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The composition and signaling of the IL-35 receptor are unconventional

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Abstract

Interleukin-35 (IL-35) belongs to the IL-12 family of heterodimeric cytokines but has a distinct functional profile. IL-35 suppresses T cell proliferation and converts naïve T cells into IL-35-producing iTr35. Here we show that IL-35 signals through a unique IL-12Rβ2:gp130 heterodimer or via homodimers. Conventional T cells are sensitive to IL-35-mediated suppression in the absence of one but not both receptor chains, whereas signaling through both chains is required for IL-35 expression and iTr35 conversion. IL35R signaling requires the transcription factors STAT1 and STAT4, which form a unique heterodimer that binds to distinct sites within the *Il12a* and *Ebi3* promoters. This unconventional mode of signaling, which is distinct from other members of the IL-12 family, may broaden the spectrum and specificity of IL-35-mediated suppression.

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AUTHOR CONTRIBUTIONS

L.W.C. designed (with help from D.A.A.V) and executed a substantial proportion of the experiments, analyzed data and wrote the manuscript; G.M.D. performed STAT co-IP and ChIP/ChIP-reChIP experiments, cytokine-receptor co-IP experiments, some of the pSTAT analysis and functional assays and wrote the manuscript; C.Guy performed confocal microscopy/FRET experiments; K.M.V. generated all the constructs used in the project; V.C. performed T_H1/T_H2 polarization for receptor analysis; D.F., A.R.S., C.A.H. and C.G.D. provided mice; K.C.G performed structural modeling of cytokine receptor complexes; P.M. performed phospho-STAT Western blot; C.A.H., P.M., K.C.G, C.G.D. and K.M.V. commented on the manuscript, and D.A.A.V. conceptualized the research, directed the study, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

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Lethal autoimmune diseases can develop in the absence of effective immunosuppressive mediators. Regulatory T (T_{reg}) cells are necessary for immune homeostasis and to prevent autoimmunity, yet also limit anti-tumor immunity[1,2]. T_{reg} cells utilize a broad range of mechanisms to suppress immunity including suppressive cytokines such as IL-35, IL-10 and TGF-β. IL-35, belongs to the IL-12 cytokine family, which also includes IL-12, IL-23, and IL-27. The latter are heterodimeric proteins composed of shared α chains (p19 [*Il23a*], p28 [*Il27*] and p35 [*Il12a*]) and β chains (p40 [*Il12b*] and Ebi3 [*Ebi3*]), are produced by antigen producing cells, and have immunomodulatory activities. IL-12 and IL-23 are pro-inflammatory cytokines that can promote T_H1 and T_H17 differentiation and function[3]. IL-27 serves to initiate T_H1 polarization but exhibits a host of immunomodulatory activities including limiting T_H2 activity[4], blocking T_H17 differentiation and TGFβ-driven induced T_{reg} (iT_{reg}) formation, and IL-10 production[5–7]. IL-35 is distinct from other family members in that it is produced by T_{reg} cell populations and is suppressive. *In vitro* and *in vivo*, IL-35 has two known biological effects; suppression of conventional T (T_{conv}) cell proliferation and conversion of naive T_{conv} cells into a strongly suppressive induced T_{reg} cell population, termed iTr35, which function via IL-35[8–10]. Thus a primary downstream target of IL-35 receptor (IL-35R) signaling is induction of *Ebi3* and *Il12a* transcription.

Chain sharing extends beyond the cytokines to their receptors and signaling pathways. IL-12Rβ1 (*Il12rb1*) and IL-12Rβ2 (*Il12rb2*) form the IL-12 receptor[11], while IL-23 signals through IL-12Rβ1 paired with the IL-23R (*Il23r*)[12]. In contrast, IL-27 utilizes gp130 (*Il6st*) and IL-27R, also known as WSX-1 or TCCR (*Il27ra*)[13]. Although IL-12Rβ1, IL-12Rβ2, IL-23R and IL-27R usage is restricted to the IL-12 cytokine receptor family, gp130 is ubiquitously expressed and is a component of receptors for many cytokines including IL-6, IL-11, leukocyte inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1) and cardiotrophin-like cytokine (CLC)[14].

Following receptor engagement, signaling is initiated by activation of members of the Janus kinase (Jak) family followed by phosphorylation and nuclear localization of members of the signal transducer and activator of transcription (STAT) family[12, 15]. A major conundrum in cytokine signaling is explaining how so many cytokines (over 50) can mediate such diverse functional events via only 7 STAT proteins[16]. While multiple STAT proteins can be phosphorylated following receptor engagement, usually only one or two are essential. For instance, although STAT1 (*Stat1*), STAT3 (*Stat3*), STAT4, (*Stat4*) and STAT5 (*Stat5*) can all be activated by IL-12 signaling, only STAT4 is required to mediate IL-12's effects[15, 17]. Although STAT phosphorylation following IL-23 signaling is similar to that of IL-12, STAT3, but not STAT4, appears to be the critical signal transducer for IL-23[12]. Similarly, IL-27 receptor engagement activates STAT1, STAT2, STAT3 and STAT5 to varying degrees but STAT1 and STAT3 seem most important for complete IL-27 bioactivity[18, 19].

While most receptor chains can contribute to both cytokine binding and downstream signaling, for some cytokine receptors one chain predominates in mediating signaling. For instance, *Il12rb2*$^{-/-}$ T cells are defective in their ability to phosphorylate STAT4, generate IFNγ and promote T_H1 differentiation despite the presence of high affinity IL-12 binding sites on IL-12Rβ1[20]. The relative contributions of gp130 and WSX1 in IL-27 signaling are more obscure. Although both receptor chains are necessary for STAT phosphorylation, IL-27 function can be abrogated with anti-gp130 mAb[13].

Given that IL-35 is comprised of chains shared with IL-12 and IL-27, we hypothesized that the IL-35R chains and the STATs that mediate its signal transduction might also be components used to mediate IL-12 and IL-27 signaling. However, as IL-35 mediates distinct biological events from other members of the IL-12 family, the use of alternate receptors and signaling pathways could not be ruled out. In this study we addressed four important

questions: (1) what receptor chains comprise the IL-35R, (2) how is IL-35R expression induced, (3) which STATs mediate IL-35R signaling, and (4) how does IL-35R signaling uniquely induce IL-35 secretion and iTr35 induction?

RESULTS

Suppression by IL-35 occurs through IL-12Rβ2 or gp130

IL-35 mediates two distinct functions in T cells: suppression of proliferation and conversion to IL-35-producing iTr35 cells[8, 10]. Thus, two anticipated functional phenotypes of genetic manipulation of IL-35R expression are resistance to IL-35-mediated suppression and prevention of iTr35 conversion. We first asked whether IL-35 could suppress the proliferation of $CD4^+CD45RB^{hi}CD25^-$ naïve $CD4^+$ T_{conv} cells that lacked expression of each of the IL-12 family receptor chains. For these experiments we either used HEK293T-derived recombinant IL-35 (rIL-35) or, to remove any potential effects of 293T supernatant, Protein G beads coupled with either IL-35 conjugated via a non-neutralizing anti-p35 specific monoclonal antibody (mAb) or isotype control mAb[8, 10]. The ability of IL-35 to suppress *Il27ra*$^{-/-}$ and *Il12rb1*$^{-/-}$ T_{conv} cells was comparable to that seen with wild-type T_{conv} cells (Supplementary Fig. 1a). In contrast, T_{conv} cells that lacked expression of either IL-12Rβ2 (*Il12rb2*$^{-/-}$) or gp130 ($CD4^{cre}$ × *Il6st*$^{fl/fl}$ referred to herein as *Il6st*$^{\Delta T}$) were partially resistant to IL-35-mediated suppression (Fig. 1a and Supplementary Fig. 2a). However, T cells that lacked expression of both gp130 and IL-12Rβ2 (*Il12rb2*$^{-/-}$ × $CD4^{cre}$ × *Il6st*$^{fl/fl}$ mice, referred to herein as IL-35R$^{\Delta T}$) were completely resistant to either soluble or bead-bound IL-35.

Next, we asked if IL-35R-deficient T_{conv} cells are also resistant to suppression mediated by regulatory T cell populations expressing a natural source of IL-35 (iTr35). The induced T_{reg} population, iTr35 suppresses proliferation of T_{conv} cells exclusively via IL-35 [8]. Both exogenously added IL-35 and IL-35-producing T_{reg} cells induced conversion of T_{conv} cells to iTr35 *in vitro* and *in vivo* under inflammatory conditions. As expected, neutralizing IL-35 activity with anti-IL-35, but not isotype control, blocked suppression of wild-type T_{conv} cells by iTr35 (Fig. 1b). Consistent with the rIL-35 observations, T_{conv} cells that lacked expression of IL-12Rβ2 or gp130 alone also appeared partially resistant to iTr35-mediated suppression. However, loss of both chains of the IL-35R (IL-35R$^{\Delta T}$) rendered cells completely resistant to iTr35-mediated suppression (Fig. 1b).

Natural T_{reg} cells that lack IL-35 expression (*Ebi3*$^{-/-}$ or *Il12a*$^{-/-}$) have partially defective function *in vitro* and *in vivo*[10]. As anticipated, *Il12rb2*$^{-/-}$, *Il6st*$^{\Delta T}$ and IL-35R$^{\Delta T}$ T_{conv} cells were partially resistant to natural T_{reg} (nT_{reg})-mediated suppression (Supplementary Fig. 1b). Nevertheless, this defect is limited due to the multitude of alternative regulatory mechanisms available to nT_{reg} cells[1]. However, nT_{reg} cells activated in the presence of T_{conv} cells in the upper chamber of a Transwell plate can mediate potent IL-35-dependent, contact-independent suppression of T_{conv} cells in the bottom well[9]. Whereas wild-type T_{conv} cells were potently suppressed by nT_{reg}-T_{conv} co-cultures, IL-35R$^{\Delta T}$ T_{conv} cells were completely resistant (Supplementary Fig. 1c). Taken together these data suggest that T_{conv} cells lacking both gp130 and IL-12Rβ2, but not IL-12Rβ1 and WSX1, are resistant to IL-35-mediated suppression regardless of source. Surprisingly, T_{conv} cells lacking just one chain of the putative IL-35R were partially resistant in *in vitro* suppression assays suggesting that signaling can occur with only one chain of the IL-35R expressed.

iTr35 conversion requires both gp130 and IL-12Rβ2

In addition to suppressing T cell proliferation, IL-35 also can induce *Ebi1* and *Il12a* expression, and the conversion of T_{conv} cells into IL-35-producing iTr35 cells. We

determined if loss of one or both chains of the putative IL-35R could impact the ability of receptor chain deficient T_{conv} cells to convert to iTr35. Although stimulation of naive wild-type T_{conv} cells in the presence of IL-35 resulted in the strong upregulation of *Ebi3* and *Il12a* mRNA, deficiency in either receptor chain blocked IL-35 upregulation (Fig. 2a). Further, while wild-type cells differentiated into iTr35 were much more suppressive than cells differentiated to iTrCon cells, *Il12rb2*$^{-/-}$ and *Il6st*$^{\Delta T}$ cells differentiated in the presence of IL-35 failed to develop a suppressive iTr35 phenotype (Fig. 2b). Despite the fact that *Il12rb2*$^{-/-}$ and *Il6st*$^{\Delta T}$ T_{conv} cells are only partially resistant to IL-35-mediated suppression *in vitro*, they failed to convert into suppressive iTr35 cells. These data suggest that single-chain signaling is sufficient for partial IL-35-mediated suppression, but a complete IL-35R (IL-12Rβ2 + gp130) is required to induce IL-35 production.

