HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2012 March 15.

Published in final edited form as:

Nat Immunol. 2011 June ; 12(6): 551–559. doi:10.1038/ni.2030.

Cytokine receptor modulation by interleukin-2 broadly regulates T helper cell lineage differentiation

Wei Liao, **Jian-Xin Lin**, **Lu Wang**, **Peng Li**, and **Warren J. Leonard**[1]

Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892-1674

Abstract

T helper (T_H) cells control host-defense to pathogens. The receptors for IL-12, IL-4, and IL-6 are required for T_H1, T_H2, and T_H17 differentiation, respectively. IL-2 signaling via the transcription factor STAT5 controls T_H2 differentiation by regulating the T_H2 cytokine gene cluster and *Il4ra* expression. Here we show that IL-2 regulates T_H1 differentiation, inducing STAT5-dependent IL-12Rβ2 and T-bet expression, with impaired human T_H1 differentiation when IL-2 was blocked. T_H1 differentiation was also impaired in mouse *Il2*$^{-/-}$ T cells but restored by IL-12Rβ2 expression. Consistent with IL-2's inhibition of T_H17 differentiation, IL-2 decreased *Il6ra* and *Il6st* expression, and *Il6st* augmented T_H17 differentiation even when IL-2 was present. Thus, IL-2 influences T_H cell differentiation by modulating cytokine receptor expression to help specify and maintain differentiated states.

The process of T helper cell (T_H) differentiation critically controls host defense for an array of pathogens[1]. Naïve CD4$^+$ T cells can differentiate into a range of T_H cells, and T_H differentiation is determined in part via key cytokine receptor regulation and inclusion in the immunological synapse[2]. IL-12Rβ2 expression is required for T_H1 differentiation[3], IL-4Rα for T_H2 differentiation[4], and IL-6Rα and IL-6 signal transducer (IL-6ST; also known as gp130) for T_H17 differentiation[5, 6], allowing responsiveness to IL-12, IL-4, and IL-6, respectively. For example, IL-12 and STAT4 together with T-bet promote differentiation into T_H1 cells, which preferentially produce interferon-γ (IFN-γ)[7]; IL-4 via STAT6 and GATA3 promote differentiation into T_H2 cells, which produce IL-4, IL-5, and IL-13[1, 8]; and TGF-β, IL-6, IL-23, IL-21 via STAT3 and RORγt together promote differentiation into T_H17 cells, which produce IL-17A, IL-17F, and IL-22[6].

IL-2 is a pleiotropic cytokine with a broad array of actions[9]. Along with IL-4, IL-7, IL-9, IL-15, and IL-21, it shares the common cytokine receptor γ chain, γ_c, which is mutated in

Users may view, print, copy, download and text and data- mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

[1]Correspondence: W.J.L. at Bldg. 10, Rm. 7B05, NHLBI, NIH, Bethesda, MD 20892-1674.

AUTHOR CONTRIBUTIONS

W.L. and J.X.L. planned studies, did experiments, and analyzed data, and wrote the manuscript, L.W. did experiments, P. L. analyzed data and wrote the manuscript, and W.J.L. supervised the project, planned studies, analyzed data, and wrote the manuscript.

Accession codes. GEO: ChIP-Seq data, GSE27158.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

humans with X-linked severe combined immunodeficiency[10], as a critical receptor component. IL-2 signals via JAK1 and JAK3 to activate STAT5A and STAT5B, and additionally uses Ras-MAP kinase and phosphoinositol 3-kinase dependent signaling pathways[9]. Among its many actions, IL-2 is a potent T cell growth factor, induces lymphokine-activated killer activity, boosts the cytolytic activity of NK cells, can augment immunoglobulin production, is a mediator of activation-induced cell death, and is an essential factor for the development of regulatory T (T_{reg}) cells[11]. IL-2 is also known to critically regulate T_H2 differentiation in a STAT5-dependent manner, acting early at the *Il4ra* locus to induce IL-4Rα expression[4] and later to open chromatin accessibility at the *Il4-Il13-Rad50* T_H2 locus[4, 12]. Given the role of IL-2 in T_H2 differentiation, as well as the ability of IL-2 to induce IFN-γ expression[13, 14] and to decrease IL-17 production during T_H17 differentiation[15], we decided to investigate whether IL-2 is more broadly involved in T_H differentiation by regulating corresponding cytokine receptors. IL-2 induced expression of IL-12R, and retroviral transduction of *Il12rb2* reversed the defective T_H1 differentiation in *Il2*$^{-/-}$ T cells. IL-2 also negatively regulated expression of *Il6ra* and of *Il6st* as at least a partial mechanism for negative regulation of T_H17 differentiation. Therefore our data reveal that IL-2 regulates T_H1, T_H2, and T_H17 differentiation based at least in part on its modulation of expression of key cytokine receptors.

RESULTS

Defective T_H1 differentiation in *Il2*$^{-/-}$ T cells

We initially focused on the role of IL-2 in T_H1 differentiation, a process that is driven by IL-12. IL-12 induces not only *Ifng* expression[1] but also T-bet[7], which promotes the survival and proliferation of differentiating T_H1 cells[16]. T-bet induces *Ifng*[7, 16] and IL-12Rβ2 expression[17], while inhibiting GATA3 binding to target genes, including the *Il4* gene[18], and thus T_H2 differentiation. Naïve CD4$^+$ T cells do not respond to IL-12 because they lack a key receptor component, IL-12Rβ2[3]. Like T_H2 differentiation, T_H1 differentiation requires activation via the T-cell receptor (TCR), which induces production of IL-2. We therefore examined the effect of TCR stimulation on IFN-γ production in wild-type (WT) and *Il2*$^{-/-}$ T cells. We stimulated *Il2*$^{+/+}$ or *Il2*$^{-/-}$ CD4$^+$ T cells with anti-CD3 + anti-CD28, gating on proliferating cells based on FSC-SSC (Fig. 1a, upper two panels). Naïve *Il2*$^{-/-}$ CD4$^+$ T cells stimulated with anti-CD3 + anti-CD28 had far fewer IFN-γ$^+$ cells than did *Il2*$^{+/+}$ CD4$^+$ T cells (Fig. 1a, second pair of panels), consistent with previous observations that IL-2 induces IFN-γ production[13, 14]. Moreover, after two rounds of T_H1 polarization, a lower percentage of *Il2*$^{-/-}$ CD4$^+$ T cells expressed IFN-γ$^+$ as compared to *Il2*$^{+/+}$ *cells* (Fig. 1a**,** lower two sets of panels). Although IL-2 contributes to survival and accordingly the *Il2*$^{-/-}$ population had more dead cells than the *Il2*$^{+/+}$ population after T_H1 differentiation (Supplementary Fig. 1a,b), in Fig. 1a, we gated on viable cells (see Annexin V and 7AAD staining in Supplementary Fig. 1a); thus the decrease in IFN-γ$^+$ cells among *Il2*$^{-/-}$ T cells could not be attributed solely to diminished viability.

Because IL-2 is a T-cell growth factor, and because IFN-γ expression is known to increase with successive rounds of cell division[19], we tested whether the role of IL-2 in T_H1 differentiation was due solely to its effects on proliferation or whether it also had

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

proliferation-independent effects on IFN-γ production. To follow cell division we labeled cells with CFSE and incubated them under T_H1 conditions for 72 h. We observed the same number of rounds of cell division, but as anticipated, the *Il2*$^{-/-}$ T cells were delayed in their division relative to the *Il2*$^{+/+}$ T cells (Fig. 1b**,** left panels). We compared the percentage of IFN-γ-producing cells in each division in the *Il2*$^{+/+}$ and *Il2*$^{-/-}$ cell populations. As anticipated, IFN-γ expression increased with cell division in *Il2*$^{+/+}$ cells, but there was a decrease in the percentage of IFN-γ-producing cells at each cell division in the *Il2*$^{-/-}$ T cells relative to the *Il2*$^{+/+}$ T cells (Fig. 1b**;** summarized in Fig. 1c). Thus, even when *Il2*$^{-/-}$ T cells underwent the same number of divisions as *Il2*$^{+/+}$ T cells, they had diminished production of IFN-γ, suggesting both proliferation-dependent and proliferation-independent effects of IL-2 in T_H1 differentiation.

