding chromatin fragments. The adjusted reads were then summed in 400 bp windows and displayed as custom tracks on the UCSC genome browser. ChIP was performed with IgG as a control for STAT5A- and STAT5B-specific antibodies. Schematics of the *Il4ra* (**a-c**) and *Il4-Il13-Il5* (**d-f**) loci with standard conservation tracks from the UCSC genome browser indicating the areas of highest conservation among 17 vertebrate species are shown in blue at the bottom of each panel. The experiment was preformed three independent times, with similar results. (**g**) T_H2 cells polarized for 2 rounds were incubated in the presence of 10 μg/ml each of anti-IL-2 (S4B6), anti-IL-2Rα (PC61) and anti-IL-2Rβ, all from BD Bioscience, for an extra 18 h and ChIP was preformed to assess STAT5B binding to indicated gene regions. **(h)** IL-2-induced binding of STAT5A and STAT5B to

indicated gene regions, as measured by ChIP. This is representative of two similar experiments. See Supplementary Table 10 for sequences or primers used in ChIP experiments.

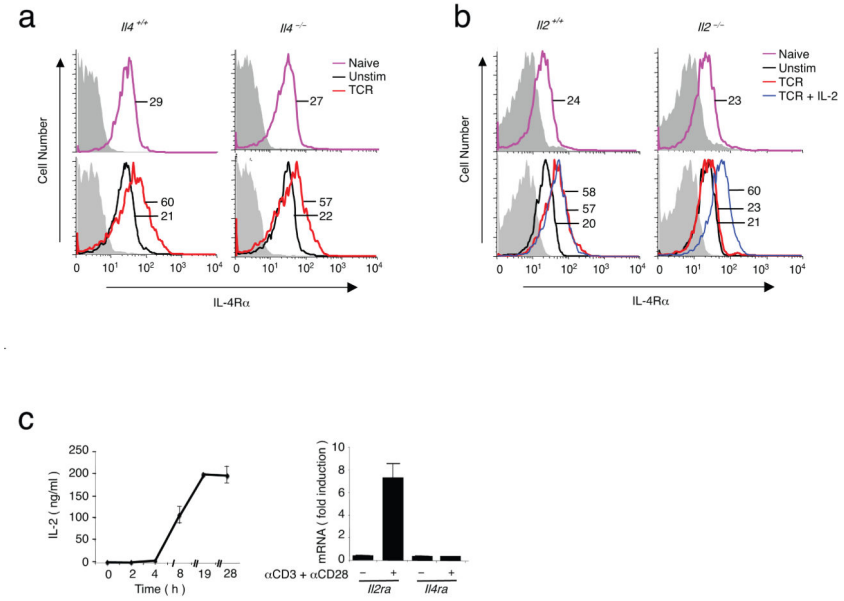

Figure 6.

IL-2 is important for TCR-induced IL-4Rα expression. (**a**) Basal IL-4Rα expression in splenic CD4$^+$ T cells freshly isolated from *Il4*$^{+/+}$ and *Il4*$^{-/-}$ mice (top) and after activation with anti-CD3 and anti-CD28 for approximately 20 h (bottom). (**b**) As in (**a**) except *Il2*$^{-/-}$ instead of *Il4*$^{-/-}$ mice were used and cells were stimulated with anti-CD3 and anti-CD28 alone or with 100 U/ml of IL-2. Experiments were repeated three times with six mice each, with similar results in each case. (**c**) Left, time course of IL-2 protein production by splenic CD4$^+$ T cells from Balb/c mice that were treated with anti-CD3 and anti-CD28. IL-2 was measured by double antibody ELISA. Right, purified splenic CD4$^+$ T cells from C57BL/6 mice were not treated or treated with anti-CD3 and anti-CD28 for 4 h, after which RNA was extracted, and *Il4ra* and *Il2ra* mRNA expression was measured by real-time PCR. The experiment shown is representative of three independent experiments.

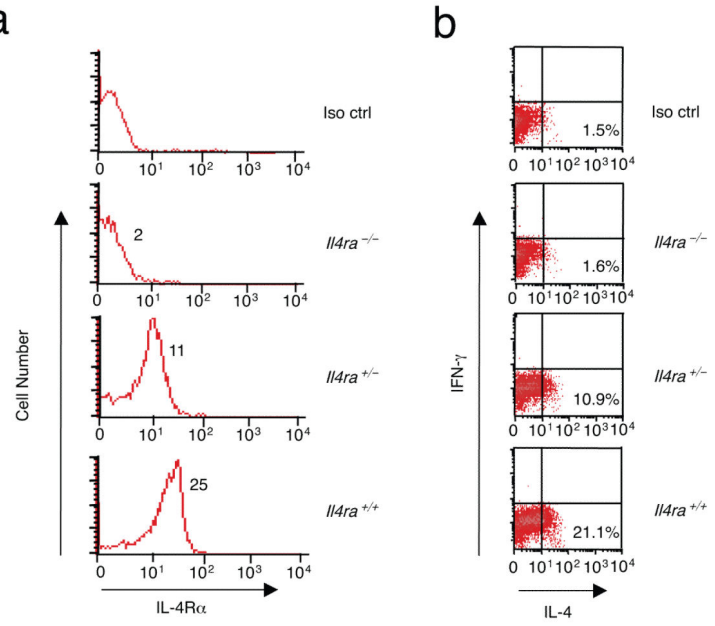

Figure 7.