IL-35R-deficient T cells are resistant to IL-35 *in vivo*

Given that iTr35 mediate suppression entirely via IL-35[8], we utilized two different models to assess the requirement of IL-12Rβ2 and gp130 in IL-35-mediated suppression *in vivo*. First, iTr35 cells regulate the homeostatic expansion of T_{conv} cells in the lymphopenic environment of recombination activating gene-1 (*Rag1*)$^{-/-}$ mice. Purified wild-type, *Il6st*$^{\Delta T}$, *Il12rb2*$^{-/-}$ or IL-35R$^{\Delta T}$ Thy1.2$^+$ T_{conv} cells, either alone or in the presence of Thy1.1$^+$ iTr35 cells, were adoptively transferred into *Rag1*$^{-/-}$ mice. Seven days after transfer, suppression of T_{conv} cell expansion was monitored by determining Thy1.2$^+$ T_{conv} cell numbers. Whereas iTr35 cells significantly limited the proliferation of wild-type, *Il6st*$^{\Delta T}$ and *Il12rb2*$^{-/-}$ Thy1.2$^+$ T_{conv} cells, they failed to block the expansion of IL-35R$^{\Delta T}$ Thy1.2$^+$ T_{conv} cells (Fig. 3a).

Second, iTr35 can also block the anti-tumor CD8$^+$ T cell response against B16 melanoma, thereby facilitating tumor growth. Wild-type, *Il6st*$^{\Delta T}$, *Il12rb2*$^{-/-}$ or IL-35R$^{\Delta T}$ CD4$^+$ and CD8$^+$ T cells, either alone or together with iTr35 cells, were adoptively transferred into *Rag1*$^{-/-}$ mice. The following day, mice were inoculated intradermally with B16 melanoma cells and tumor size determined after 14 days. In the absence of iTr35, tumor burden was similar between mice receiving CD4$^+$ and CD8$^+$ T cells, regardless of genotype (Fig. 3b). When present, iTr35 blocked anti-tumor immunity in wild-type, *Il6st*$^{\Delta T}$ or *Il12rb2*$^{-/-}$ CD4$^+$ and CD8$^+$ T cell recipients, which consequently developed large tumors. In contrast, IL-35R$^{\Delta T}$ CD4$^+$ and CD8$^+$ T cell recipients were resistant to iTr35-mediated suppression, resulting in limited tumor growth. Recipient antigen presenting cells (APCs) contribute to tumor immunity and may express the IL35R. However, the impact of IL-35 on APCs has not been examined here as only the transferred CD4$^+$ and CD8$^+$ T cells lack receptor expression. Also, tumor growth is equally determined by IL-35R$^{\Delta T}$ CD4$^+$ or CD8$^+$ T cells in the presence of iTr35 suggesting that an effect of IL-35 on APCs in this setting is at most limited. Collectively, these data demonstrate that IL-35R-deficient T cells are resistant to IL-35-mediated suppression *in vivo*. In both *Rag1*$^{-/-}$ and tumor models T cells lacking just one of the IL-35R chains (IL-12Rβ2 or gp130) could still be suppressed by IL-35 in a manner indistinguishable from wild-type cells, further suggesting that IL-35 can mediate signaling via a single receptor chain *in vivo*, contrasting observations showing that *in vivo* sensitivity to IL-12 and IL-27 are completely abolished following loss of one of the chains of their respective receptors[20–22].

IL-35 induces gp130 and IL-12Rβ2 hetero- and homodimers

In order to obtain direct physical evidence that IL-35 binds to gp130 and IL-12Rβ2, we first employed laser scanning spinning disc confocal microscopy combined with total internal reflection fluorescence (TIRF) illumination and fluorescence resonance energy transfer (FRET)-based detection. mCherry-tagged IL-12Rβ2 plus either (GFP-tagged IL-12Rβ1 or GFP-tagged gp130 were transiently expressed on HEK293T cells. As expected, a significant

increase in the normalized FRET (nFRET) signal was observed with cells expressing the IL-12R (IL-12Rβ2+IL-12Rβ1) following addition of IL-12 but not IL-35 (Fig. 4a). Similarly, a significant increase in nFRET was observed with cells expressing the putative IL-35R (IL-12Rβ2+gp130) following addition of IL-35 but not a negative control (Fig. 4b). The most likely mechanism of IL-35-induced signaling via a single chain is homodimerization. However, we could not detect gp130 or IL-12Rβ2 homodimerization using this approach (i.e. with gp130-GFP + gp130-mCherry; data not shown). It is possible that homodimeric receptors associate with reduced affinity and/or kinetics or an altered mode of assembly (distance and/or rotation) which may limit FRET efficiency.

As an alternate approach, we used the fluorescent protein-tagged receptor chains in co-immunoprecipitation experiments. Various combinations of receptor constructs were co-transfected into HEK293T cells and treated with either 293T-derived IL-12 or IL-35. Surface receptors were cross-linked and subjected to immunoprecipitation using mCherry (DsRed) -specific antibodies, followed by GFP detection by immunoblotting. As expected, IL-12 induced heterodimerization of IL-12R β1 and IL-12Rβ2, but none of the other receptor chain combinations tested (Fig. 4c,d). Consistent with our FRET observations, IL-35 induced the homodimerization and heterodimerization of gp130 and IL-12Rβ2 (Fig. 4c,d). These results suggest that IL-35 can bind to three receptor combinations, providing mechanistic insight into the partial resistance to IL-35-mediated suppression observed following single-chain deletion. It should be noted that while we use the term dimerization here as the minimal likely mode of receptor association, we cannot rule out the possibility of high order assembly structures being induced by IL-35 as has been suggested for IL-6[23]. Taken together with the functional analysis above, these data confirm that IL-12Rβ2 and gp130 constitute the most plausible components of the IL-35R. However, additional molecules might facilitate cytokine binding or downstream signaling, although this would be unprecedented within the IL-6R and IL-12R families[14, 24].

IL-35R expression is induced by IL-2 and IL-27

We next assessed the pattern of IL-35R expression and how this might correlate with and predict cellular sensitivity to IL-35. *Il6st* and, thus, gp130 surface expression is fairly ubiquitous on resting T cells, changing modestly following activation[25, 26]. Optimal responsiveness of T_{conv} cells to IL-12 requires activation in the presence of IL-2 or IL-27, which up-regulate expression of the IL-12β2 receptor chain[27–29]. Consistent with these previous observations, T_{conv} cell activation in the presence of increasing doses of IL-2 or IL-27 induced increased expression of *Il12rb2* (Fig. 5a). Addition of neutralizing IL-2 antibodies resulted in little to no *Il12rb2* upregulation upon T cell receptor (TCR) stimulation, suggesting that autocrine IL-2 was required for increased receptor expression upon T cell activation. In our system, *Il12rb1* and *Il6st* levels did not change appreciably upon activation with either of these cytokines (Fig. 5a).

To examine the biological consequences of receptor upregulation, T_{conv} cells were activated in the presence of IL-2, IL-27, or neutralizing antibodies to IL-2, rested, and these previously activated T cells (T_{act}) restimulated in the presence of titered amounts of IL-35, with fresh T_{conv} cells included for comparison. T_{conv} cells activated in the presence of IL-2 or IL-27 were significantly more sensitive to IL-35 treatment than T_{conv} cells activated in their absence or fresh T_{conv} cells (Fig. 5b, c).

Analysis of T_H1, T_H2 and T_H17 cells revealed variable IL-35R chain expression (Supplementary Fig. 2). Notably, T_H1 cells express significantly higher levels of *Il12rb2* than T_{conv} cells, T_H2 or T_H17 cells, while *Il6st* is more highly expressed on T_H1 and T_H2 cells compared to T_{conv} cells and T_H17. T_{conv} cells, T_H1 and T_H2 cells are all susceptible to IL-35-mediated suppression while T_H17 cells are resistant (VC, LC, DAAV et al,

unpublished results). Thus, while IL-35R chain expression is likely to influence susceptibility to IL-35-mediated suppression, other factors may also contribute. Nevertheless, our data implicate IL-2 and IL-27 as important inducers of IL-35R upregulation and thereby sensitization to IL-35-mediated suppression.

IL-35 signals through STAT1 and STAT4

Given that the IL-35R utilizes IL-12Rβ2 and gp130, it seemed logical that signaling would be mediated via the STAT family of transcription factors. Consistent with previous reports[27, 28], IL-12 treatment of T_{conv} cells resulted in intracellular phosphorylation of STAT4 while IL-27 signaling induced STAT1 and STAT3 phosphorylation (Fig. 6a). IL-35 treatment of wild-type T_{conv} cells resulted in phosphorylation of STAT1 and STAT4, but no activation of either STAT3 or STAT5 above background. Moreover, no induction of pSTAT1 or pSTAT4 was observed in T_{conv} cells that lacked the IL-35 receptor. Immunoblotting confirmed the induction of substantial pSTAT1, but somewhat weaker pSTAT4 activation, in T_{conv} cell blasts treated with IL-35 (Fig. 6b).

Given that T cells expressing only one IL-35R chain were still resistant to IL-35-mediated suppression, we asked which STATs associated with signaling via what receptor chain. Intracellular phospho-flow cytometry and immunoblot analysis showed that T cells that express IL-12Rβ2, but not gp130, only induce pSTAT4 in response to IL-35, while T cells that express gp130, but not IL-12Rβ2, only induce pSTAT1 (Fig. 6c,d). These data further confirm the ability of IL-35 to mediate signaling in the presence of only one of the IL-35R chains.