IL-2 augments *Il12rb2* and *Tbx21* expression

To clarify the role of IL-2 in T_H1 differentiation, we examined *Il2*$^{-/-}$ T_H1 differentiated cells for T_H1-associated genes. These cells expressed less *Ifng* as well as less *Il12rb2* and *Tbx21* (encoding T-bet) mRNA than did WT T_H1 cells (Fig. 2a); *Ccnd3* (encoding cyclin D3) is shown as a control gene whose expression was not altered (Fig. 2a). Because IFN-γ was reported to drive expression of both *Il12rb2* and *Tbx21* genes and initiate T_H1 differentiation[3, 20], we next investigated whether the defective T_H1 differentiation of *Il2*$^{-/-}$ T cells was due to defective IL-2-dependent IFN-γ production. Whereas IL-2 substantially reversed the diminished IFN-γ production observed in the *Il2*$^{-/-}$ cells (Fig. 2b), the addition of IFN-γ had a smaller effect, as shown by intracellular staining and secreted cytokine (Fig. 2b, Supplementary Fig. 2). Interestingly, in this setting, IL-2 substantially restored *Il12rb2* mRNA expression, whereas IFN-γ only had a modest effect (Fig. 2c, upper panel). In contrast, IFN-γ appeared slightly better than IL-2 in increasing *Tbx21* mRNA expression (Fig. 2c, middle panel). We also examined the effect on mRNA encoding *Il21rb1*, the other component of the IL-12 receptor. Although *Il12rb1* expression was not decreased in *Il2*$^{-/-}$ T cells, the addition of IL-2 increased its expression (Fig. 2c, lower panel), suggesting that while *Il12rb1* expression was not limiting, its induction by IL-2 could potentially further increase IL-12 responsiveness. Consistent with prior reports of a role for IFN-γ in the induction of both the *Il12rb2* and *Tbx21* genes[3, 20], IL-2-induced *Il12rb2* and *Tbx21* mRNA levels were lower in *Ifng*$^{-/-}$ $CD4^+$ T cells (Fig. 2d), but the fold induction was not decreased (Fig. 2d), indicating IFN-γ-independent induction by IL-2; thus, the effect of IL-2 in T_H1 differentiation is at least partially independent of IFN-γ. This conclusion was further supported by the ability of IL-2 to induce *Il12rb2* and *Tbx21* mRNA expression in *Stat1*$^{-/-}$ T cells (Fig. 2e); the basal levels were lower than in the *Stat1*$^{+/+}$ T cells, but IL-2 still induced expression. IL-2 also induced *Il12rb2* mRNA in *Tbx21*$^{-/-}$ T cells (Fig. 2f), indicating that it can act independently of T-bet. For unclear reasons, there was increased basal *Il12rb2* mRNA expression in the *Tbx21*$^{-/-}$ T cells (Fig. 2f). IL-2 and IL-12 were previously shown to cooperatively induce *Il12rb2*[21], our data now indicate a direct IL-12-independent effect of IL-2. Consistent with effects of both IL-2 and IFN-γ on T_H1 differentiation, antibodies to IL-2Rα and IL-2Rβ or to IFN-γ decreased *Il12rb2* and *Tbx21* mRNA expression (Fig. 2g); the antibodies cooperatively inhibited *Tbx21* expression, whereas blocking IL-2 or IFN-γ alone decreased *Il12rb2* mRNA (Fig. 2g).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

IL-2 also induced human *IL12RB2* and *TBX21* gene (Fig. 3a) and protein (Fig. 3b,c) expression, whereas IFN-γ had little if any effect at 4 h (Fig. 3a-c). We confirmed there was no reproducible significant effect of IFN-γ even at 8 or 24 h time points (Supplementary Fig. 3). Thus in both mice and humans, IL-2 promotes expression of IL-12Rβ2 and T-bet. To examine the role of IL-2 in human T_H1 differentiation, we used blocking antibodies to IL-2Rα (anti-Tac[22]) and IL-2Rβ (Mikβ1[23]). When human peripheral blood naïve ($CD45RA^{high}CD25^{-}HLA^{-}DR^{-}$) $CD4^{+}$ T cells were cultured under T_H1-polarizing conditions, the anti-IL-2R antibodies decreased *IL12RB2, TBX21*, and *IFNG* mRNA induction (Fig. 3d) as well as the percentage of $IFN\gamma^{+}$ $CD4^{+}$ T cells (Fig. 3e). The addition of exogenous IFN-γ only slightly reversed this effect (Fig. 3e). Similar to mouse T_H1 differentiation, during human T_H1 differentiation, T cells undergo cell division (Fig. 3f, left panel) and the percentage of IFN-γ^{+} cells tended to increase with each division (Fig. 3g, left panel). As expected, because IL-2 is a T cell growth factor, the addition of anti-IL-2Rα or IL-2Rβ during T_H1 differentiation decreased proliferation (Fig. 3f, right panel), but this treatment also markedly diminished the percentage of IFN-γ^{+} cells in each division (Fig. 3g), indicating that analogous to mouse T_H1 differentiation, the effect of IL-2 on human T_H1 differentiation is partially independent of its proliferative effect. Consistent with the data in Fig. 3e, IFN-γ had little if any effect in reversing the inhibition mediated by anti-IL-2R antibodies (Fig. 3g). Thus, in mice and humans, IL-2 can promote T_H1 differentiation in a manner at least partially independent of proliferation and IFN-γ.

STAT5A and STAT5B regulate *Il12rb2* and *Tbx21* expression

Because IL-2 activates STAT5A and STAT5B[9], we next investigated the role of these STAT5 proteins in *Il12rb2* and *Tbx21* gene expression. By RT-PCR and flow cytometry, following *in vitro* deletion of *Stat5a* and *Stat5b* in $CD4^{+}$ T cells from mice in which the *Stat5a*-*Stat5b* locus was floxed (Fig. 4a, first two panels) *Il12rb2* and *Tbx21* mRNA expression were partially decreased, with a greater decrease in *Il12rb2* expression (Fig. 4a, 3rd and 4th panels). Although the decrease in *Tbx21* mRNA was not statistically significant, there was a reproducibly observed decrease in T-bet protein (Fig. 4a, 5th panel). To further investigate the importance of STAT5 in T_H1 differentiation, we examined the effect of retroviral expression of a constitutively-activated form of STAT5A, denoted STAT5A-1*6[24]. When this was transduced into *Il2*$^{-/-}$ T cells, defective T_H1 differentiation was partially reversed (Fig. 4b), consistent with a role for STAT5 in T_H1 differentiation (Fig. 4b). ChIP-Seq was used to look at STAT5 interaction with the *Il12rb2* gene. IL-2 induced STAT5A and STAT5B binding to the mouse *Il12rb2* gene at multiple $TTCN_3GAA$ GAS motifs (Fig. 4c; sequences are in Supplementary Fig. 4). We cloned the TSS-proximal region spanning GAS1 and GAS2 5′ of the TSS and the GAS3 region 3′ of the TSS in the pGL4.10 luciferase reporter vector and found that reporter activity was induced by IL-2 (Fig. 4d, see GAS1,2,3 construct) and required an intact GAS1 motif (Fig. 4d). Consistent with IL-2-induced IFN-γ-independent *Il12rb2* expression (Fig. 2d), IL-2 still induced reporter activity in the presence of anti-IFN-γ (Fig. 4d). The importance of GAS1 was confirmed in preactivated human T cells, with STAT5A and STAT5B binding *IL12RB2* at the site (Fig. 4e); note the sequence conservation of the GAS1 motif in humans and mice (bottom of Fig. 4e). No IFN-γ-induced STAT1 binding was detected at this site (Fig. 4e), in keeping with little if any effect of IFN-γ on IL-12Rβ2 expression (Fig. 3a,b). Consistent

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

with the ability of IL-12 to induce expression of its own receptor (Fig. 3a), IL-12 induced *Il12rb2* reporter activity, albeit more weakly than IL-2 (Fig. 4f, GAS1,2,3 construct), and this also required an intact GAS1 motif (Fig. 4f).

Analogous to their binding to elements in the *Il12rb2* gene, STAT5A and STAT5B also bound to the mouse *Tbx21* gene (Fig. 5a), ≈12 kb 5′ of the TSS at a region that binds STAT4[25], and this region mediated IL-2-induced luciferase reporter activity (Fig. 5b). As expected, anti-IFN-γ had only a partial effect on reporter activity (Fig. 5b and Supplementary Fig. 5), consistent with IFN-γ-independent effects of IL-2 on T_H1 differentiation, as we observed above. Whereas the non-canonical motifs may partially contribute to reporter activity, mutation of the canonical GAS motif essentially abrogated activity (Fig. 5b, mut3 vs. mut1 and mut2). In the human *TBX21* gene, STAT5A and STAT5B bound the same region as STAT4 (Fig. 5c) at a site conserved between human and mouse (Fig. 5d), suggesting the possibility that these different STATs can compete for binding to the same site. STAT1 exhibited only very low binding at this site (Fig. 5c), consistent with higher induction of *TBX21* by IL-2 and IL-12 than by IFN-γ (Fig. 3e). In contrast, STAT1 as well as STAT4, STAT5A, and STAT5B bound to the promoter region of human *IRF1* (Fig. 5e), another transcription factor important for T_H1 differentiation[26]. When we deleted *Stat5a* and *Stat5b* by *Cre*-mediated deletion *in vitro* using mouse cells there was a slight decrease in *Irf1* mRNA as well as in *Il12rb1* mRNA (Supplementary Fig. 6), consistent with *Il12rb1* being regulated by *Irf1*[26]. Whereas IL-2 more strongly induced *Il12rb2* promoter activity than did IL-12 (Fig. 4f), it less strongly induced *Tbx21* reporter activity (Fig. 5f). Both cytokines required the same GAS motif for *Tbx21* induction (Fig. 5f, mut3); thus, IL-2 and IL-12 act at least in part via shared elements to regulate expression of both T-bet and IL-12Rβ2. Antibodies to IL-2, IL-2Rα, and IL-2Rβ lowered IL-12-induced *Tbx21* activity (Fig. 5g), presumably at least in part by their inhibition of IL-2-induced IL-12Rβ2 expression.

