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

Abstract

T helper (T<sub>H</sub>) cells control host-defense to pathogens. The receptors for IL-12, IL-4, and IL-6 are required for T<sub>H1</sub>, T<sub>H2</sub>, and T<sub>H17</sub> differentiation, respectively. IL-2 signaling via the transcription factor STAT5 controls T<sub>H2</sub> differentiation by regulating the T<sub>H2</sub> cytokine gene cluster and Il4ra expression. Here we show that IL-2 regulates T<sub>H1</sub> differentiation, inducing STAT5-dependent IL-12Rβ2 and T-bet expression, with impaired human T<sub>H1</sub> differentiation when IL-2 was blocked. T<sub>H1</sub> differentiation was also impaired in mouse Il2<sup>−/−</sup> T cells but restored by IL-12Rβ2 expression. Consistent with IL-2's inhibition of T<sub>H17</sub> differentiation, IL-2 decreased Il6ra and Il6st expression, and Il6st augmented T<sub>H17</sub> differentiation even when IL-2 was present. Thus, IL-2 influences T<sub>H</sub> cell differentiation by modulating cytokine receptor expression to help specify and maintain differentiated states.
humans with X-linked severe combined immunodeficiency\textsuperscript{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\textsuperscript{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\textsubscript{reg}) cells\textsuperscript{11}. IL-2 is also known to critically regulate T\textsubscript{H}2 differentiation in a STAT5-dependent manner, acting early at the Il4ra locus to induce IL-4Ra expression\textsuperscript{4} and later to open chromatin accessibility at the Il4-Ill3-Rad50 T\textsubscript{H}2 locus\textsuperscript{4,12}. Given the role of IL-2 in T\textsubscript{H}2 differentiation, as well as the ability of IL-2 to induce IFN-\gamma expression\textsuperscript{13,14} and to decrease IL-17 production during T\textsubscript{H}17 differentiation\textsuperscript{15}, we decided to investigate whether IL-2 is more broadly involved in T\textsubscript{H} differentiation by regulating corresponding cytokine receptors. IL-2 induced expression of IL-12R, and retroviral transduction of Il12rb2 reversed the defective T\textsubscript{H}1 differentiation in Il2\textsuperscript{−/−} T cells. IL-2 also negatively regulated expression of Il6ra and of Il6st as at least a partial mechanism for negative regulation of T\textsubscript{H}17 differentiation. Therefore our data reveal that IL-2 regulates T\textsubscript{H}1, T\textsubscript{H}2, and T\textsubscript{H}17 differentiation based at least in part on its modulation of expression of key cytokine receptors.

RESULTS

Defective T\textsubscript{H}1 differentiation in Il2\textsuperscript{−/−} T cells

We initially focused on the role of IL-2 in T\textsubscript{H}1 differentiation, a process that is driven by IL-12. IL-12 induces not only Ifng expression\textsuperscript{1} but also T-bet\textsuperscript{7}, which promotes the survival and proliferation of differentiating T\textsubscript{H}1 cells\textsuperscript{16}. T-bet induces Ifng\textsuperscript{7,16} and IL-12R\beta2 expression\textsuperscript{17}, while inhibiting GATA3 binding to target genes, including the Il4 gene\textsuperscript{18}, and thus T\textsubscript{H}2 differentiation. Naïve CD4\textsuperscript{+} T cells do not respond to IL-12 because they lack a key receptor component, IL-12R\beta2\textsuperscript{3}. Like T\textsubscript{H}2 differentiation, T\textsubscript{H}1 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-\gamma production in wild-type (WT) and Il2\textsuperscript{−/−} T cells. We stimulated Il2\textsuperscript{+/+} or Il2\textsuperscript{−/−} CD4\textsuperscript{+} T cells with anti-CD3 + anti-CD28, gating on proliferating cells based on FSC-SSC (Fig. 1a, upper two panels). Naïve CD4\textsuperscript{+} T cells do not respond to IL-12 because they lack a key receptor component, IL-12R\beta2\textsuperscript{3}. Like T\textsubscript{H}2 differentiation, T\textsubscript{H}1 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-\gamma production in wild-type (WT) and Il2\textsuperscript{−/−} T cells. We stimulated Il2\textsuperscript{+/+} or Il2\textsuperscript{−/−} CD4\textsuperscript{+} T cells with anti-CD3 + anti-CD28, gating on proliferating cells based on FSC-SSC (Fig. 1a, upper two panels). Naïve Il2\textsuperscript{−/−} CD4\textsuperscript{+} T cells stimulated with anti-CD3 + anti-CD28 had far fewer IFN-\gamma\textsuperscript{+} cells than did Il2\textsuperscript{+/+} CD4\textsuperscript{+} T cells (Fig. 1a, second pair of panels), consistent with previous observations that IL-2 induces IFN-\gamma production\textsuperscript{13,14}. Moreover, after two rounds of T\textsubscript{H}1 polarization, a lower percentage of Il2\textsuperscript{−/−} CD4\textsuperscript{+} T cells expressed IFN-\gamma\textsuperscript{+} as compared to Il2\textsuperscript{+/+} cells (Fig. 1a, lower two sets of panels). Although IL-2 contributes to survival and accordingly the Il2\textsuperscript{−/−} population had more dead cells than the Il2\textsuperscript{+/+} population after T\textsubscript{H}1 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-\gamma\textsuperscript{+} cells among Il2\textsuperscript{−/−} T cells could not be attributed solely to diminished viability.