To determine which STATs were required to mediate IL-35 signaling, we examined the susceptibility of T_{conv} cells that lacked STAT1, STAT3 or STAT4 to IL-35-mediated suppression. Whereas IL-35 suppressed the proliferation of *Stat3*$^{\Delta T}$ T_{conv} cells (CD4^{cre} × *Stat3*$^{fl/fl}$) to a degree comparable to wild-type T_{conv} cells, suppression of *Stat1*$^{-/-}$ and *Stat4* $^{-/-}$ T_{conv} cells was significantly reduced (Fig. 6e). Similarly, nT_{reg}-mediated suppression of *Stat1*$^{-/-}$ and *Stat4*$^{-/-}$, but not *Stat3*$^{\Delta T}$, T_{conv} cells was also impaired (Supplementary Fig. 3). Importantly, *Il12rb2* and *Il6st* receptor mRNA expression was comparable in *Stat1*$^{-/-}$, *Stat4* $^{-/-}$ and wild-type T_{conv} cells (data not shown). Collectively, these data suggest that both STAT1 and STAT4 are required for maximal IL-35-mediated suppression.

IL-35 utilizes a unique STAT1:STAT4 heterodimer

IL-35 can convert T_{conv} cells into hypo-responsive, strongly suppressive iTr35[8]. Thus IL-35 signaling induces *Ebi3* and *Il12a* expression, IL-35 production and the generation of iTr35. Mutants deficient in one chain of the IL-35R that were only able to activate one of the two required STAT proteins, failed to convert to iTr35 (Fig. 2). This suggested that both STAT1 and STAT4 signaling downstream of the IL-35R were required to induce transcription of *Ebi3* and *Il12a*, a prerequisite for conversion of T cells into iTr35. IL-35 induced significantly less *Ebi3* and *Il12a* transcription in STAT1- and STAT4-deficient T_{conv} cells, implicating a coordinated role for these transcription factors in mediating IL-35 production and iTr35 conversion (Supplementary Fig. 4a). Considering the rapid increase in *Ebi3* and *Il12a* expression after stimulation with IL-35 (1–3h), it is unlikely that signaling is occurring through an intermediate step (Supplementary Fig. 4b).

Several cytokine receptors activate STAT1 and STAT4 to drive pro-inflammatory T_H1-type responses, so it is unclear how their activation via the IL-35R could give rise to a very different outcome. Two well-known activators of STAT1 and STAT4 are IFN-γ and IL-12, respectively[17, 30]. Treatment of activated T_{conv} cells with IL-12 + IFN-γ or IL-35 alone

induced STAT1 and STAT4 activation (Fig. 7a). However, T_{conv} cells stimulated with IL-12 + IFN-γ did not upregulate *Ebi3* or *Il12a*, and thus would not secrete IL-35 and become iTr35 (Fig. 7b). Thus, the activation of STAT1 and STAT4 by IL-35 leads to a distinct outcome.

Although STAT proteins generally transduce cytokine signals by homodimerization, which promotes their translocation to the nucleus, STAT1:STAT2 and STAT1:STAT3 heterodimerization has been implicated in interferon and IL-6 receptor signaling, respectively[31]. Reciprocal co-immunoprecipitation and immunoblot analysis demonstrated that IL-35 induces the formation of a STAT1:STAT4 heterodimer 30 min after stimulation (Fig. 7c). In contrast, treatment of activated T_{conv} cells with IL-12 + IFN-γ failed to induce appreciable formation of STAT1:STAT4 heterodimers despite potent activation of both STAT proteins (Fig. 7a,c). These data suggest that IL-35 can mediate distinct signaling consequences compared with IFN-γ and IL-12 by inducing the formation of a STAT1:STAT4 heterodimer.

Given that *Ebi3-Il12a* transcription is an exclusive direct downstream consequence of IL-35R signaling, we sought to determine if STAT1:STAT4 heterodimers possessed a unique binding pattern within these promoters. *In silico* analysis of the *Il12a* and *Ebi3* promoters using a proposed consensus STAT motif (TTC-N_{1-5}-AGA, allowing for mismatch) identified nine potential binding sites (Fig. 7d)[32, 33]. We designed primers to interrogate six of these sites by chromatin immunoprecipitation (ChIP) analysis using antibodies to STAT1 and STAT4. As controls we also included known STAT1 and STAT4 binding sites within the *Irf1* and *Il18ra* promoters, respectively[34, 35]. T cells, four days post-activation, were stimulated with IL-12, IFN-γ or IL-35 and assayed for STAT1 and STAT4 binding by ChIP. As expected, IL-12 induced STAT4 binding to *Il18ra*-750 and IFN-γ induced STAT1 binding to *Irf1*-250 (Fig. 7e,f). However, IL-35 did not induce STAT binding to either site. Although IL-12 or IFN-γ induced minimal STAT1 and STAT4 binding to the *Il12a* and *Ebi3* promoters, IL-35 induced STAT binding to multiple sites. The pattern of STAT1 and STAT4 binding following IL-35 treatment was very similar implicating the use of a heterodimer. To directly address this possibility, we conducted ChIP-reChIP analysis in which the anti-STAT4 ChIP product was subjected to a second ChIP with anti-STAT1, and vice versa. T cells stimulated with IL-12 + IFN-γ induced minimal STAT1:STAT4 interaction with the *Il12a* and *Ebi3* promoter STAT consensus sites. However, T cells stimulated with IL-35 showed strong enrichment of STAT1 and STAT4 binding to *Il12a*-250 and *Ebi3*-500, as well as other sites in the promoters. Although the amplicons used contain only one putative STAT binding consensus sequence, we cannot rule out the possibly that STAT 1 and STAT4 might bind at adjacent sites that are either within this amplicon or in the fragments generated during DNA shearing. These data implicate a STAT1:STAT4 heterodimer as the unique biochemical effector of IL-35 signaling, which appears to specifically bind to multiple sites within *Il12a* and *Ebi3* promoters.

Potential configurations of IL-35 receptors

The composition of the IL-35R presents a conundrum regarding the mode of IL-35:IL-35R interaction and assembly when considered in light of the site 1, 2 and 3 architectural paradigm originally established for IL-6[36]. In this blueprint, site 3 of the cytokine engages the N-terminal Ig-domain (IgD) of the signaling receptor, thus the presence of an N-terminal IgD is required for it to act as a site 3 binder (Fig. 8). Absence of the N-terminal IgD as seen, for example, in WSX-1 and IL-12Rβ1 would appear to relegate them to site 2 binding. While there are no known crystal structures of IL-12, IL-27 or IL-23 receptor signaling complexes, current models of the IL-12:IL-12R interaction suggest that the IL-12Rβ2 IgD domain binds to site 3 on p35, while also interacting with the p40 subunit via its N-terminal

domain[20, 21, 24, 37] (Fig. 8b). However, in the IL-27:IL-27R complex gp130 is also predicted to bind to site 3 in the p35 homologue p27[24]. This presents a conundrum as IL-12Rβ2 and gp130 both contain N-terminal IgD that could serve as the site 3 interacting domain, yet clearly both cannot be binding to the same site and suggests differences in the formation of the IL-35:IL-35R complex compared with the IL-12 and IL-27 receptor complexes. There are three possibilities (Fig. 8c). First, gp130 binds to site 2 in the IL-6, CNTF and LIF complexes[24] and thus could do so in the IL-35:IL-35R complex, leaving IL-12Rβ2 to bind to site 3. Second, the IL-35:IL-35R complex could form symmetric homo-hexameric assemblies (2:2:2) analogous to the IL-6R complex[23], thus allowing IL-12Rβ2 and gp130 to each bind to site 3 on p35. Third, gp130 and IL-12Rβ2 could both be capable of site 2 and site 3 binding, and therefore exist in an interchanging equlibrium of hetero-tetrameric complexes (1:1:1:1, gp130: IL-12Rβ2:EBI3:P35) (Fig. 8c - two 'hetero' models). Indeed, studies on the IL-6 complex suggest that it may be able to signal as tetrameric or hexameric assembly[23]. Finally, a completely novel mode of binding may exist that utilizes the site 1/2/3 model in a manner not predicted from the existing structural information on IL-6, LIF, and CNTF[24, 36, 38]. While a definitive answer will require detailed IL-35:IL-35R structural analysis, it is interesting to note that the IL-35 neutralizing antibodies we have described, which are specific for Ebi3, can immunoprecipitate IL-27 and IL-35 but only neutralize IL-35, while those specific for p35 can immunoprecipitate IL-12 and IL-35 but only neutralize IL-35. This further supports the notion that IL-35:IL-35R complex formation is distinct and differs from the IL-12 and IL-27 receptor complexes.

DISCUSSION

The family of gp130-related shared cytokine receptors has been the most actively growing sub-group of the Type I cytokine family, with the most recent addition being IL-35. Important similarities and differences between IL-12, IL-27 and IL-35 signaling have been revealed in this study. Given that IL-35 is part of the IL-12 cytokine family, it was reasonable to presume that the IL-35R might share usage of receptor chains and utilize common signaling molecules. However, since the biological activity of IL-35 contrasts with that of its cytokine family members, commonality in signaling could not be assumed. We found that the IL-35 receptor and signaling pathway does overlap with IL-12, by using IL-12Rβ2 which is part of the IL-12R, and IL-27, by using gp130 which is part of the IL-27R. However, in contrast to IL-12 and IL-27, IL-35 can signal through a single receptor chain with gp130 alone inducing pSTAT1 and IL-12Rβ2 inducing pSTAT4. Indeed, in the absence of either one of the IL-35 receptor chains *in* vivo, the suppressive effects of IL-35 remain intact. To completely abrogate IL-35 activity *in vivo*, loss of both IL-12Rβ2 and gp130 was required. Given our current understanding of receptor signaling and data presented here, IL-35 appears to mediate the homodimerization of either IL-12Rβ2 or gp130, as well as IL-12Rβ2:gp130 heterodimerization. However, we cannot entirely rule out the possibility that the various forms of the IL-35R may form higher order multimers of these receptor chains. gp130 can signal as either a homodimer (e.g. with IL-6:sIL6Rα or viral IL-6) or a heterodimer with LIF-R (e.g. CNTF and LIF). This is also a formal possibility for IL-12Rβ2, which so far is only known in a heterodimeric context in the IL-12 receptor complex. Whether signaling induced by IL-35 can be productively mediated via a single IL-35R chain in all physiological conditions and disease scenarios remains to be determined. However the complete IL-12Rβ2:gp130 receptor is required for maximal suppression, IL-35 induction and iTr35 conversion.