Analysis of ChIP-Seq libraries from T_H1-differentiated cells revealed that the STAT5A and STAT5B peaks seen at the GAS1, GAS2, and GAS3 motifs in the *Il12rb2* locus in T cells stimulated with IL-2 (Fig. 4c) were also evident in T_H1 cells (Fig. 6a). STAT4, STAT5A, and STAT5B ChIP-Seq data revealed that both STAT5B and STAT5A exhibited binding at the *Il12rb2* GAS1 site that was relatively stronger than that of STAT4; conversely, STAT4 exhibited very strong binding upstream of the *Tbx21* gene (the 11th strongest peak out of 7318 peaks in the STAT4 library), whereas STAT5A and STAT5B binding were relatively weak at this site. Because these data were from the same ChIP-Seq libraries, we can conclude that the relative binding of STAT5A and STAT5B is weaker than STAT4 binding at the *Tbx21* locus but stronger than that of STAT4 at the *Il12rb2* locus in T_H1 differentiated cells (**Fig. 6, b** vs. **a**). Coupled to the functional reporter assays (**Fig.** 4f**,** 5f), these data suggest that IL-2 via STAT5 is the dominant signal for *Il12rb2* induction, whereas IL-12 via STAT4 is more critical for *Tbx21* regulation.

Il12rb2 rescues T_H1 differentiation in *Il2*$^{-/-}$ T cells

We next investigated whether enforced expression of *Il12rb2* or *Tbx21* could rescue defective T_H1 differentiation in *Il2*$^{-/-}$ cells. As expected, *Il12rb2* retroviral transduction had

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

little effect in *Il2*$^{+/+}$ cells, which already express IL-12Rβ2; however, transduction of *Il2*$^{-/-}$ CD4^{+} T cells with the *Il12rb2* retrovirus markedly increased IFN-γ protein expression (Fig. 6c,d). In contrast, transduction of *Tbx21* did not rescue impaired T_H1 differentiation in *Il2*$^{-/-}$ CD4^{+} T cells (Fig. 6e), suggesting IL-2-induced T-bet is not sufficient for T_H1 differentiation. Nevertheless, retroviral transduction of *Tbx21* increased IFN-γ production in both T_H17-differentiated *Il2*$^{+/+}$ *and Il2*$^{-/-}$ T cells (Fig. 6e), confirming the functionality of the retrovirally-transduced protein, although the basis for the different effect of the *Tbx21* retrovirus in T_H1 vs. T_H17-differentiated cells is unclear. Interestingly, transduction of the *Il12rb2* retrovirus substantially increased *Il12rb2* and *Tbx21* mRNA, as compared to a control virus in both *Il2*$^{+/+}$ and *Il2*$^{-/-}$ T cells (Fig. 6f). In contrast, the *Tbx21* retrovirus significantly increased *Tbx21* mRNA but if anything slightly decreased *Il12rb2* mRNA expression in *Il2*$^{+/+}$ T cells and only modestly increased *Il12rb2* mRNA in *Il2*$^{-/-}$ T cells (Fig. 6f). At the protein level, both viruses induced T-bet expression (Fig. 6g). In contrast, for IL-12Rβ2 expression, retroviral transduction of *Il12rb2* had a modest shift whereas transduction of *Tbx21* had very little if any discernible shift (Fig. 6h).

IL-2 is required for T_H1 responses *in vivo*

The above data collectively revealed a critical role for IL-2 in T_H1 differentiation *in vitro*. We next sought to extend these findings by examining an antigen-specific response *in vivo*. Mice immunized with pigeon cytochrome C (PCC) exhibited a marked increase in cell surface IL-12Rβ2 expression on *Il2*$^{+/+}$ CD4^{+} T cells, but there was little effect in T cells from *Il2*$^{-/-}$ mice (Fig. 7a right vs. left panels; Fig. 7b). Similarly, less *Tbx21* mRNA was detected in cells from *Il2*$^{-/-}$ mice (Fig. 7c) and PCC-induced IFN-γ protein (Fig. 7d) and mRNA (Fig. 7e) expression was also diminished. Thus, IL-2 is required for normal *Il12rb2* and *Tbx21* expression *in vivo*.

IL-2 decreases gp130 expression

The above data and a prior study[4] together establish key roles for IL-2 in T_H1 and T_H2 differentiation, at least in part based on cytokine receptor modulation. IL-2 can also negatively regulate T_H17 differentiation[15]. Given the key role of IL-6 in T_H17 differentiation, we investigated whether IL-2 might inhibit expression of IL-6Rα and/or gp130 (encoded by *Il6ra* and *Il6st*, respectively), and indeed found that IL-2 stimulation decreased *Il6ra* and *Il6st* mRNA (Fig. 8a) and protein (Fig. 8b and Fig. 8c) expression. Consistent with this, under T_H17 conditions, both *Il6ra* and *Il6st* mRNA expression were higher in *Il2*$^{-/-}$ than in WT T cells (Fig. 8d). The increased expression correlated with a higher level of *Il17a* mRNA (Fig. 8d). *Il12rb2* was expressed even in T_H17-differentiated cells in an IL-2-dependent fashion (Fig. 8d), which might explain the ability of T_H17 cells to produce IFN-γ when IL-12 is present and thus for the cells to exhibit T_H plasticity[27]. Because IL-2 is required for normal IFN-γ production (Fig. 1a) and IFN-γ negatively regulates the generation of T_H17 cells[6], it was conceivable that increased IL-17 protein in the absence of IL-2 resulted from the loss of IFN-γ production by these cells. However, we could not detect IFN-γ production by the T_H17 cells in either the WT or *Il2*$^{-/-}$ background (Fig. 8e, left panel), indicating that IFN-γ by itself cannot explain the difference in IL-17 production in the *Il2*$^{+/+}$ and *Il2*$^{-/-}$ T cells (Fig. 8e **right panel**). When *Il6ra* and *Il6st* were

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

separately introduced into $CD4^+$ T cells by retroviral transduction (Fig. 8f), only *Il6st* could partially rescue the defective T_H17 differentiation caused by IL-2, with an increase in IL-17A protein expression (Fig. 8g). The fact that *Il6st* retroviral transduction increased IL-17A expression in WT mice (Fig. 8g, iii vs. i) indicated that the level of gp130 is limiting. This was underscored by the fact that IL-2 treatment diminished *Il6st* expression (Fig. 8a) and that retroviral expression of *Il6st* into IL-2-treated cells increased T_H17 differentiation (Fig. 8g, vi vs. iv). However, given that IL-2 inhibited T_H17 differentiation even when *Il6st* was constitutively expressed (Fig. 8g, vi vs. iii), the role of IL-2 extends beyond the regulation of gp130.

DISCUSSION

IL-2 is a pleiotropic cytokine with a very broad array of actions, including its abilities to drive T-cell proliferation, augment cytolytic activity, promote T_{reg} cell development, and mediate activation-induced cell death. IL-2 can also increase IL-4Rα expression, thereby priming T cells for T_H2 differentiation. We now show that IL-2 also regulates other key cytokine receptors, inducing IL-12Rβ2 to allow responsiveness to IL-12 and promoting T_H1 differentiation, while repressing IL-6Rα and gp130 and thus IL-6 responsiveness, thereby inhibiting T_H17 differentiation.

Previously it was suggested that JAK3, which is a kinase that associates with the common cytokine receptor γ chain[28], could contribute to T_H1 differentiation by mediating IL-2-induced STAT5 binding to the *Ifng* gene[29]. Our findings herein show broader roles for IL-2 in T_H1 differentiation, with IL-2-induced STAT5A and STAT5B binding to key elements in both the *Il12rb2* and *Tbx21* genes. Not only was expression of *Il12rb2* and *Tbx21* diminished in *Il2*$^{-/-}$ mice, but IL-2 could induce expression of each of these genes. The induction of *Il12rb2* by IL-2 appears to be more important for T_H1 differentiation than the induction of *Tbx21* given that retroviral transduction of *I12rb2* but not *Tbx21* could rescue T_H1 differentiation in *Il2*$^{-/-}$ cells. Although *Il12rb1* was not diminished in *Il2*$^{-/-}$ mice, IL-2 also induced *Il12rb1* expression, which potentially can further enhance responsiveness to IL-12. Interestingly, IL-2 and IL-12 appear to have overlapping actions for T_H1 differentiation, with IL-2 via STAT5 and IL-12 via STAT4 both contributing to the regulation of *Il12rb2* and *Tbx21* expression. Although retroviral transduction of *Tbx21,* unlike *Il12rb2*, could not rescue T_H1 differentiation in *Il2*$^{-/-}$ T cells, *Tbx21* was previously shown to be capable of promoting T_H1 differentiation in *Stat4*$^{-/-}$ cells[16], and we also show herein that the *Tbx21* retrovirus could increase IFN-γ production by T_H17 cells. T-bet can also repress T_H17 differentiation [30] and our data confirm this observation, which may help to explain why IFN-γ production is facilitated in this setting. Thus, *Tbx21* has a vital role but presumably there are essential signals provided by IL-2 for T_H1 differentiation for which T-bet alone cannot compensate.