Because IL-2 is a T-cell growth factor, and because IFN-\gamma expression is known to increase with successive rounds of cell division\textsuperscript{19}, we tested whether the role of IL-2 in T\textsubscript{H}1 differentiation was due solely to its effects on proliferation or whether it also had

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proliferation-independent effects on IFN-γ production. To follow cell division we labeled cells with CFSE and incubated them under Th1 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 Th1 differentiation.

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

To clarify the role of IL-2 in Th1 differentiation, we examined Il2−/− Th1 differentiated cells for Th1-associated genes. These cells expressed less Ifng as well as less Il12rb2 and Tbx21 (encoding T-bet) mRNA than did WT Th1 cells (Fig. 2a); Ccnld3 (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 Th1 differentiation, we next investigated whether the defective Th1 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. 2b, 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, 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 Th1 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 cooperate in the induction of Il12rb2, our data now indicate a direct IL-12-independent effect of IL-2. Consistent with effects of both IL-2 and IFN-γ on Th1 differentiation, antibodies to IL-2Rα and IL-2Rβ or to IFN-γ decreased Il12rb2 and Tbx21 mRNA expression (Fig. 2g); the antibodies cooperatively inhibited Th21 expression, whereas blocking IL-2 or IFN-γ alone decreased Il12rb2 mRNA (Fig. 2g).
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 TH1 differentiation, we used blocking antibodies to IL-2Rα (anti-Tac) and IL-2Rβ (Mikβ1). When human peripheral blood naïve (CD45RAhiCD25−HLA-DR−) CD4+ T cells were cultured under TH1-polarizing conditions, the anti-IL-2R antibodies decreased IL12RB2, TBX21, and IFNG mRNA induction (Fig. 3d) as well as the percentage of IFNγ+ CD4+ T cells (Fig. 3e). The addition of exogenous IFN-γ only slightly reversed this effect (Fig. 3e). Similar to mouse TH1 differentiation, during human TH1 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 TH1 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 TH1 differentiation, the effect of IL-2 on human TH1 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 TH1 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 STAT5B9, 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 TH1 differentiation, we examined the effect of retroviral expression of a constitutively-activated form of STAT5A, denoted STAT5A-1*6. When this was transduced into Il2−/− T cells, defective TH1 differentiation was partially reversed (Fig. 4b), consistent with a role for STAT5 in TH1 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 TTCN3GAG 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).
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), \( \approx 12 \) 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-\( \gamma \) had only a partial effect on reporter activity (Fig. 5b and Supplementary Fig. 5), consistent with IFN-\( \gamma \)-independent effects of IL-2 on Th1 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-\( \gamma \) (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 Th1 differentiation\(^26\).

When we deleted Stat5a and Stat5b by Cre-mediated deletion \textit{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-12R\( \beta \)2. Antibodies to IL-2, IL-2R\( \alpha \), and IL-2R\( \beta \) lowered IL-12-induced Tbx21 activity (Fig. 5g), presumably at least in part by their inhibition of IL-2-induced IL-12R\( \beta \)2 expression.