IL-27 activates a heterogeneous mixture of STAT proteins depending on the cell type in question, although in T cells STAT1 and STAT3 dominate[22, 27, 28, 39]. Like IL-27, IL-35 signals through two different STAT proteins, STAT1 and STAT4. However, STAT3 appears dispensable for IL-35 signaling, an interesting observation given its importance for

IL-27 signaling, which is downstream of gp130 engagement. IL-12 family cytokine signaling involves phosphorylation of many different STAT proteins, which can homodimerize, thereby acting alone to influence gene transcription, or can heterodimerize to work in concert to elicit an alternate response. The intensity as well as the kinetics of STAT activation has previously been shown to influence downstream signaling events, gene expression and the nature of the biological response. For instance, IFNα signaling in myeloid cells can either promote or inhibit immunity through phosphorylation of STAT1, STAT2, and STAT3[31]. A primarily STAT1:STAT2 response initiates the anti-viral, pro-inflammatory response. When STAT3 expression dominates, STAT1-dependent gene expression is inhibited, directly suppressing immunity. In addition, dominant STAT3 can indirectly alter the IFNα signal by dimerizing with STAT1, thereby inhibiting homodimerization of STAT1 and the biological activities that are induced in response to STAT1 homodimers[31]. By this mechanism, the IFNα signal is shifted from a pro-inflammatory to an anti-inflammatory response as a result of changing the relative expression and activation of STAT proteins as well as their dimerization capacity. In addition, STAT1:STAT3 heterodimers have been previously described, therefore dominance of one STAT protein over another may influence not only the activity of the STAT protein in question, but also its ability to homo- and heterodimerize to mediate distinct biological outcomes. We have shown here that IL-35 induces the formation of a STAT1:STAT4 heterodimer, promoting a distinct mode of *Il12a* and *Ebi3* promoter binding and gene activation. It is possible that STAT heterodimers may be generally linked to anti-inflammatory programs.

Since IL-35 appears to utilize receptor chains and STATs that are similar to those used by other IL-12 family members, another important question is how a T cell can translate potentially similar signals into such distinct biological outcomes[16]. This enigma is certainly seen with IL-10 and IL-6. Although both cytokines signal primarily through STAT3, the outcome of their signaling and their biological activity is vastly different and has been a focus of intense research for more than a decade[30]. Thus, the utilization of STAT1:STAT4 heterodimers gives biochemical distinction to IL-35 signaling. However, it is still unclear why a STAT1:STAT4 heterodimer predominates upon IL-35 stimulation and not after simultaneous stimulation with STAT1 and STAT4 activators, such as IFN-γ and IL-12 respectively. It is clear that gp130 and IL12Rβ2 homodimers cannot induce both pSTAT1 and pSTAT4, suggesting that a heterodimer only forms after ‘canonical’ gp130:IL12Rβ2 receptor activation. It is possible that bringing these two receptor chains into close proximity favors STAT heterodimerization that may not occur following ligation of two spatially distinct receptors. Receptors that activate STAT1 or STAT4 may be spatially segregated, preventing the formation of an anti-inflammatory STAT1:STAT4 heterodimer. Indeed, the IFN-γR tracks readily to the immunologic synapse following TCR activation, while the IL-12R has a more diffuse localization[40, 41]. One might speculate that this is required to negate the close proximity of these receptors during T cell activation and thus prevent inadvertent STAT1:STAT4 heterodimer formation.

The expression pattern of the IL-35 receptor will provide insight into potential IL-35 target cell types. While gp130 is fairly ubiquitously expressed, IL-12Rβ2 is expressed mainly by activated T cells and natural killer cells[21]. In T cells, the expression of IL-12Rβ2 is most highly expressed by T_H1 cells, and its expression correlates with responsiveness to IL-12[42] and IL-35. Expression of IL-12Rβ2 has also been shown on other cell types, such as dendritic cells[43], which may affect the spectrum of IL-35 bioactivity in the immune system. IL-12Rβ2 is undetectable on most resting T cells, but can be rapidly upregulated by exposure to IL-2, IL-12, IL-27, IFN-y, tumor-necrosis factor (TNF) and co-stimulation through CD28. Indeed, we have shown here that IL-2 or IL-27 pretreatment increases T cell sensitivity to IL-35 mediated suppression. Thus, IL-35 might have biological effects on a

variety of cellular targets and under a variety of disease conditions. IL-27 is of particular interest given its capacity to exhibit inhibitory activity. Although it is thought that this is mediated, in part, via its induction of IL-10[44, 45], it is also possible that IL-27-mediated induction of IL-12Rβ2 may sensitize cells to IL-35-mediated suppression and thus may contribute to the perceived inhibitory activity of IL-27. The capacity of IL-35 to mediate suppression via IL-12Rβ2:gp130 heterodimers and IL-12Rβ2 and gp130 homodimers could have profound implications for its range and breadth of regulatory control. This is especially relevant for gp130, which is broadly expressed[21]. Indeed, utilization of gp130 homodimers may provide a mechanism for IL-35 to suppress naïve T cells that do not express IL-12Rβ2.

Given the potent immunosuppressive capacity of IL-35, blocking its function may be therapeutically beneficial in a number of disease settings. We have previously shown that inhibiting IL-35 activity with a neutralizing anti-IL-35 mAb has significant *in vivo* efficacy[8]. The unique IL-35 receptor composition of IL-12Rβ2 and gp130 presents some interesting considerations and therapeutic opportunities. Animals lacking gp130 are not viable, likely due to the large number of cytokines that utilize gp130. For this reason it is difficult to infer a lack of IL-35 signaling as causative of this phenotype. In contrast, mice lacking IL-12Rβ2 have a mild phenotype, with reports suggesting that they are more susceptible to autoimmunity and B cell malignancies[46]. Interestingly, several autoimmune and inflammatory diseases have been linked to IL-12Rβ2 including atopic dermatitis, primary biliary cirrhosis, type 1 diabetes, and Behçet's disease[47–49]. Taken together, these observations suggest that the clinical consequences of gp130 and IL-12Rβ2 dysfunction may affect immune regulation via the IL-35:IL-35R signaling axis, suggesting that further studies are required to assess the importance of the IL-35 signaling pathway in human disease.

ONLINE METHODS

Mice

Spleens and lymph nodes from *Il12rb1*$^{-/-}$ mice were provided D. Fairweather and J.A. Frisancho (Johns Hopkins University), CD4^{cre} × *Il6st*$^{fl/fl}$ (*Il6st*$^{\Delta T}$) mice by M. Karin and S. Grivennikov (University of California at San Diego), *Il27ra*$^{-/-}$ mice by C. Hunter and J. Stumhofer (University of Pennsylvania), *Stat1*$^{-/-}$ mice by A. Satoskar and P. Reville (Ohio State University), and *Stat3*$^{\Delta T}$ (CD4 Cre × STAT3 fl/fl) mice by C. Drake and H.R. Yen (Johns Hopkins University). *Il12rb2*$^{-/-}$, *Stat4*$^{-/-}$, *Rag1*$^{-/-}$, C57BL/6, B6.PL and Balb/c mice were purchased from Jackson Laboratory. Some experiments with CD4^{cre} × *Il6st*$^{fl/fl}$ mice were performed with a colony established at St. Jude from mice obtained from Rodger McEver (University of Oklahoma Health Sciences Center). These were crossed with *Il12rb2*$^{-/-}$ mice to generate IL-35$^{\Delta T}$ mice (CD4^{cre} × *Il6st*$^{fl/fl}$ × *Il12rb2*$^{-/-}$). All receptor and STAT deficient mice are on a C57BL/6 background, with the exception of *Stat4*$^{-/-}$ mice which are on a Balb/c background. In all experiments, wild type mice used were of the appropriate genetic background. Animal experiments were performed in American Association for the Accreditation of Laboratory Animal Care-accredited, specific-pathogen-free facilities in the St. Jude Animal Resource Center. Animal protocols were approved by the St Jude Animal Care and Use Committee.

Neutralizing IL-35 mAb

Neutralizing IL-35 mAb was developed with recombinant murine Ebi3 protein, as described.[8]

Transfection of HEK293T cells for IL-35 protein generation

IL-35 constructs were generated by recombinant PCR as described[10]. Constructs containing *Ebi3* and *Il12a* linked by a 2A "self-cleaving" peptide or a flexible linker were used for

IL-35 generation and an empty vector containing an IRES-GFP construct was used as a control. 1.0×10^6 293T cells were seeded into culture plates, then transfected using Trans-IT reagent (Mirus Biotechnologies). After 16 h media was changed and supernatant collected for 48 h. Supernatants were filtered, concentrated, and reconstituted into fresh RPMI. Each lot of supernatant is screened for its ability to inhibit T cell proliferation and validated extensively as previously described (see Ref. [8]; Supplementary Fig. 2 and 4).

T_{conv} purification and iTr35 conversion

Naïve T_{conv} ($CD4^+CD25^-CD45RB^{hi}$) and nT_{reg} ($CD4^+CD25^+CD45RB^{lo}$) cells were purified as previously described[8, 10]. Splenic and lymph node suspensions depleted of non-T cells by magnetic sorting were purified flow cytometrically. iTr35 cells were generated as previously described[8].

Immunoprecipitation and Immnoblotting

Following 18h activation with anti-CD3 + anti-CD28 coated beads, cells were treated with 100ng/ml IL-12, IL-27 or IL-35. Cells were resuspended in lysis buffer and subjected to immunoblotting for pSTAT1 and pSTAT4 (Cell Signaling Technology and Santa Cruz Biotechnology). For immunoprecipitations, cells were resuspended in lysis buffer, precleared with Protein G beads, incubated with antibodies overnight, and immunoprecipitated using Protein G beads. Blots were developed using ECL (Amersham Biosciences).

Microscopic detection of IL-35R FRET

IL-12 family receptor chains cloned into modified MSCV retroviral vectors yielded receptor chains tagged with either GFP (donor) or mCherry (acceptor) (IL-12Rβ1-GFP, IL-12Rβ2-mCherry, and GP130-GFP). Specific details regarding primers and constructs can be obtained from Kate Vignali (Kate.Vignali@st.jude.org). 293T cells were transfected 24 h prior to plating on Poly-L-lysine-coated 8-well tissue culture slides. Cells were analyzed using confocal microscopy and TIRF illumination (Intelligent Imaging Innovations). Images were acquired at 2 sec intervals for a total time of 30 sec to calculate basal FRET values, followed by addition of rIL-12 [25 ng/ml] or mIL-35 [25 ng/ml] and continued image acquisition at 2 sec intervals. 293T cells were transfected with single GFP or mCherry constructs for determination of donor bleedthrough fluorescence (average Fd/Dd = 0.0004) as well as direct excitation of the acceptor (average Fa/Aa = 0.003). Three color mathematical correction was applied to generate a FRET channel, where Fc = Transfer − Fa/Aa*Acceptor-Fd/Dd*Donor. Normalized FRET (nFRET) represents the ratio of corrected FRET signal detected prior to and following cytokine, with basal FRET signals arbitrarily set to 1.

mRNA isolation

RNA isolated using Qiagen microRNA extraction kits (QIAGEN) or Trizol (Life Technologies) was reverse-transcribed using the cDNA archival kit (Applied Biosystems) as previously described[8, 10].