Consistent with an even broader role for IL-2 in T_H differentiation via regulation of cytokine receptors, IL-2 down-regulated *Il6ra* and *Il6st* and inhibition of T_H17 differentiation by IL-2 could be partially rescued by forced expression of *Il6st*, although our data also indicate that IL-2 can act via an *Il6st*–independent mechanism as well.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

In summary, our findings reveal a broader role for IL-2 in T_H differentiation than was previously anticipated, underscoring its ability to modulate expression of key cytokine receptors to control responsiveness to a range of cytokines after antigen encounter. It is striking that IL-2 increases IL-12Rβ2, IL-4Rα[4], and IL-2Rβ[31] expression but decreases gp130 expression, modulating signals by IL-12, IL-4, IL-2, and IL-6, and thereby for T_H1, T_H2, T_{reg}, and T_H17 cell differentiation (this study and refs. 4 and 32). Thus, IL-2 can function as a master regulator that modulates a range of key cytokine receptors to influence cell fate decisions, both priming for differentiation and helping to maintain a differentiated state. Previously, studies revealed that IL-2 potently represses IL-7Rα expression[33], thereby diminishing survival signals and potentially facilitating activation-induced cell death and the elimination of cells during the contraction phase following viral infection. Collectively, our findings indicate that IL-2 can prime T cells for a range of effector functions, depending on the particular antigen or pathogen that is encountered and the cellular milieu. Moreover, these results indicate mechanisms and provide rational approaches by which one can potentially modulate IL-2 responses to control T_H differentiation to either facilitate the clearance of pathogens or diminish damage caused by immune responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Drs. Gertraud Robinson and Lothar Hennighausen (NIDDKD, NIH) for mice in which the *Stat5a* to *Stat5b* region was floxed, Drs. Keji Zhao and Dustin Schones (NHLBI, NIH) for valuable discussions, Drs. Ronald H. Schwartz (NIAID, NIH), Rosanne Spolski (NHLBI, NIH), and Yrina Rochman (NHLBI, NIH) for critical comments, Dr. Kairong Cui (NHLBI, NIH) for help in generating ChIP-Seq data, Dr. Ken Murphy (Washington University in St. Louis) for the pRV-*Il12rb2* retroviral construct, and Dr. Jinfang Zhu (NIAID, NIH) for the pRV-*Tbx21* construct. This worked was supported by the Division of Intramural Research, NHLBI, NIH.

Appendix

METHODS

Mouse and human lymphocytes

B10.A *Il2*$^{-/-}$ mice on a *Rag2*$^{-/-}$/5C.C7 TCR transgenic background (line 110) and control mice (*Rag2*$^{-/-}$/5C.C7, line 94) were from the NIAID/NIH contract barrier at Taconic Farms. Line 94 mice lack endogenous T and B cells but have mature peripheral $CD4^+$ T cells expressing the 5C.C7 transgene, as well as a small number of $CD8^+$ T cells (information from Taconic). Line 110 has similar cellular distributions, including similar NK cell numbers. The 129S6 *Stat1*$^{-/-}$ and control littermates were from Taconic Farms. BALB/c, C57BL/6, C57BL/6 *Ifng*$^{-/-}$, and C57BL/6 *Tbx21*$^{-/-}$ mice were from Jackson Laboratory. Animal protocols were approved by the NHLBI Animal Care and Use Committee and followed the NIH Guidelines "Using Animals in Intramural Research." Naïve $CD4^+$ T cells from spleen and lymph nodes were purified from 5-12 week old mice using $CD4^+CD62^+$ T cell Isolation Kit II (Miltenyi). Human $CD45RA^+CD25^-HLA\text{-}DR^-CD4^+$ (For T_H1 differentiation) naïve cells were sorted from buffy coats from normal donors at the NIH Blood Bank, or human $CD45RA^+CD4^+$ cells were purified using the naïve human $CD4^+$

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

isolation kit II (Miltenyi). Cells were cultured in RPMI 1640 medium containing 10 mM Hepes (pH 7.0), 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (complete medium), including 50 μM 2-ME (for mice).

Quantitative RT-PCR

mRNA levels were determined by RT-PCR, performed as described[4]. Primers and probes were from ABI. Expression was normalized to *Rpl7*.

ChIP-Seq analysis

ChIP-Seq assays were performed as described[4], using antibodies to STAT5A, STAT5B, or STAT4 (each from R & D Systems), STAT1 (Santa Cruz), or normal rabbit serum. Given high homology of human and mouse STATs, the same antibodies could be used for both species. Sequence reads were mapped to the mouse genome (mm8, February 2006 Assembly) and human genome (hg18, March 2006 Assembly) using the Illumina GA data analysis pipeline. Reads mapping with 2 mismatches were retained, centered on corresponding chromatin fragments, and reads were summed in 400 bp sliding windows that are displayed as custom tracks on the UCSC genome browser. Microarray data and short reads from ChIP-Seq data will be available via Gene Expression Omnibus (GEO), accession number GSE27158 at www.ncbi.nlm.nih.gov/geo. MACS (Model-based Analysis for ChIP-Seq)[34] was used to detect binding sites (peaks) and evaluate the significance of the binding for each STAT. The parameters were set as follows: mfold=25, p-value=1e-5, lambdaset=[1000,5000,10000] and gsize=2.3e+9. mfold is the high-confidence fold-enrichment used for assigning candidate peaks in the treatment library as compared to the control library; *p*-value is a user-defined threshold, and candidate peaks with *p*-values below this threshold are considered as meaningful binding sites; gsize is the mappable genome size and lambdaset is a dynamic parameter that is used to capture local bias and calculate *p*-values. For Fig. 6, we ran MACS on STAT4, STAT5A, and STAT5B ChIP-Seq data and evaluated the significance of the binding by calculating *p*-values of dynamic Poisson distributions and ranking peaks within each ChIP-Seq library. The lower the p-value, the more significant the peak. We divided the peaks into three categories: (a) Weak peaks if 1e-5 > *p*-value >1e-10, (b) Significant peaks if 1e-10 > *p*-value > 1e-20, and (c) Very significant peaks of *p*-value < 1e-20. For STAT GAS peaks related to the *Il12rb2* gene, we found that STAT4, STAT5A and STAT5B significantly bound to the GAS1 site (*p*-values = 1e-28, 1e-29, and 1e-68, respectively, and peaks were ranked in the libraries as 810/7318, 754/6973 and 237/6897). STAT5A and STAT5B binding on GAS1 site is relatively stronger than STAT4. For *Tbx21* binding sites, STAT4 exhibited very strong binding upstream of the gene (*p*-value = 1e-150, and this peak was ranked 11th out of 7318 peaks in the STAT4 library). By comparison, STAT5A and STAT5B binding were weak (p-values ~ 1e-3 and ~ 1e-7, respectively; the STAT5B peak is ranked 5492 out of 6897 peaks) at the *Tbx21* locus.

Flow cytometric analyses

Mouse cells were stained with appropriate isotype control antibodies or with Cy-chrome-anti-CD4 (L3T4), allophycocyanin-anti-CD8 (Ly2), PE-anti-IL-4 (11B11), FITC or APC-anti-IFN-γ (XMG1.2), or PE-anti-CD126 (D7715A7) (all above are from BD Biosciences),

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

APC-anti-mgp130 (125623) from R & D Systems, or Alexa Flour 647 anti-IL-17A (eBio17B7) from eBioscience. Human CD4$^+$ T cells were stained with PE-cy5 or FITC-anti-CD4 (RPA-T4), FITC or PE-anti-IFN-γ (4S.B3), PE-anti-IL-4 (8D4-8) (BD Bioscience), APC-anti-IL-12Rβ2 (305719) (R & D Systems), or Alexa Flour 647-anti-mouse/human T-bet (eBiosience). Cells were analyzed on FACSCalibur or FACSCanto II flow cytometers (Becton Dickinson) using FlowJo software (Tree Star, Inc).

Th polarization

Mouse or human naïve CD4$^+$ T cells were activated with 2 μg/ml anti-CD3 + 1 μg/ml anti-CD28 (PharMingen). For "no priming" Th0 conditions, no cytokines or other antibodies were added. For mouse T_H1 conditions, anti-IL-4 (11B11, 10 μg/ml) plus IL-12 (10 ng/ml) were added. For human T_H1 conditions, anti-IL-4 (MP4-25D2, 10 μg/ml) plus IL-12 (10 ng/ml) were added. For mouse T_H17 conditions, 5 ng/ml of IL-6, 1 ng/ml of TGFβ, 10 μg/ml of anti-IL-4, and 10 μg/ml of anti-IFNγ were added. Mouse IL-12, IFNγ, and IL-4 were from PeproTech, human IL-6 from R & D Systems, and human IL-12 and IFNγ from PeproTech or R & D Systems. Neutralizing antibodies to mouse IFNγ (XMG1.2), mouse IL-2 (S4B6), mouse IL-2Rα (PC61), mouse IL-2Rβ (TM–β1), and human IFNγ (B27) were from BD bioscience; anti-human IL-2Rα (anti-Tac)[22] and IL-2Rβ (Mikβ1)[23] were from Dr. Thomas Waldmann (NCI).