Analysis of ChIP-Seq libraries from Th1-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 Th1 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 11\(^{th}\) 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 Th1 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.

\textit{Il12rb2 rescues Th1 differentiation in Il2\(-/-\) T cells}

We next investigated whether enforced expression of Il12rb2 or Tbx21 could rescue defective Th1 differentiation in Il2\(-/-\) cells. As expected, Il12rb2 retroviral transduction had
little effect in $I\!\!L2^{+/-}$ cells, which already express IL-12Rβ2; however, transduction of $I\!\!L2^{-/-}$ 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 Th1 differentiation in $I\!\!L2^{-/-}$ CD4+ T cells (Fig. 6e), suggesting IL-2-induced T-bet is not sufficient for Th1 differentiation. Nevertheless, retroviral transduction of Tbx21 increased IFN-γ production in both Th17-differentiated $I\!\!L2^{+/-}$ and $I\!\!L2^{-/-}$ T cells (Fig. 6e), confirming the functionality of the retrovirally-transduced protein, although the basis for the different effect of the Tbx21 retrovirus in Th1 vs. Th17-differentiated cells is unclear. Interestingly, transduction of the Il12rb2 retrovirus substantially increased Il12rb2 and Tbx21 mRNA, as compared to a control virus in both $I\!\!L2^{+/-}$ and $I\!\!L2^{-/-}$ T cells (Fig. 6f). In contrast, the Tbx21 retrovirus significantly increased Tbx21 mRNA but if anything slightly decreased Il12rb2 mRNA expression in $I\!\!L2^{+/-}$ T cells and only modestly increased Il12rb2 mRNA in $I\!\!L2^{-/-}$ T cells (Fig. 6f). At the protein level, both viruses induced T-bet expression (Fig. 6g). In contrast, 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 Th1 responses in vivo**

The above data collectively revealed a critical role for IL-2 in Th1 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 $I\!\!L2^{+/-}$ CD4+ T cells, but there was little effect in T cells from $I\!\!L2^{-/-}$ mice (Fig. 7a right vs. left panels; Fig. 7b). Similarly, less Tbx21 mRNA was detected in cells from $I\!\!L2^{-/-}$ 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 study4 to gether establish key roles for IL-2 in Th1 and Th2 differentiation, at least in part based on cytokine receptor modulation. IL-2 can also negatively regulate Th17 differentiation15. Given the key role of IL-6 in Th17 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 Th17 conditions, both Il6ra and Il6st mRNA expression were higher in $I\!\!L2^{-/-}$ 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 Th17-differentiated cells in an IL-2-dependent fashion (Fig. 8d), which might explain the ability of Th17 cells to produce IFN-γ when IL-12 is present and thus for the cells to exhibit Th plasticity27. Because IL-2 is required for normal IFN-γ production (Fig. 1a) and IFN-γ negatively regulates the generation of Th17 cells6, 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 Th17 cells in either the WT or $I\!\!L2^{-/-}$ background (Fig. 8e, left panel), indicating that IFN-γ by itself cannot explain the difference in IL-17 production in the $I\!\!L2^{+/-}$ and $I\!\!L2^{-/-}$ T cells (Fig. 8e right panel). When Il6ra and Il6st were

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separately introduced into CD4+ T cells by retroviral transduction (Fig. 8f), only Il6st could partially rescue the defective Th17 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 Th17 differentiation (Fig. 8g, vi vs. iv). However, given that IL-2 inhibited Th17 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 Treg cell development, and mediate activation-induced cell death. IL-2 can also increase IL-4Rα expression, thereby priming T cells for Th2 differentiation. We now show that IL-2 also regulates other key cytokine receptors, inducing IL-12Rβ2 to allow responsiveness to IL-12 and promoting Th1 differentiation, while repressing IL-6Rα and gp130 and thus IL-6 responsiveness, thereby inhibiting Th17 differentiation.