In vitro suppression assays

Anti-CD3- + anti-CD28-coated latex beads were generated as previously described[8, 10]. In some experiments, superparamagnetic anti-CD3 + anti-CD28 beads were used (Dynabeads, Life Technologies). To determine suppression, 1.25×10^4 or 2.5×10^4 cells were activated with anti-CD3- + anti-CD28-coated beads with IL-35 supernatants, IL-35 beads, or iTr35 for 72 h; proliferation was determined by [^{3}H]-thymidine incorporation.

"% suppression" is calculated as: 100 – 100*[(CPM of "suppressed" Teff)/(average CPM of Teff alone)] to normalize data across experiments (CPM can vary significantly from experiment to experiment). Ranges of CPM values obtained with T_{conv} cells activated alone for each set of experiments are detailed in the figure legends (typically ranged from 20,500–91,000).

Homeostatic expansion

Homeostasis assays were performed as described[10]. Briefly, WT or knockout naive Thy1.2$^+$ T_{conv} cells were purified and used as "responder" cells in adoptive transfer. Thy 1.1$^+$ iTr35 were generated and used as "suppressor" cells. Splenic T cell numbers were determined by flow cytometry seven days after transfer.

B16 tumor model

Rag1$^{-/-}$ mice received indicated cells i.v. on day −1 of experiment. Wild type or receptor-deficient naïve CD4$^+$CD25$^-$ (9×10^6/mouse) and CD8$^+$ T cells (6×10^6/mouse) alone or in combination with iTr35 cells (10^6/mouse) were adoptively transferred. B16-F10 melanoma was a gift from Mary Jo Turk (Dartmouth College, Hanover, NH) and cultured as described[8].

Chromatin Immunoprecipitation

Naïve T_{conv} cells were stimulated with plate-bound anti-CD3 (3 μg/mL) and anti-CD28 (2 μg/mL) for 48 h in the presence of 100 U/mL of rhIL-2, then expanded 48h in IL-2 without TCR or CD28 stimulation. 25×10^6 cells were washed, rested 2 h on ice in serum-free media, then stimulated with cytokines (rIL-12: 25ng/mL, IFN-γ: 100ng/mL, rIL-35: 25% culture volume of 293T supernatant) for 90 min. Cells were crosslinked with 1% formaldehyde for 30 minutes, followed by a 15 minute quench with glycine. Chromatin was prepared using SDS lysis, and sheared to 250–750bp fragments with a Diagenode Bioruptor (Belgium) using 15 × 30s pulses. Chromatin was precleared using ssDNA-(Sigma) and BSA-blocked Protein G beads (GE Healthcare). STAT1 and STAT4 or isotype-matched IgG control antibodies (Cell Signaling, Danvers, MA) were added and rotated overnight. Protein-DNA complexes were immunoprecipitated using blocked beads, washed in low salt, high salt, LiCl, and TE buffers, and eluted with $NaHCO_3$. For ChIP-reChIP, complexes were first eluted with 0.1M DTT and subjected to a second round of immunoprecipitation, washes, and elution with $NaHCO_3$. Eluates were reverse-crosslinked, proteinase K and RNase A treated, and DNA purified using Qiaquick DNA Purification columns (QIAGEN). DNA was assayed by real-time PCR using SYBR Green (Applied Biosystems).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol. 2008; 8:523–532. [PubMed: 18566595]
2. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol. 2006; 6:295–307. [PubMed: 16557261]
3. O'Shea JJ, Paul WE. Regulation of T(H)1 differentiation--controlling the controllers. Nat Immunol. 2002; 3:506–508. [PubMed: 12032561]
4. Artis D, et al. The IL-27 receptor (WSX-1) is an inhibitor of innate and adaptive elements of type 2 immunity. J Immunol. 2004; 173:5626–5634. [PubMed: 15494513]
5. Awasthi A, et al. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat Immunol. 2007; 8:1380–1389. [PubMed: 17994022]
6. Fitzgerald DC, et al. Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. Nat Immunol. 2007; 8:1372–1379. [PubMed: 17994023]
7. Stumhofer JS, et al. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. Nat Immunol. 2007; 8:1363–1371. [PubMed: 17994025]
8. Collison LW, et al. IL-35-mediated induction of a potent regulatory T cell population. Nat Immunol. 2010; 11:1093–1101. [PubMed: 20953201]
9. Collison LW, Pillai MR, Chaturvedi V, Vignali DA. Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. J Immunol. 2009; 182:6121–6128. [PubMed: 19414764]
10. Collison LW, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature. 2007; 450:566–569. [PubMed: 18033300]
11. Chua AO, Wilkinson VL, Presky DH, Gubler U. Cloning and characterization of a mouse IL-12 receptor-beta component. J Immunol. 1995; 155:4286–4294. [PubMed: 7594587]
12. Parham C, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. J Immunol. 2002; 168:5699–5708. [PubMed: 12023369]
13. Pflanz S, et al. WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. J Immunol. 2004; 172:2225–2231. [PubMed: 14764690]
14. Silver JS, Hunter CA. gp130 at the nexus of inflammation, autoimmunity, and cancer. J Leukoc Biol. 2010; 88:1145–1156. [PubMed: 20610800]
15. Bacon CM, et al. Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. Proc Natl Acad Sci U S A. 1995; 92:7307–7311. [PubMed: 7638186]
16. Delgoffe GM, Murray PJ, Vignali DA. Interpreting mixed signals: the cell's cytokine conundrum. Curr Opin Immunol. 2011
17. Thierfelder WE, et al. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature. 1996; 382:171–174. [PubMed: 8700208]
18. Huber M, et al. IL-27 inhibits the development of regulatory T cells via STAT3. Int Immunol. 2008; 20:223–234. [PubMed: 18156621]
19. Owaki T, et al. STAT3 is indispensable to IL-27-mediated cell proliferation but not to IL-27-induced Th1 differentiation and suppression of proinflammatory cytokine production. J Immunol. 2008; 180:2903–2911. [PubMed: 18292512]
20. Wu C, et al. IL-12 receptor beta 2 (IL-12R beta 2)-deficient mice are defective in IL-12-mediated signaling despite the presence of high affinity IL-12 binding sites. J Immunol. 2000; 165:6221–6228. [PubMed: 11086056]
21. Presky DH, et al. A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. Proc Natl Acad Sci U S A. 1996; 93:14002–14007. [PubMed: 8943050]
22. Villarino A, et al. The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection. Immunity. 2003; 19:645–655. [PubMed: 14614852]

23. Schroers A, et al. Dynamics of the gp130 cytokine complex: a model for assembly on the cellular membrane. Protein Sci. 2005; 14:783–790. [PubMed: 15722452]
24. Skiniotis G, Lupardus PJ, Martick M, Walz T, Garcia KC. Structural organization of a full-length gp130/LIF-R cytokine receptor transmembrane complex. Mol Cell. 2008; 31:737–748. [PubMed: 18775332]
25. Betz UA, Muller W. Regulated expression of gp130 and IL-6 receptor alpha chain in T cell maturation and activation. Int Immunol. 1998; 10:1175–1184. [PubMed: 9723704]
26. Charlot-Rabiega P, Bardel E, Dietrich C, Kastelein R, Devergne O. Signaling events involved in interleukin 27 (IL-27)-induced proliferation of human naive CD4+ T cells and B cells. J Biol Chem. 2011; 286:27350–27362. [PubMed: 21669870]
27. Hibbert L, Pflanz S, De Waal Malefyt R, Kastelein RA. IL-27 and IFN-alpha signal via Stat1 and Stat3 and induce T-Bet and IL-12Rbeta2 in naive T cells. J Interferon Cytokine Res. 2003; 23:513–522. [PubMed: 14565860]
28. Takeda A, et al. Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. J Immunol. 2003; 170:4886–4890. [PubMed: 12734330]
29. Liao W, Lin JX, Wang L, Li P, Leonard WJ. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. Nat Immunol. 1–2:551–559.
30. O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. Immunity. 2008; 28:477–487. [PubMed: 18400190]
31. Ho HH, Ivashkiv LB. Role of STAT3 in type I interferon responses. Negative regulation of STAT1-dependent inflammatory gene activation. J Biol Chem. 2006; 281:14111–14118. [PubMed: 16571725]
32. Yamamoto K, Miura O, Hirosawa S, Miyasaka N. Binding sequence of STAT4: STAT4 complex recognizes the IFN-gamma activation site (GAS)-like sequence (T/A)TTCC(C/G)GGAA(T/A). Biochem Biophys Res Commun. 1997; 233:126–132. [PubMed: 9144409]
33. Ehret GB, et al. DNA binding specificity of different STAT proteins. Comparison of in vitro specificity with natural target sites. J Biol Chem. 2001; 276:6675–6688. [PubMed: 11053426]
34. Yu Q, Thieu VT, Kaplan MH. Stat4 limits DNA methyltransferase recruitment and DNA methylation of the IL-18Ralpha gene during Th1 differentiation. EMBO J. 2007; 26:2052–2060. [PubMed: 17380127]
35. Ramsauer K, et al. Distinct modes of action applied by transcription factors STAT1 and IRF1 to initiate transcription of the IFN-gamma-inducible gbp2 gene. Proc Natl Acad Sci U S A. 2007; 104:2849–2854. [PubMed: 17293456]
36. Boulanger MJ, Chow DC, Brevnova EE, Garcia KC. Hexameric structure and assembly of the interleukin-6/IL-6 alpha-receptor/gp130 complex. Science. 2003; 300:2101–2104. [PubMed: 12829785]
37. Stern AS, Gubler U, Presky DH, Magram J. Structural and functional aspects of the IL-12 receptor complex. Chem Immunol. 1997; 68:23–37. [PubMed: 9329214]
38. Huyton T, et al. An unusual cytokine:Ig-domain interaction revealed in the crystal structure of leukemia inhibitory factor (LIF) in complex with the LIF receptor. Proc Natl Acad Sci U S A. 2007; 104:12737–12742. [PubMed: 17652170]
39. Lucas S, Ghilardi N, Li J, de Sauvage FJ. IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms. Proc Natl Acad Sci U S A. 2003; 100:15047–15052. [PubMed: 14657353]
40. Canda-Sanchez A, et al. Differential distribution of both IL-12Rbeta chains in the plasma membrane of human T cells. J Membr Biol. 2009; 227:1–12. [PubMed: 19067026]
41. Maldonado RA, Irvine DJ, Schreiber R, Glimcher LH. A role for the immunological synapse in lineage commitment of CD4 lymphocytes. Nature. 2004; 431:527–532. [PubMed: 15386021]
42. 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]
43. Grohmann U, et al. IL-12 acts directly o-n DC to promote nuclear localization of NF-kappaB and primes DC for IL-12 production. Immunity. 1998; 9:315–323. [PubMed: 9768751]