Expression of mouse *Il12rb2, Tbx21, Il6ra*, or *Il6st* retroviruses

Il12rb2, Tbx21, Il6ra, or *Il6st* were cloned in pRV, a GFP-expressing retroviral vector, and these constructs or control pRV were transfected together with the pCl-ECO packaging plasmid into 293T cells. Retroviral supernatant was mixed with 8 μg/ml polybrene and viruses introduced by centrifugation at 3,500 rpm for 45 minutes at 30°C into mouse CD4$^+$ CD62$^+$ T cells that had been preactivated under appropriate Th differentiation conditions. The supernatant was replaced with new medium, cells cultured as indicated for 3 days, and stimulated with 50 ng/ml PMA, 500 ng/ml ionomycin, and BD GolgiPlug (BD Bioscience) for 4 h. Cells were incubated with permeabilization buffer (BD Bioscience) and antibodies for 40 min. Data were analyzed with FlowJo. The % cytokine-producing cells was measured by gating on live CD4$^+$ T cells with forward vs. side scatter profiles and GFP$^+$ staining. pRV-m*Il6ra* and pRV-*mgIl6st* were generated by Origene (Rockville, MD).

Generation of *Il12rb2* promoter constructs

To generate *Il12rb2* promoter constructs, we cloned the PCR-generated region spanning GAS1, GAS2, and the *Il12rb2* promoter region 5′ to the luciferase gene in pGL4.10 and the PCR-generated GAS3 region 3′ of the luciferase gene, including 100-200 bp of 5′ and 3′ flanking sequence (≈300-400 bp for GAS1/*Il12rb2* and 800 bp for GAS3 regions); we also generated GAS motif mutants (Supplementary Fig. 4).

Generation of *Stat5a*- and *Stat5b*-deficient T cells

Stat5$^{fl/fl}$ CD4$^+$ T cells were pre-activated with 2 μg/ml anti-CD3 + 1 μg/ml anti-CD28 for 48 h, rested overnight, and the *Stat5a-Stat5b* locus deleted with a *Cre* retrovirus that also drives GFP expression. GFP$^+$ cells were then sorted to isolate cells deficient in *Stat5a* and *Stat5b*.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Statistical analyses

Statistical analyses for mRNA expression were performed by One-Way ANOVA.

References

1. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood. 2008; 112:1557–1569. [PubMed: 18725574]
2. Maldonado RA, et al. Control of T helper cell differentiation through cytokine receptor inclusion in the immunological synapse. J Exp Med. 2009; 206:877–892. [PubMed: 19349465]
3. Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. J Exp Med. 1997; 185:817–824. [PubMed: 9120387]
4. Liao W, et al. Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression. Nat Immunol. 2008; 9:1288–1296. [PubMed: 18820682]
5. Nishihara M, et al. IL-6-gp130-STAT3 in T cells directs the development of IL-17+ Th with a minimum effect on that of Treg in the steady state. Int Immunol. 2007; 19:695–702. [PubMed: 17493959]
6. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009; 27:485–517. [PubMed: 19132915]
7. Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating Th1 immune responses. Annu Rev Immunol. 2003; 21:713–758. [PubMed: 12500979]
8. Ansel KM, Djuretic I, Tanasa B, Rao A. Regulation of Th2 differentiation and Il4 locus accessibility. Annu Rev Immunol. 2006; 24:607–656. [PubMed: 16551261]
9. Lin JX, Leonard WJ. The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. Oncogene. 2000; 19:2566–2576. [PubMed: 10851055]
10. Leonard WJ. Cytokines and immunodeficiency diseases. Nat Rev Immunol. 2001; 1:200–208. [PubMed: 11905829]
11. Kim HP, Imbert J, Leonard WJ. Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. Cytokine Growth Factor Rev. 2006; 17:349–366. [PubMed: 16911870]
12. Cote-Sierra J, et al. Interleukin 2 plays a central role in Th2 differentiation. Proc Natl Acad Sci U S A. 2004; 101:3880–3885. [PubMed: 15004274]
13. Bream JH, et al. A distal region in the interferon-gamma gene is a site of epigenetic remodeling and transcriptional regulation by interleukin-2. J Biol Chem. 2004; 279:41249–41257. [PubMed: 15271977]
14. Reem GH, Yeh NH. Interleukin 2 regulates expression of its receptor and synthesis of gamma interferon by human T lymphocytes. Science. 1984; 225:429–430. [PubMed: 6429853]
15. Laurence A, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity. 2007; 26:371–381. [PubMed: 17363300]
16. Mullen AC, et al. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. Science. 2001; 292:1907–1910. [PubMed: 11397944]
17. Afkarian M, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. Nat Immunol. 2002; 3:549–557. [PubMed: 12006974]
18. Hwang ES, Szabo SJ, Schwartzberg PL, Glimcher LH. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. Science. 2005; 307:430–433. [PubMed: 15662016]
19. Bird JJ, et al. Helper T cell differentiation is controlled by the cell cycle. Immunity. 1998; 9:229–237. [PubMed: 9729043]
20. Lighvani AA, et al. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. Proc Natl Acad Sci U S A. 2001; 98:15137–15142. [PubMed: 11752460]
21. Chang JT, Segal BM, Shevach EM. Role of costimulation in the induction of the IL-12/IL-12 receptor pathway and the development of autoimmunity. J Immunol. 2000; 164:100–106. [PubMed: 10604999]

22. Leonard WJ, et al. A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor. Nature. 1982; 300:267–269. [PubMed: 6815536]
23. Tsudo M, Kitamura F, Miyasaka M. Characterization of the interleukin 2 receptor beta chain using three distinct monoclonal antibodies. Proc Natl Acad Sci U S A. 1989; 86:1982–1986. [PubMed: 2467293]
24. Onishi M, et al. Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. Mol Cell Biol. 1998; 18:3871–3879. [PubMed: 9632771]
25. Yang Y, Ochando JC, Bromberg JS, Ding Y. Identification of a distant T-bet enhancer responsive to IL-12/Stat4 and IFNgamma/Stat1 signals. Blood. 2007; 110:2494–2500. [PubMed: 17575072]
26. Kano S, et al. The contribution of transcription factor IRF1 to the interferon-gamma-interleukin 12 signaling axis and TH1 versus TH-17 differentiation of CD4+ T cells. Nat Immunol. 2008; 9:34–41. [PubMed: 18059273]
27. Lee YK, et al. Late developmental plasticity in the T helper 17 lineage. Immunity. 2009; 30:92–107. [PubMed: 19119024]
28. Leonard WJ, O'Shea JJ. Jaks and STATs: biological implications. Annu Rev Immunol. 1998; 16:293–322. [PubMed: 9597132]
29. Shi M, Lin TH, Appell KC, Berg LJ. Janus-kinase-3-dependent signals induce chromatin remodeling at the Ifng locus during T helper 1 cell differentiation. Immunity. 2008; 28:763–773. [PubMed: 18549798]
30. Lazarevic V, et al. T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat. Nat Immunol. 12:96–104. [PubMed: 21151104]
31. Imada K, et al. Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. J Exp Med. 1998; 188:2067–2074. [PubMed: 9841920]
32. Malek TR. The biology of interleukin-2. Annu Rev Immunol. 2008; 26:453–479. [PubMed: 18062768]
33. Xue HH, et al. IL-2 negatively regulates IL-7 receptor alpha chain expression in activated T lymphocytes. Proc Natl Acad Sci U S A. 2002; 99:13759–13764. [PubMed: 12354940]
34. Zhang Y, et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 2008; 9:R137. [PubMed: 18798982]
35. Ben-Sasson SZ, et al. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. Proc Natl Acad Sci U S A. 2009; 106:7119–7124. [PubMed: 19359475]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

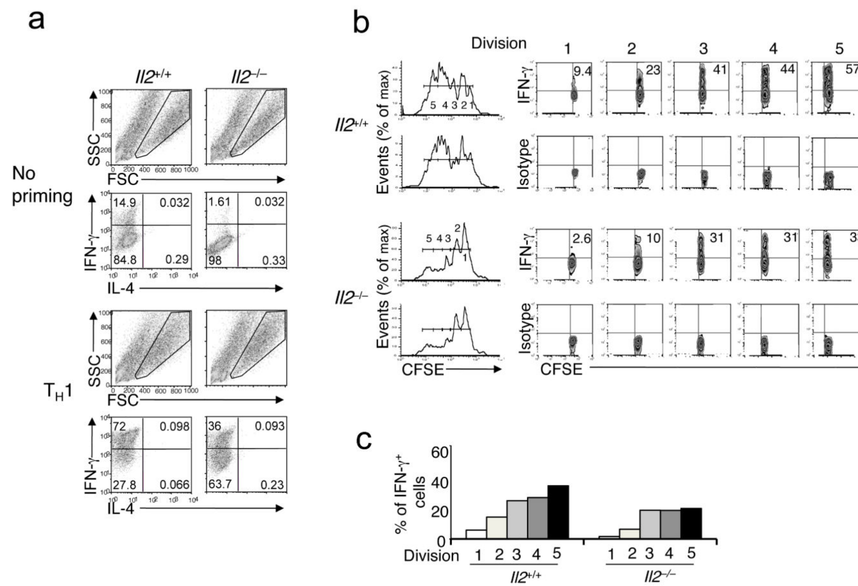

Figure 1. IL-2 has proliferation-dependent and independent effects for T_H1 differentiation