Extent of IL-4Rα expression influences T_H2 cell differentiation. $CD4^+$ T cells from *Il4ra*$^{+/+}$, *Il4ra*$^{+/-}$, and *Il4ra*$^{-/-}$ Balb/c mice were activated with 2 μg/ml anti-CD3 and 1 μg/ml anti-CD28 for 92 h. (**a,b**) IL-4Rα surface expression was analyzed by flow cytometry (**a**) or cells were cultured under T_H2 conditions for 92 h and intracellular IL-4 and IFN-g were measured (**b**). In (**a**), the numbers indicate MFI and in (**b**) they indicate the percent of cells producing IL-4. The experiment was performed three times with 2 to 4 mice per group in each experiment.

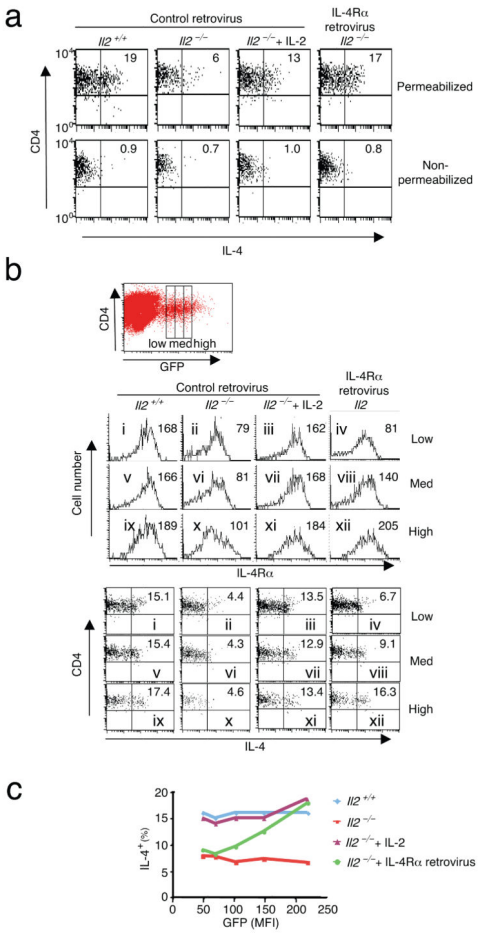

Figure 8.

Retrovirus-mediated expression of IL-4Rα rescues T_H2 differentiation of *Il2*$^{-/-}$ CD4$^+$ T cells. **(a-c)** *Il2*$^{+/+}$ and *Il2*$^{-/-}$ 5C.C7 TCRtg CD4$^+$ T cells were activated under T_H2 conditions for 24 h. Activated T cells were then infected with control or pGFP-RV-m*Il4ra* retroviruses, cultured under T_H2 conditions, and restimulated with PMA and ionomycin for 6 h. (**a**) Total (permeabilized) and cell surface (non-permeabilized) staining was performed on infected CD4$^+$ T cells (GFP$^+$CD4$^+$ cells). Shown is representative intracellular cytokine staining from 5 independent experiments. (**b**) Cells expressing low, medium, and high amounts of GFP (as a measure of retroviral transduction) were subjected to staining for IL-4Rα (numbers represent MFI) and IL-4 (% IL-4-expressing cells). (**c**) GFP$^+$ cells from indicated samples were separated based on GFP MFI, and each subset was stained for IL-4 expression. Graph depicts proportion of cells producing IL-4 within each subset.

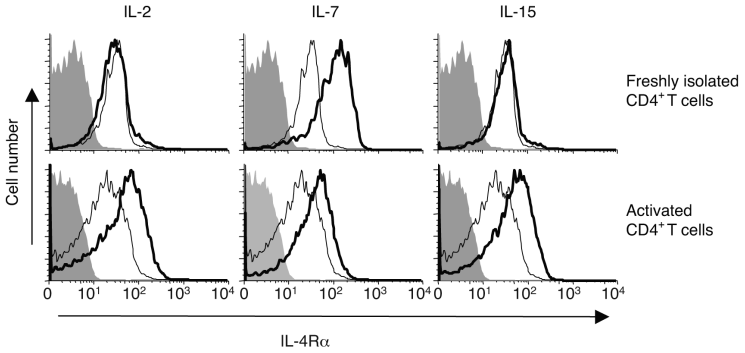

Figure 9.

Other STAT5-activatingn cytokines can increase IL-4Rα expression. Freshly isolated T cells (top) and T cells pre-activated with anti-CD3 and anti-CD28 (bottom) were treated with the indicated cytokines and stained for IL-4Rα surface expression (thin line, no cytokine; thick line, cytokine). The experiment shown is representative of four independent experiments.

Condition	Top motif	Sensitivity (%)	Specifity (%)
8 h Stat5a		66	64
8 h Stat5b		40	94
13 h Stat5a		48	70
13 h Stat5b		61	64
2 rounds diff Stat5a		73	66
2 rounds diff Stat5b		65	63