44. Andersson J, et al. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. J Exp Med. 2008; 205:1975–1981. [PubMed: 18710931]

45. Pot C, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. J Immunol. 2009; 183:797–801. [PubMed: 19570826]

46. Pistoia V, Cocco C, Airoldi I. Interleukin-12 receptor beta2: from cytokine receptor to gatekeeper gene in human B-cell malignancies. J Clin Oncol. 2009; 27:4809–4816. [PubMed: 19720917]

47. Hirschfield GM, et al. Primary biliary cirrhosis associated with HLA, IL12A, and IL12RB2 variants. N Engl J Med. 2009; 360:2544–2555. [PubMed: 19458352]

48. Matsui E, et al. Mutations of the IL-12 receptor beta2 chain gene in atopic subjects. Biochem Biophys Res Commun. 1999; 266:551–555. [PubMed: 10600539]

49. Remmers EF, et al. Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behcet's disease. Nat Genet. 2010; 42:698–702. [PubMed: 20622878]

50. Xu Y, et al. Crystal structure of the entire ectodomain of gp130: insights into the molecular assembly of the tall cytokine receptor complexes. J Biol Chem. 2010; 285:21214–21218. [PubMed: 20489211]

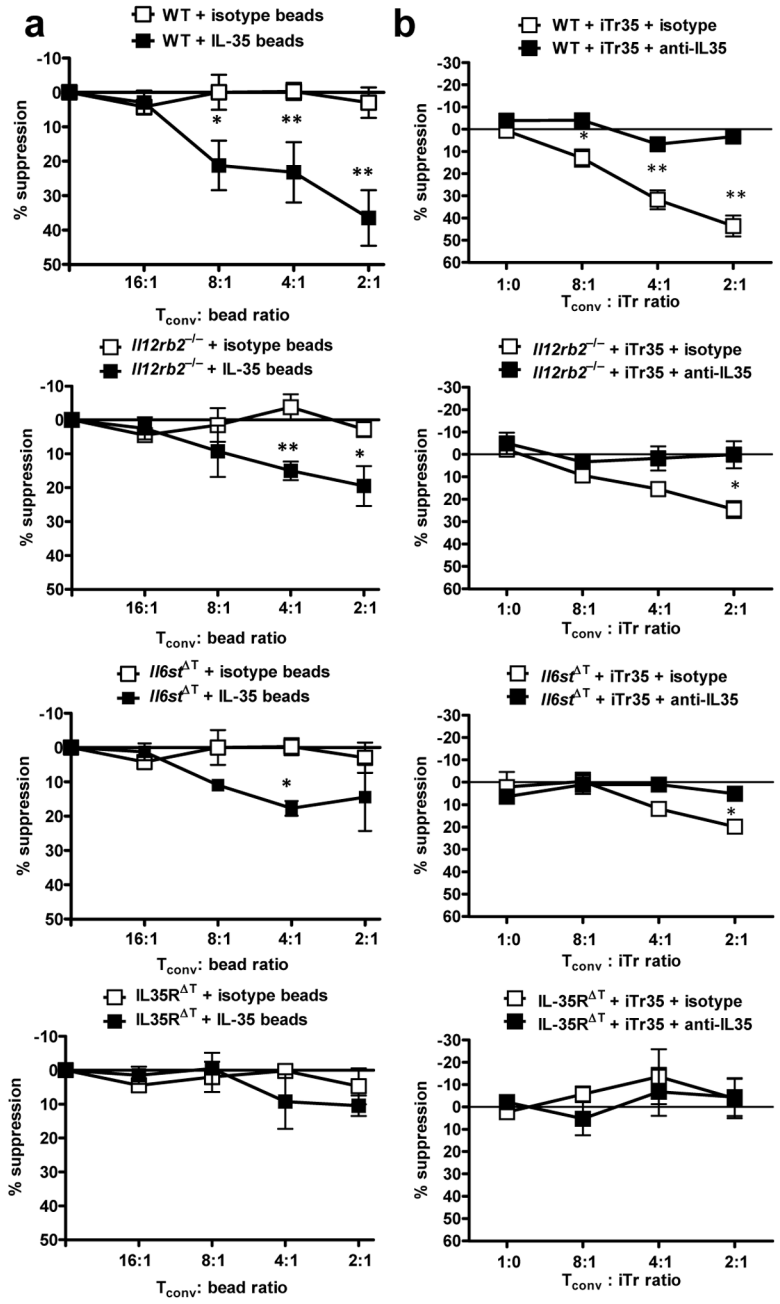

Figure 1. IL-12Rβ2 or gp130 are sufficient for IL-35-mediate suppression

(**a–b**) T_{conv} cells purified by flow sorting from wild-type (WT - C57BL/6), *Il12rb2* $^{-/-}$, CD4^{cre} × *Il6st*$^{fl/fl}$ (abbreviated *Il6st*$^{\Delta T}$), or *Il12rb2* $^{-/-}$ × CD4^{cre} × *Il6st*$^{fl/fl}$ (abbreviated IL-35R$^{\Delta T}$) mice were activated with anti-CD3- + anti-CD28-coated beads for 3 days in the presence of indicated concentrations of IL-35-coated beads (isotype control or non-neutralizing anti-IL-35 mAb were incubated with IL-35 supernatant and then coupled with protein G beads) (**a**), or iT$_R$35 in combination with neutralizing IL-35 mAb or isotype control mAb (**b**). Proliferation was determined by [^{3}H]-thymidine incorporation. Counts per minute of T_{conv} cells activated alone, in the absence of any suppression, counts ranged from

22,000–65,000. Data represent the mean ± SEM of (**a**) 5 and (**b**) 3 (representative of 8) independent experiments. (* $p < 0.05$, ** $p < 0.01$ by unpaired t-test).

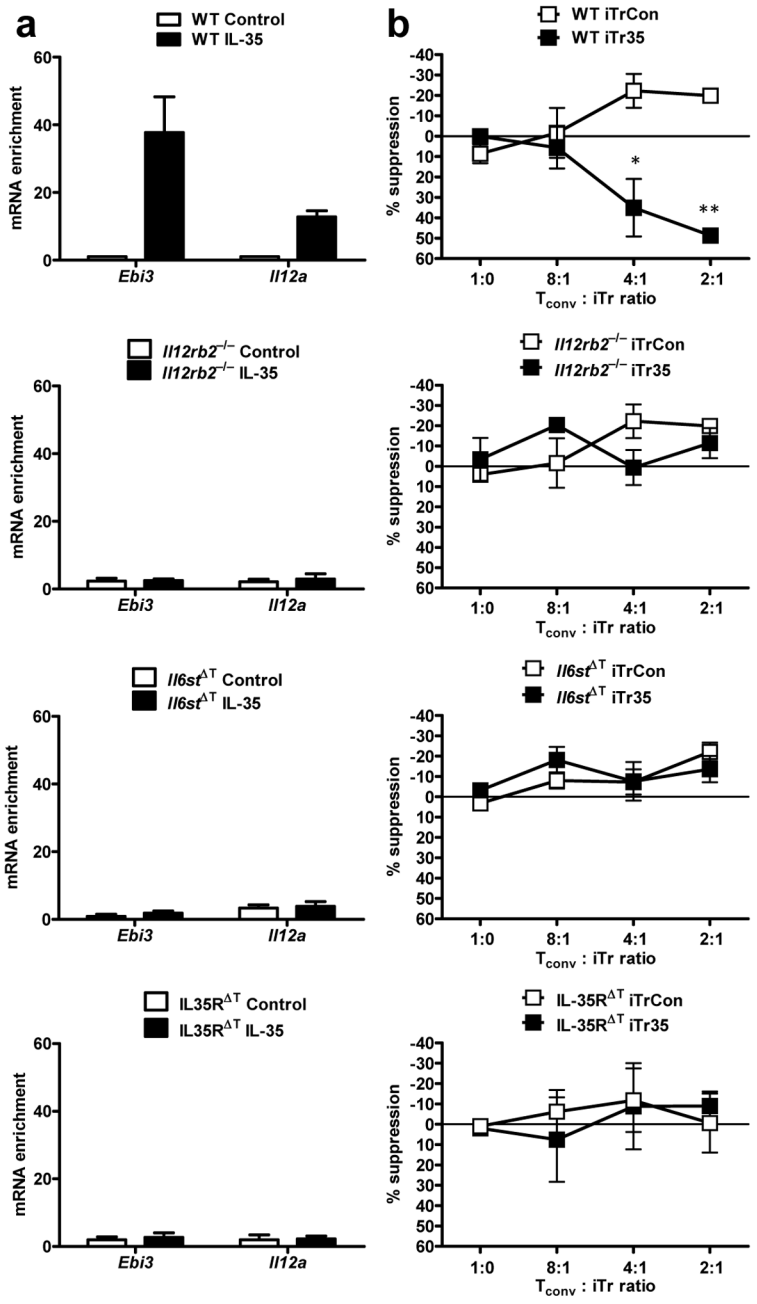

Figure 2. Both IL-12Rb2 and gp130 are required for *Ebi3* and *Il12a* expression and iTr35 conversion

(**a**) T_{conv} cells were purified from WT, *Il6st* $^{\Delta T}$, *Il12rb2* $^{-/-}$, or IL-35R $^{\Delta T}$ mice and activated in the presence of T_{conv} control or IL-35 supernatant, then assayed for *Ebi3* and *Il12a* mRNA upregulation by qPCR. Results are normalized to *Actb* expression and scaled to WT cells stimulated with control supernatant. (**b**) T_{conv} purified by flow sorting from WT mice were activated with anti-CD3- + anti-CD28 coated beads for 3 days in the presence of iTrCon (cells differentiated in control supernatant) or iTr35 cells generated from WT, *Il6st* $^{\Delta T}$, *Il12rb2* $^{-/-}$, or IL-35R$^{\Delta T}$ mice. Proliferation was measured by [^{3}H]-thymidine incorporation. CPM of T_{conv} cells activated alone ranged from 20,500–45,000. Data

represent the mean of (**a**) 3 or (**b**) 3 [of 6] independent experiments (* $p < 0.05$, ** $p < 0.01$ by unpaired t-test).