(**a**) Naïve splenic and lymph node CD4+ T cells from *Il2*$^{+/+}$ or *Il2*$^{-/-}$ B10.A 5C.C7TCR transgenic-*Rag2*$^{-/-}$ mice (lines 94 and 110, respectively) were cultured under no-priming or T_H1 conditions for 3 days, washed, and incubated without cytokines or antibodies (no-priming) or with 10 ng/ml IL-12 + 10 μg/ml anti-IL-4 (T_H1) for 2 days, re-incubated under the original no-priming or T_H1 conditions for 3 days, cultured for 5 h with PMA+ ionomycin and GolgiStop, and stained intracellularly for IFN-γ and IL-4. Shown is 1 of 3 experiments. (**b,c**) Naïve splenic and lymph node CD4+ T cells from *Il2*$^{+/+}$ or *Il2*$^{-/-}$ B10.A 5C.C7TCR transgenic/*Rag2*$^{-/-}$ mice were labeled with 5 μM CFSE and cultured under T_H1 conditions for 3 days, re-stimulated with PMA + ionomycin in the presence or GolgiStop for 5 h, and stained intracellularly for IFNγ. Cells were divided into 5 groups according to CFSE dilution (**b**) and the percentage of IFN-γ-producing cells was determined by flow cytometry (**c**). Data are representive of 3 independent experiments.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

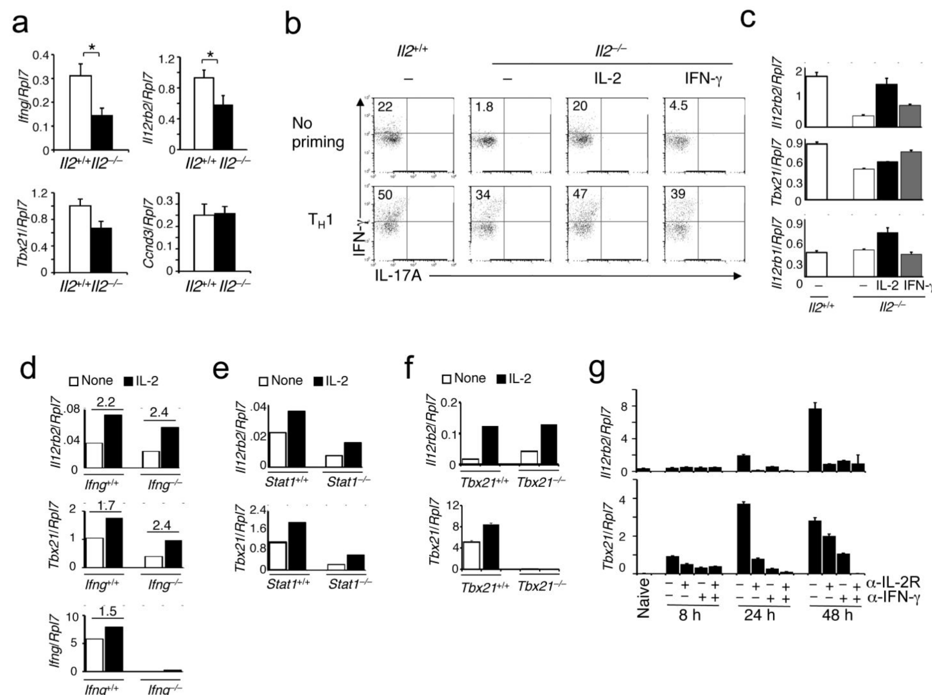

Figure 2. IL-2 induces *Il12rb2* and *Tbx21* expression and is required for normal T_H1 differentiation

(**a**) T_H1 cells in Fig. 1a were analyzed by RT-PCR for *Ifng, Il12rb2, Tbx21*, and *Ccnd3* mRNA, normalized to *Rpl7* expression (means ± S.E.M; data representative of 3 independent experiments). (**b,c**) Naïve splenic and lymph node $CD4^+$ T cells from $Il2^{+/+}$ or $Il2^{-/-}$ B10.A 5C.C7 TCR transgenic $Rag2^{-/-}$ mice were cultured under T_H1 conditions with indicated cytokines for 3 days, re-stimulated with PMA + ionomycin in the presence of GolgiStop for 5 h, and stained intracellularly for IFN-γ and IL-17A (**b**) or analyzed for *Il12rb2, Tbx21,* and *Il12rb1* mRNA (**c**). The experiment was performed 3 times for **b** and twice for **c**. (**d**) $CD4^+$ lymph node T cells from C57BL/6 $Ifng^{-/-}$ and WT mice were activated with plate-bound anti-CD3 + anti-CD28 for 8 h with or without IL-2 and mRNA analyzed. (**e,f**) $CD4^+$ lymph node T cells from 129 strain $Stat1^{-/-}$ (**e**), or $Tbx21^{-/-}$ (**f**) and WT controls were activated with plate-bound anti-CD3 + anti-CD28 for 48 h, rested overnight, stimulated with or without IL-2 for 4 h, and *Il12rb2* and *Tbx21* mRNA analyzed. In **d-f**, data were normalized to *Rpl7* expression and represent 1 of 3 similar experiments. (**g**) Naïve $CD4^+$ T cells from BALB/c mice were cultured under T_H1 conditions with indicated antibodies. *Il12rb2* and *Tbx21* mRNAs were measured 8, 24, and 48 h (means ± S.E.M.). Shown is 1 of 2 experiments with 3 mice per group.

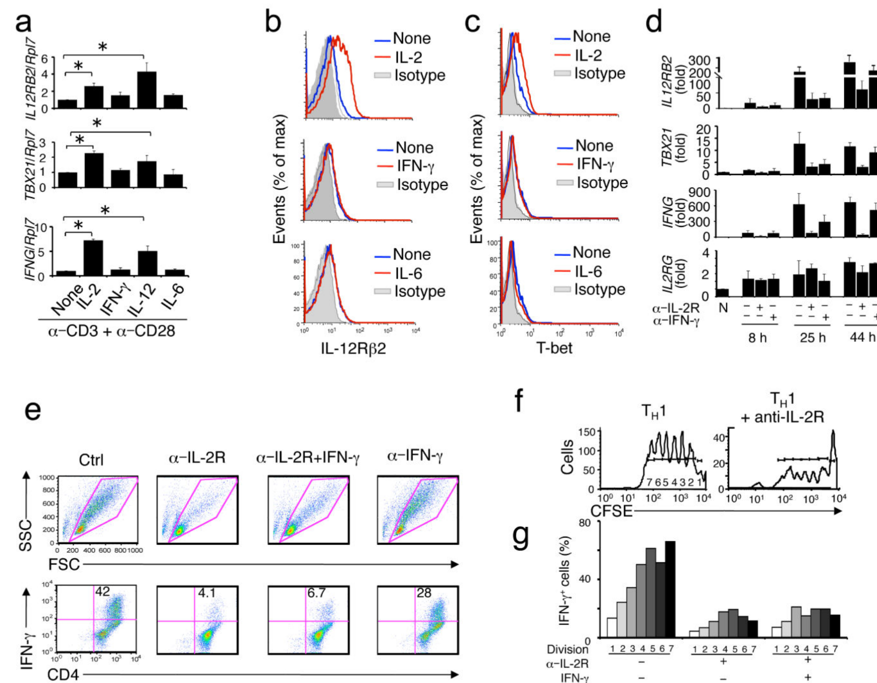

Figure 3. IL-2 induces *IL12RB2* and *TBX21* during human T_H1 differentiation

(**a-c**) Human peripheral blood CD4+ T cells were activated by plate-bound anti-CD3 + anti-CD28 for 48 h, rested overnight, and stimulated with the indicated cytokines for 4 h (**a**) or 20 h (**b, c**). (**a**) *IL12RB2, TBX21*, and *IFNG* mRNA expression (means ± S.E.M from 1 of 3 experiments). In (**b,c**), IL-12Rβ2 and T-bet expression were measured by flow cytometry; representative of 3 experiments. *, $p < 0.05$. (**d**) Naïve human peripheral blood CD4+ T cells were cultured under T_H1 conditions with the indicated antibodies. *IL12RB2, TBX21*, and *IFNG* mRNA expression was determined at 8, 25, and 44 h. Shown is 1 of 3 experiments with 3 donors per group. α-IL-2R indicates α-IL-2Rα + α-IL-2Rβ. (**e**) Naïve human peripheral blood CD4+ T cells were cultured under T_H1 conditions with IFN-γ and/or antibodies as indicated for 3 days, re-stimulated with PMA + ionomycin in the presence of GolgiStop for 5 h, and stained intracellularly for CD4 and IFN-γ. Shown is 1 of 3 experiments. (**f,g**) IL-2 has proliferation-dependent and proliferation-independent effects for T_H1 differentiation. Naïve human peripheral blood CD4+ T cells were labeled with 5 μM CFSE and cultured as indicated for 5 days. Cytokines and antibodies (except anti-CD3 and anti-CD28) were replenished every 2 days. Cells were divided into 7 groups according to CFSE dilution (**f**), and intracellular IFN-γ expression analyzed by flow cytometry (**g**). Shown is one donor representative of two independent experiments, with 2 donors per experiment.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