Previously it was suggested that JAK3, which is a kinase that associates with the common cytokine receptor γ chain28, could contribute to Th1 differentiation by mediating IL-2-induced STAT5 binding to the Ifng gene29. Our findings herein show broader roles for IL-2 in Th1 differentiation, with IL-2-induced STAT5A and STAT5B binding to key elements in both the Il2rb2 and Tbx21 genes. Not only was expression of Il2rb2 and Tbx21 diminished in Il2−/− mice, but IL-2 could induce expression of each of these genes. The induction of Il2rb2 by IL-2 appears to be more important for Th1 differentiation than the induction of Tbx21 given that retroviral transduction of Il2rb2 but not Tbx21 could rescue Th1 differentiation in Il2−/− cells. Although Il2rb1 was not diminished in Il2−/− mice, IL-2 also induced Il2rb1 expression, which potentially can further enhance responsiveness to IL-12. Interestingly, IL-2 and IL-12 appear to have overlapping actions for Th1 differentiation, with IL-2 via STAT5 and IL-12 via STAT4 both contributing to the regulation of Il2rb2 and Tbx21 expression. Although retroviral transduction of Tbx21, unlike Il2rb2, could not rescue Th1 differentiation in Il2−/− T cells, Tbx21 was previously shown to be capable of promoting Th1 differentiation in Stat4−/− cells16, and we also show herein that the Tbx21 retrovirus could increase IFN-γ production by Th17 cells. T-bet can also repress Th17 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 Th1 differentiation for which T-bet alone cannot compensate.

Consistent with an even broader role for IL-2 in Th differentiation via regulation of cytokine receptors, IL-2 down-regulated Il6ra and Il6st and inhibition of Th17 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.
In summary, our findings reveal a broader role for IL-2 in TH 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-4Rα4, and IL-2Rβ31 expression but decreases gp130 expression, modulating signals by IL-12, IL-4, IL-2, and IL-6, and thereby for TH1, TH2, Treg, and TH17 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, 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 TH 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.

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Appendix

METHODS

Mouse and human lymphocytes

B10.A Il2−/− mice on a Rag2−/−/Sc.C7 TCR transgenic background (line 110) and control mice (Rag2−/−/Sc.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-DR−CD4+ (For TH1 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+
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 Rd17.

**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](http://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 11\(^{th}\) 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),
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 (eBioscience). 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 Th1 conditions, anti-IL-4 (11B11, 10 μg/ml) plus IL-12 (10 ng/ml) were added. For human Th1 conditions, anti-IL-4 (MP4-25D2, 10 μg/ml) plus IL-12 (10 ng/ml) were added. For mouse Th17 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+ 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-mIl6ra and pRV-mglIl6st 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

Stat5fl/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.
**Statistical analyses**

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

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Figure 1. IL-2 has proliferation-dependent and independent effects for T_{H}1 differentiation

(a) Naïve splenic and lymph node CD4^{+} T cells from \textit{Il2}^{+/+} or \textit{Il2}^{-/-} B10.A 5C.C7TCR transgenic-Rag2^{-/-} mice (lines 94 and 110, respectively) were cultured under no-priming or T_{H}1 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_{H}1) for 2 days, re-incubated under the original no-priming or T_{H}1 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 \textit{Il2}^{+/+} or \textit{Il2}^{-/-} B10.A 5C.C7TCR transgenic/Rag2^{-/-} mice were labeled with 5 μM CFSE and cultured under T_{H}1 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 representative of 3 independent experiments.
Figure 2. IL-2 induces \textit{Il12rb2} and \textit{Tbx21} expression and is required for normal T\textsubscript{H1} differentiation