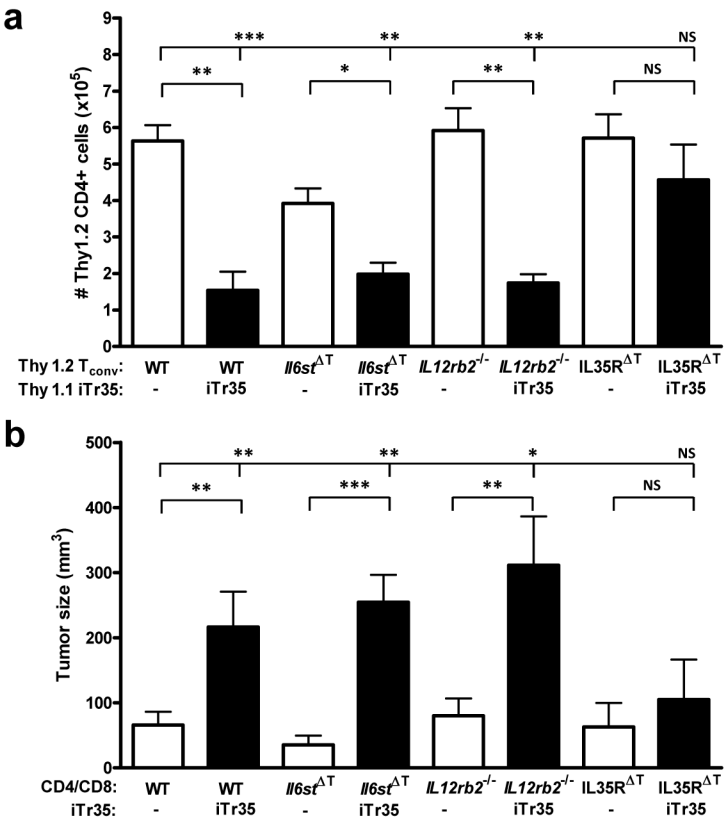

Figure 3. IL-35R-deficient T_{conv} cells are resistant to IL-35-mediated suppression *in vivo*

(**a**) Homeostatic expansion was monitored by i.v. injection of Thy1.2$^+$ T_{conv} cells from WT (C57BL/6), *gp130* $^{\Delta T}$, *Il12rb2* $^{-/-}$, or IL-35R$^{\Delta T}$ mice alone or with Thy1.1$^+$ iTr35 cells (as regulatory cells) into *Rag1* $^{-/-}$ mice. Seven days after transfer, splenic T cell numbers were determined by flow cytometry. (**b**) *Rag1* $^{-/-}$ mice received CD4$^+$ and CD8$^+$ T cells from WT (C57BL/6), *gp130* $^{\Delta T}$, *Il12rb2* $^{-/-}$, or IL-35R$^{\Delta T}$ mice alone or with iTr35 cells via the tail vein on day −1 of the experiment. On day 0, all were injected with 120,000 B16 cells i.d. in the right flank. Tumor volume was measured 14 days as after inoculation (mm^3). Data represent the mean ± SEM of (**a**) 5–12 and (**b**) 6–10 mice per group.

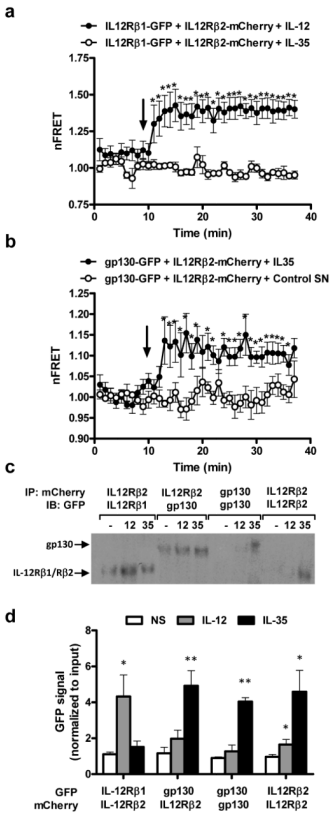

Figure 4. IL-12Rβ2 and gp130 associate in the presence of IL-35 to form three receptors

(**a**) Interaction of IL-12 with its receptor was determined using laser scanning spinning disc confocal microscopy combined with TIRF illumination and FRET-based detection. GFP-tagged IL-12Rβ1 and mCherry-tagged IL-12Rβ2 were transiently expressed on 293T cells. FRET excitation of the mCherry-IL-12Rβ2 acceptor following excitation of the GFP-IL-12Rβ1 donor was determined prior to and following the addition of IL-12 or IL-35 (arrow) by time lapsed imaging. FRET signals were mathematically corrected for donor bleed-through and direct excitation of the acceptor, and were normalized based on the FRET signal (nFRET) detected for the interval preceding addition of cytokine. (**b**) Similar analyses were performed using a GFP-tagged gp130 receptor as donor and mCherry-IL-12Rβ2 as acceptor following addition of IL-35 or control (arrow). Data represent the mean of (**a**) 2 and (**b**) 8 experiments, where significant increases in nFRET signals following addition of cytokine were considered to be significantly increased above control levels as noted: (**a**) * = $p<0.001$; (**b**) * = $p<0.05$. (**c**) Co-immunoprecipitation of receptor chains in response to cytokines. GFP- or mCherry-tagged receptor constructs were transiently co-expressed in 293T cells and then stimulated with IL-12 or IL-35. Cells were cross-linked with a cleavable cross-linker and lysed. Receptors were immunoprecipitated using anti-DsRed antibodies and interrogated for GFP signal by immunoblot analysis. (**d**) Densitometry scanning of four independent experiments as in **b**. GFP signal was normalized to the GFP signal from input lanes and scaled to vector-treated control (set to 1, * = $p < 0.05$, ** = $p < 0.01$, unpaired t-test).

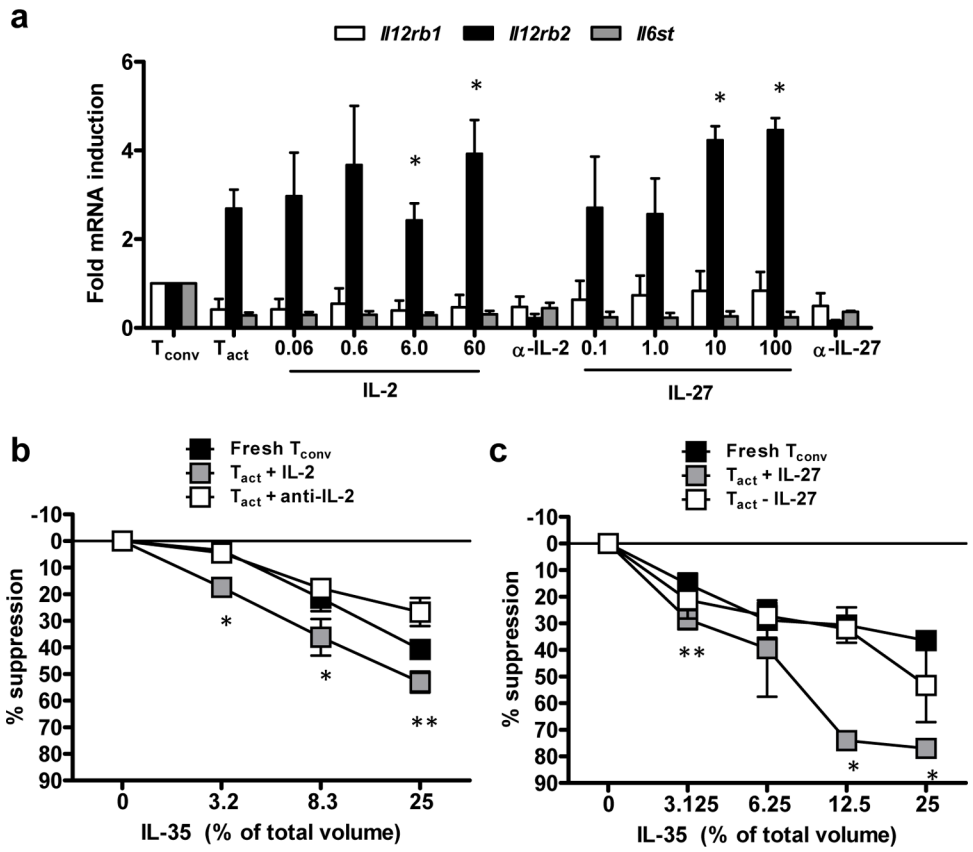

Figure 5. IL35R chain expression can be induced IL-2 and IL-27

(**a**) T_{conv} cells were stimulated in the presence of varying doses of IL-2, IL-27, or neutralizing antibodies to IL-2 or IL-27p28. RNA was extracted and cDNA generated. *Il6st*, *Il12rb1* and *Il12rb2* mRNA expression was normalized to *Actb* expression and scaled to signal from naïve T cells (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, unpaired t-test). (**b,c**) T cells were activated 48 h in the presence of indicated cytokines or anti-cytokine antibodies, rested 48 h, and restimulated with indicated concentrations of IL-35 for 3 days. Proliferation was determined by [^{3}H]-thymidine incorporation. CPM of T_{conv} cells activated alone ranged from 31,000–75,000. Data represent the mean ± SEM of (**a**) 3, (**b**) 3, (**c**) 2 [of 6] independent experiments.