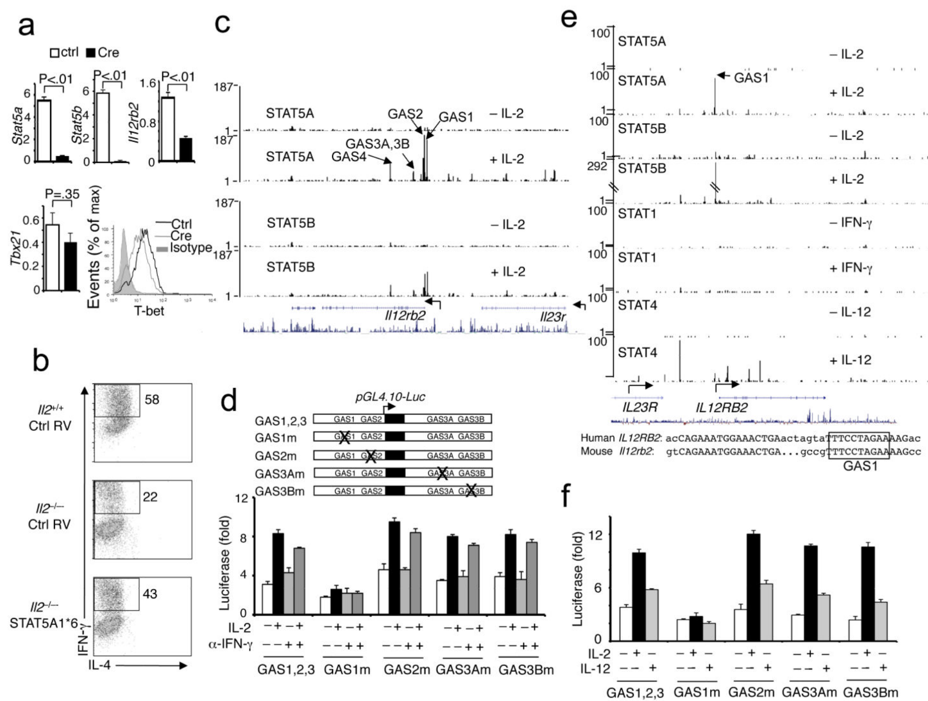

Figure 4. IL-2-induced STAT5-dependent *Il12rb2* expression

(**a**) *Stat5*$^{fl/fl}$ CD4^{+} T cells were subjected to *Cre*-mediated recombination to delete the *Stat5* locus, and *Stat5a*, *Stat5b, Il12Rb2,* and *Tbx21* mRNAs determined 2 days later; T-bet expression was evaluated by flow cytometry (performed 3 times). (**b**) After 1 day of T_H1 conditions, *Il2*$^{-/-}$ and control naïve CD4^{+} T cells were infected by control or *Stat5a*-1*6 retroviruses and cultured under T_H1 conditions for 3 days. Intracellular IFNγ and IL-4 were measured in GFP^{+} cells. Shown is 1 of 3 experiments. (**c**) STAT5A and STAT5B ChIP-Seq analysis in BALB/c T cells activated with anti-CD3 + anti-CD28 for 72 h, rested overnight, and stimulated without or with IL-2 for 1 h. Bottom, schematic of the *Il12rb2* locus, indicating areas of conservation among 17 vertebrate species. The experiment was performed 3 times. (**d**) Pre-activated BALB/c CD4^{+} T cells were transfected with indicated *Il12rb2* reporter constructs, treated as indicated with 100 U/ml IL-2 or 10 μg/ml anti-IFN-γ for 6 h, and luciferase activity analyzed (experiment performed 3 times). (**e**) ChIP-Seq analysis of STAT5A, STAT5B, STAT1, and STAT4 binding at the *IL12RB2* locus in pre-activated human CD4^{+} T cells stimulated for 1 h with IL-2, IL-12, or IFNγ. Bottom, human and mouse sequences spanning the GAS motif. (**f**) Pre-activated BALB/c CD4^{+} T cells transfected with *Il12rb2* reporter constructs, stimulated with IL-2 or IL-12 for 6 h, and luciferase activity determined (mean ± S.E.M.; experiment performed 3 times). Similar results were obtained in C57BL/6 CD4^{+} T cells (not shown).

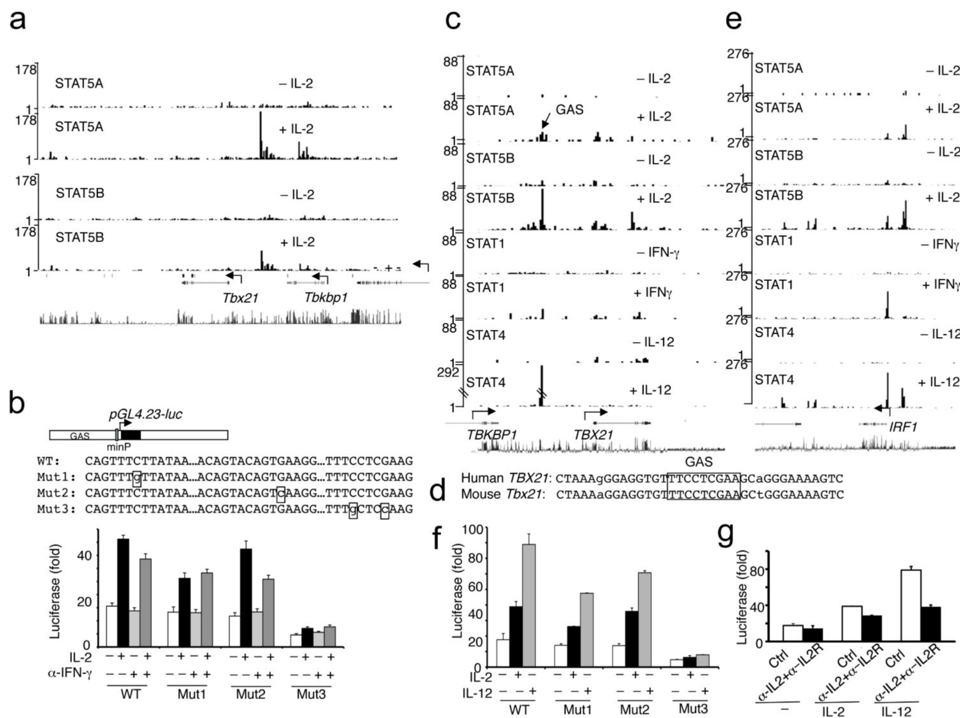

Figure 5. IL-2-induced STAT5-dependent *Tbx21* expression

(**a**) STAT5A and STAT5B binding at the *Tbx21* locus. The data are from the same ChIP-Seq libraries as in Fig. 4a. (**b**) An 800 bp DNA fragment containing the STAT5 binding region with one GAS and two GAS-like motifs corresponding to the upstream *Tbx21* region was inserted into the pGL4.23. luciferase reporter vector 5′ to the SV40 minimal promoter. Luciferase activity (means ± S.E.M.) of WT and GAS mutant constructs were measured. Experiments were repeated 3 times. (**c**) ChIP-Seq data at the human *TBX21* gene, analogous to the *IL12RB2* data in Fig. 2e. (**d**) Human and mouse sequences spanning the conserved *TBX21* GAS motif (boxed). (**e**) ChIP-Seq data at the human *IRF1* locus. (**f, g**) WT and mutant *Tbx21* luciferase constructs were transfected as in **b** into pre-activated BALB/c CD4+ T cells, stimulated with IL-2 or IL-12 for 6 h in the presence of control or anti-IL-2R antibodies as indicated, and cell lysates analyzed for luciferase activity (means ± S.E.M.). Three independent experiments were performed. Similar results were obtained in C57BL/6 CD4+ T cells (data not shown).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