(a) T\textsubscript{H1} cells in Fig. 1a were analyzed by RT-PCR for \textit{Ifng}, \textit{Il12rb2}, \textit{Tbx21}, and \textit{Ccnd3} mRNA, normalized to \textit{Rpl7} expression (means ± S.E.M; data representative of 3 independent experiments). (b,c) Naïve splenic and lymph node CD4\textsuperscript{+} T cells from \textit{Il2\textsuperscript{+/+}} or \textit{Il2\textsuperscript{−/−}} B10.A SC.J7 TCR transgenic \textit{Rag2\textsuperscript{−/−}} mice were cultured under T\textsubscript{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-\textgamma and IL-17A (b) or analyzed for \textit{Il12rb2}, \textit{Tbx21}, and \textit{Il12rb1} mRNA (c). The experiment was performed 3 times for b and twice for c. (d) CD4\textsuperscript{+} lymph node T cells from C57BL/6 \textit{Ifng\textsuperscript{−/−}} 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\textsuperscript{+} lymph node T cells from 129 strain \textit{Stat1\textsuperscript{−/−}} (e), or \textit{Tbx21\textsuperscript{−/−}} (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 \textit{Il12rb2} and \textit{Tbx21} mRNA analyzed. In d-f, data were normalized to \textit{Rpl7} expression and represent 1 of 3 similar experiments. (g) Naïve CD4\textsuperscript{+} T cells from BALB/c mice were cultured under T\textsubscript{H1} conditions with indicated antibodies. \textit{Il12rb2} and \textit{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-2R$\alpha + \alpha$-IL-2R$\beta$. (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.
Figure 4. IL-2-induced STAT5-dependent *Il12rb2* expression

(a) *Stat5*^f/f^ 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, *Il2rβ2*^−/−^ and control naïve CD4^+^ T cells were infected by control or *Stat5a*-1*β6* 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).
Figure 6. Rescue of Th1 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 Th1 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 Th1 conditions for 3 days. (c, d), Il12rb2 rescues Th1 differentiation in Il2−/− mice. After culturing 1 day under Th1 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 Th1 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. (e), representative mouse; (d), summary of an experiment with 4 mice. The experiment was performed 2 times. *, P<0.05. (e) T-bet cannot rescue Th2 differentiation in Il2−/− mice. After culturing 1 day under Th1 or Th17 conditions, naïve CD4+ T cells from Il2−/− (line 110) and control (line 94) mice were infected by control or Tbx21 retroviruses and cultured under Th1 or Th17 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).
Figure 7. IL-2 is required for in vivo T<sub>H1</sub> responses
(a,b) B10.A Il2<sup>−/−</sup> mice on a Rag2<sup>−/−</sup> 5C.C7 TCR transgenic background (line 110) and control mice (5C.C7/Rag2<sup>−/−</sup>, line 94) were injected i.v. with 1 mg of pigeon cytochrome C protein (pCC, Sigma) 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-12R<sub>β2</sub> staining from one animal in each group (number is MFI), gated on CD4<sup>+</sup> cells (a) and the summary of all animals in each group (b). (c) Tbx21 mRNA from purified CD4<sup>+</sup> T cells from control and Il2<sup>−/−</sup> 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<sup>+/+</sup> or Il2<sup>−/−</sup> mice were immunized and lymph nodes isolated as in (a). Cells were then directly intracellularly stained for IFN<sub>γ</sub> and IL-4 and analyzed by flow cytometry (d) or mRNA was isolated from purified CD4<sup>+</sup> T cells from control and Il2<sup>−/−</sup> mice and Ifng mRNA was determined relative to Rpl7 mRNA (e).
Figure 8. Importance of IL-2 in T<sub>H17</sub> differentiation

(a-c) BALB/c CD4<sup>+</sup> 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 representative (b,c, left panels) and summary of total (b,c, right panels).

(d) Il2<sup>+/+</sup> and Il2<sup>−/−</sub> mouse naïve cells were cultured under T<sub>H17</sub> conditions (see Methods) for 26 h, and mRNAs for the indicated genes were measured by RT-PCR. (e) Il2<sup>+/+</sup> and Il2<sup>−/−</sub> mouse naïve cells were cultured under “no priming” or T<sub>H17</sub> conditions for 3 days, IFN<sub>γ</sub> and IL17 in supernatants were measured by ELISA. Shown is 1 of 3 experiments.

(f,g) Retroviral transduction of Il6st rescues defective T<sub>H17</sub> differentiation by IL-2. Naïve T cells from BALB/c mice were cultured for 1 day under T<sub>H17</sub> conditions, infected with control, Il6ra, or gp130 retroviruses, and cultured under T<sub>H17</sub> conditions for an additional 3 days. IL-6R<sub>α</sub> or gp130 surface expression was measured on gated GFP<sup>+</sup> cells (f) and IL-17A and IFN<sub>γ</sub> were measured by intracellular staining (g). Shown is one of three independent experiments.