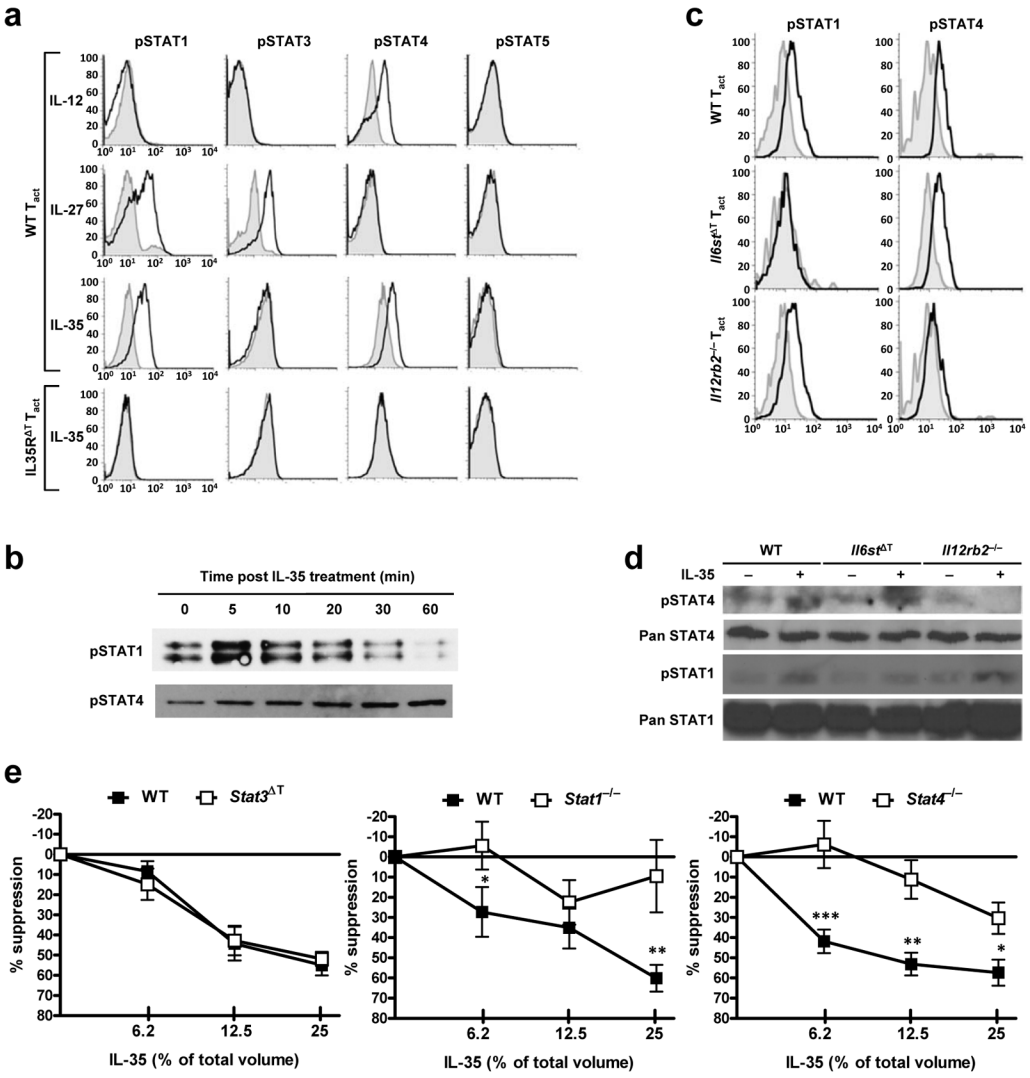

Figure 6. IL-35 signals through STAT1 and STAT4

(**a**) T_{conv} cells purified by flow sorting were treated with indicated cytokines for 30 min following 18h activation (αCD3-CD28) alone (IL-27) or activation (αCD3-CD28) in the presence of rIL-27 (IL-12 and IL-35). Intracellular staining of pSTATs was determined by flow cytometry (isotype control in grey). (**b**) T_{conv} cells purified by flow sorting were treated with IL-35 for indicated times following 24h activation (αCD3-CD28). Cells were lysed in cold RIPA buffer, resolved by SDS-PAGE and probed with anti-pSTAT Abs to identify STAT phosphorylation. (**c**) T_{conv} cells from wild-type (C57BL/6), *gp130*$^{\Delta T}$ or *Il12rb2*$^{-/-}$ mice were stimulated with IL-35 and stained for pSTAT1 and pSTAT4 as in A (no stimulation in grey). (**d**) Immunoblot analysis of T cells blasts from WT (C57BL/6), *gp130*$^{\Delta T}$ or *Il12rb2*$^{-/-}$ mice stimulated with IL-35 for 30 min and probed as in **b**. (**e**) T_{conv} cells were purified by flow sorting from WT (C57BL/6 or Balb/c), *Stat1*$^{-/-}$ (C57BL/6 background), *Stat3*$^{-/-}$ (C57BL/6 background), or *Stat4*$^{-/-}$ (Balb/c background) mice. T_{conv} cells were activated with anti-CD3- + anti-CD28-coated latex beads for 3 days in the presence of indicated concentrations of IL-35. Proliferation was determined by [^{3}H]-thymidine incorporation. Counts per minute of T_{conv} cells activated alone ranged from 24,000–91,000. Data represent the mean ± SEM of (**a,b,e**) 3–8, (**c**) 4, and (**d**) 3 independent experiments.

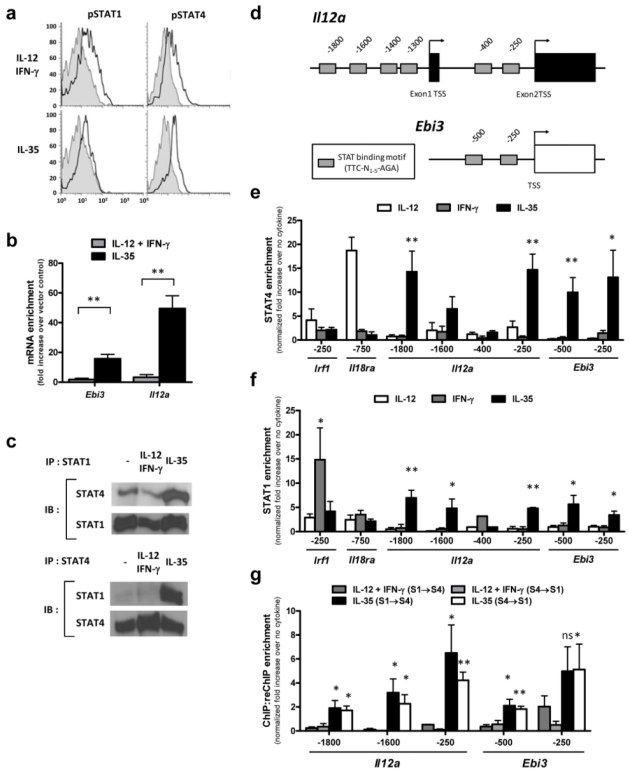

Figure 7. IL-35 utilizes a STAT1:STAT4 heterodimer to mediate *Ebi3* and *Il12a* promoter interaction

(**a**) T_{conv} cells were activated 48 h in the presence of IL-2 and IL-27, rested for 5 d in IL-2, serum starved for 3 h on ice, and stimulated with control supernatant, IL-12 + IFN-γ, or IL-35. Cells were stained intracellularly for pSTAT1 and pSTAT4. Shaded histograms represents control supernatant treatment. (**b**) T_{conv} cells activated for 48 h in the presence of control supernatant, IL-35, or IL-12 + IFN-γ were interrogated for *Ebi3* and *Il12a* mRNA by qPCR. Values represent fold increase over control supernatant normalized to *Actb* expression (* = $p < 0.05$, ** = $p < 0.01$, unpaired t-test). (**c**) T_{conv} cells were stimulated as in **a** and lysed. Lysates were precleared, then STAT1 or STAT4 was immunoprecipitated. Eluates were immunoblotted for STAT1 and STAT4. (**d**) Schematic diagram of the mouse *Il12a* and *Ebi3* promoters. Eight STAT binding sites were identified; six are interrogated here. (**e**) ChIP analysis of STAT4 binding sites in the *Il12a* and *Ebi3* promoters. T_{conv} cells were activated with anti-CD3, anti-CD28, and IL-2 for 48 h, expanded in IL-2 for 48 h, serum starved 3 h, then stimulated with control supernatant, -IL-12, IFN-γ, or IL-35 for 90 min. Chromatin was prepared from 15×10^6 T cells per stimulation and subjected to STAT4 ChIP. ChIP DNA was assayed using real-time PCR. Primers to *Irf1* and *Il18ra* promoter sites were used as controls. Results represent fold increase over control protein treated cells, which are scaled using isotype-IgG ChIP and input (* = $p < 0.05$, ** = $p < 0.01$, unpaired t-test IL-12 vs. IL-35). (**f**) ChIP analysis as in (**e**), but for STAT1 (* = $p < 0.05$, ** = $p < 0.01$, unpaired t-test, IFN-γ vs IL-35). (**g**) Expanded T_{conv} cells (30×10^6) were prepared as in **e**. The eluted chromatin from STAT4 or STAT1 ChIP was then subjected to a second IP (reChIP). Analyses were restricted to loci in which both STAT1 and STAT4 ChIP signals were enriched following IL-35 stimulation in **e** and **f** (* = $p < 0.05$, ** = $p < 0.01$, unpaired t-test, IL-12+IFN-γ vs. IL-35). Data are representative (**a,c**) or are the mean of 3 (**b,f**), 5 (**e**) independent experiments, or 2 [of 3] (**g**) independent experiments.

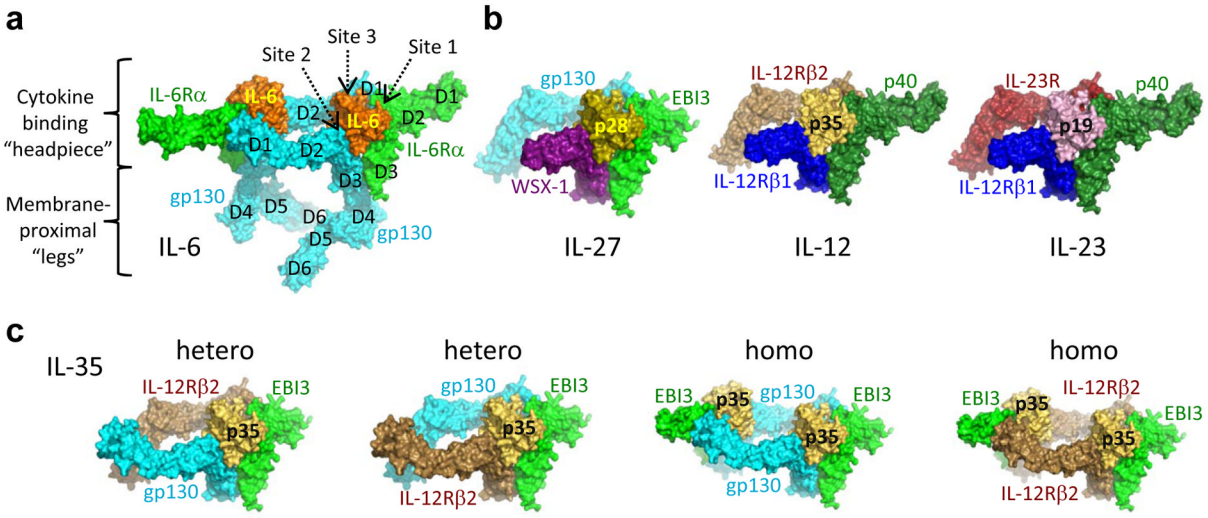

Figure 8. Structural models of the IL-12 family of cytokine receptor complexes

(**a**) Structure of the complete ectodomain receptor signaling complex for IL-6 with IL-6Ra and gp130 derived from electron microscopic and crystallographic data[24, 36, 50]. Sites 1, 2 and 3 are indicated on the model. (**b**) Models of potential IL-35 signaling complexes based on the IL-6 complex demonstrating alternative arrangements of the receptors as signaling-competent homo- or hetero-meric dimers. (**c**) Models of ectodomain complexes of IL-27, IL-12, and IL-23 based on the IL-6 complex. Models were constructed, visualized and rendered in Pymol (Schrödinger LLC The PyMOL Molecular Graphics System, Version 1.2r3pre).