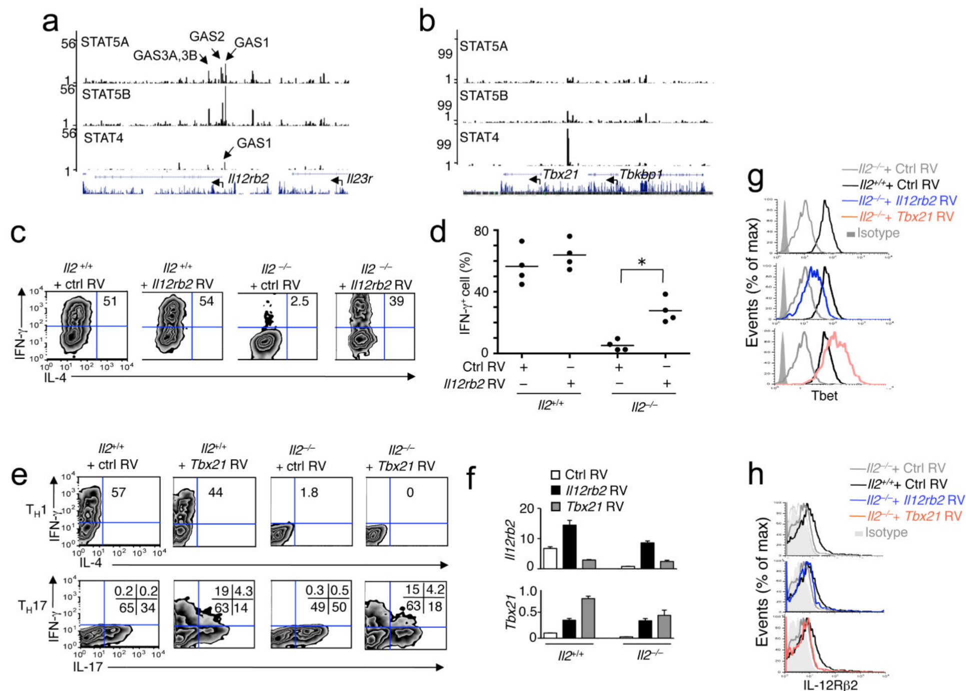

Figure 6. Rescue of T_H1 differentiation in *Il2*$^{-/-}$ mice by *Il12rb2* but not *Tbx21*

(**a,b**), ChIP-Seq analysis of STAT5A, STAT5B, and STAT4 binding at *Il12rb2* (**a**) and *Tbx21* (**b**) loci in mouse CD4$^+$ T cells cultured under T_H1 conditions for 3 days, washed and cultured with 10 ng/ml IL-12 + 10 μg/ml anti-IL-4 for 2 days, and then restimulated under T_H1 conditions for 3 days. (**c, d**), *Il12rb2* rescues T_H1 differentiation in *Il2*$^{-/-}$ mice. After culturing 1 day under T_H1 conditions, naïve CD4$^+$ T cells from B10.A *Il2*$^{-/-}$ *Rag2*$^{-/-}$ 5C.C7 TCR transgenic (line 110) and control (line 94) mice were infected by control or *Il12rb2* retroviruses and cultured under T_H1 conditions for 3 days, re-stimulated with PMA + ionomycin in the presence of GolgiStop for 5 h, and stained intracellularly for IFN-γ and IL-4, gating on GFP$^+$ cells. (**c**), representative mouse; (**d**), summary of an experiment with 4 mice. The experiment was performed 2 times. *, P<0.05. (**e**) T-bet cannot rescue T_H2 differentiation in *Il2*$^{-/-}$ mice. After culturing 1 day under T_H1 or T_H17 conditions, naïve CD4$^+$ T cells from *Il2*$^{-/-}$ (line 110) and control (line 94) mice were infected by control or *Tbx21* retroviruses and cultured under T_H1 or T_H17 conditions for 3 days. Gated GFP$^+$ cells were analyzed by intracellular staining for IFNγ, IL-4, or IL17A. Shown is a representative mouse from 6. (**f-h**) GFP$^+$ cells from (**c**) and (**e**) were sorted. *Il12rb2* or *Tbx21* was measured by RT-PCR (**f**) or flow cytometry (**g,h**).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

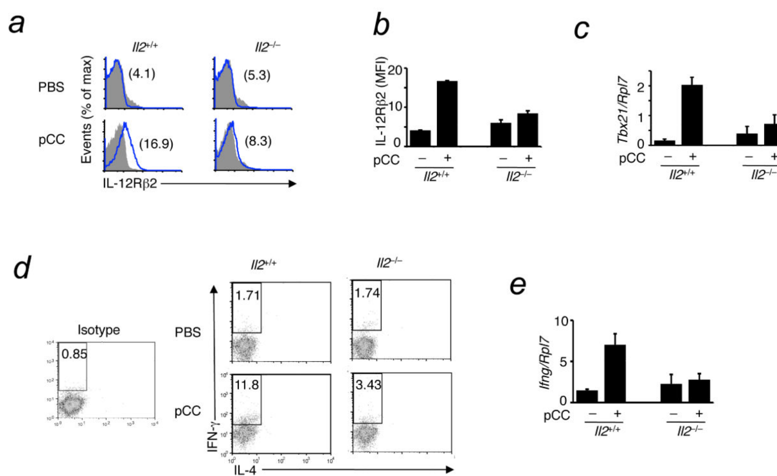

Figure 7. IL-2 is required for in vivo T_H1 responses

(a,b) B10.A *Il2*$^{-/-}$ mice on a *Rag2*$^{-/-}$ 5C.C7 TCR transgenic background (line 110) and control mice (5C.C7 /*Rag2*$^{-/-}$, line 94) were injected i.v. with 1 mg of pigeon cytochrome C protein (pCC, Sigma)[35] or an equal volume of 1 x PBS. All mice additionally were injected i.p. with 25 ug of immunostimulant R848 (GLSynthesis Inc). Lymph nodes were analyzed 16 to 20 h later. Experiments were performed twice, with 2-3 replicates per condition. Shown is IL-12Rβ2 staining from one animal in each group (number is MFI), gated on CD4$^+$ cells (**a**) and the summary of all animals in each group (**b**). (**c**) *Tbx21* mRNA from purified CD4$^+$ T cells from control and *Il2*$^{-/-}$ mice. The experiment was performed twice with 2 to 3 mice/group in each experiment, with similar results. Shown are means ± S.D. (**d,e**) *Il2*$^{+/+}$ or *Il2*$^{-/-}$ mice were immunized and lymph nodes isolated as in (**a**). Cells were then directly intracellularly stained for IFNγ and IL-4 and analyzed by flow cytometry (**d**) or mRNA was isolated from purified CD4$^+$ T cells from control and *Il2*$^{-/-}$ mice and *Ifng* mRNA was determined relatived to *Rpl7* mRNA (**e**).

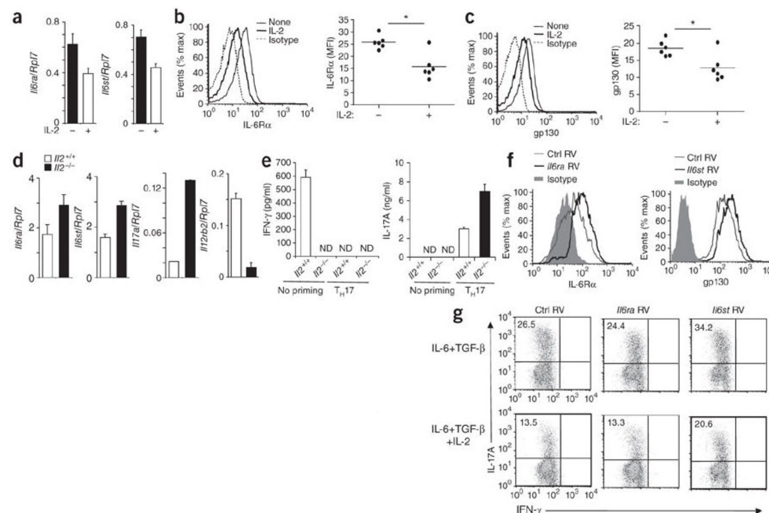

Figure 8. Importance of IL-2 in T_H17 differentiation

(a-c) BALB/c $CD4^+$ T cells were activated by plate-bound anti-CD3 + anti-CD28 for 48 h, rested overnight, and stimulated with IL-2 for 4 h (**a**) or 24 h (**b,c**) and assayed for *Il6ra* and *Il6st* mRNA (**a**) or cell surface expression (**b,c**). Experiments were performed twice, with 3 replicates Shown is a represntative (**b,c**, left panels) and summary of total (**b,c**, right panels). (**d**) *Il2*$^{+/+}$ and *Il2*$^{-/-}$ mouse naïve cells were cultured under T_H17 conditions (see Methods) for 26 h, and mRNAs for the indicated genes were measured by RT-PCR. (**e**) *Il2*$^{+/+}$ and *Il2*$^{-/-}$ mouse naïve cells were cultured under "no priming" or T_H17 conditions for 3 days, IFNγ and IL17 in supernatants were measured by ELISA. Shown is 1 of 3 experiments. (**f,g**) Retroviral transduction of *Il6st* rescues defective T_H17 differentiation by IL-2. Naïve T cells from BALB/c mice were cultured for 1 day under T_H17 conditions, infected with control, *Il6ra*, or *gp130* retroviruses, and cultured under T_H17 conditions for an additional 3 days. IL-6Rα or gp130 surface expression was measured on gated GFP^+ cells (**f**) and IL-17A and IFNγ were measured by intracellular staining (**g**). Shown is one of three independent experiments.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript