Activated T Cells Secrete an Alternatively Spliced Form of Common γ-Chain that Inhibits Cytokine Signaling and Exacerbates Inflammation

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SUMMARY

The common γ-chain (γc) plays a central role in signaling by IL-2 and other γc-dependent cytokines. Here we report that activated T cells produce an alternatively spliced form of γc mRNA that results in protein expression and secretion of the γc extracellular domain. The soluble form of γc (sγc) is present in serum and binds to IL-2Rα and IL-7Rα proteins on T cells to inhibit cytokine signaling and promote inflammation. sγc suppressed IL-7 signaling to impair naive T cell survival during homeostasis and exacerbated Th17-cell-mediated inflammation by inhibiting IL-2 signaling upon T cell activation. Reciprocally, the severity of Th17-cell-mediated inflammatory diseases was markedly diminished in mice lacking sγc. Thus, sγc expression is a naturally occurring immunomodulator that regulates γc cytokine signaling and controls T cell activation and differentiation.

INTRODUCTION

Cytokines of the common γ-chain (γc) family are critical for development of T lineage cells and depend on γc for cellular signaling (Rochman et al., 2009). γc deficiency results in a paucity of both mature B and T cells because of impaired signaling by the γc cytokine IL-7 (Noguchi et al., 1993). In fact, IL-7 signaling is necessary for developing lymphocytes to proceed through the pre-pro-B cell stage of B cell differentiation and the CD4+CD8+ double-negative (DN) stage of thymopoiesis (DiSanto et al., 1995; Peschon et al., 1994; von Freeden-Jeffry et al., 1997). Impaired IL-7 signaling also profoundly impairs mature T cell survival and homeostasis (Schluns et al., 2000; Tan et al., 2001). Because γc is also required for IL-2 and IL-15 signaling, γc deficiency impairs development of FoxP3+ regulatory T cells and NK cells (Heaney and Golde, 1996; Ma et al., 2006; Vosshenrich et al., 2005) and also alters CD4 T helper cell lineage fate and CD8 memory cell differentiation (Rochman et al., 2009). Thus, γc governs the generation, differentiation, and homeostasis of all lymphocyte subsets in the adaptive immune system.

Although γc expression is necessary for γc cytokine signaling, γc alone cannot bind cytokines and cannot trigger downstream signaling (Minami et al., 1993). Rather, γc cytokines induce γc membrane proteins to complex with proprietary cytokine receptor subunits, such as the IL-7-specific IL-7 receptor α chain (IL-7Rα) and the IL-2-specific IL-2 receptor β-chain (IL-2Rβ), to transduce cytokine receptor signals. Importantly, the magnitude and kinetics of γc cytokine responses are thought to be controlled by the proprietary cytokine-specific receptor subunits and not by γc (Rochman et al., 2009). For example, IL-7 stimulation affects IL-7Rα expression but does not affect γc expression (Park et al., 2004), and IL-2 stimulation affects IL-2Rα and IL-2Rβ without affecting γc expression (Depper et al., 1985; Siegel et al., 1987). Consequently, modulation of γc expression is thought to be irrelevant to either the kinetics or magnitude of cytokine signaling. Instead, γc expression levels are thought to be developmentally set and to remain constant during T cell activation and differentiation (Rochman et al., 2009).

In contrast to this prevailing view, we now report that modulation of γc expression actively contributes to the regulation of cytokine responses. Interestingly, γc exerts its regulatory effects not as a conventional membrane receptor protein but as a secreted protein induced by T cell stimulation. Specifically, we found that activated T cells expressed a γc mRNA splice isoform that resulted in production and secretion of soluble γc ectodomain proteins. Soluble γc inhibited cell signaling by γc cytokines and exacerbated inflammatory responses by promoting differentiation of pathogenic CD4+ Th17 cells both in vitro and in vivo. Conversely, the severity of Th17-cell-mediated inflammatory autoimmune disease was markedly diminished in mice lacking sγc. Thus, this study identifies a role for γc as an active immunoregulatory molecule whose alternatively spliced form is secreted to dampen signaling by γc cytokines and promote inflammatory T cell immune responses in vivo.
RESULTS

Identification of a γc mRNA Splice Isoform that Produces a Secreted Form of γc

T cell activation induces expression of soluble factors such as IL-2 that control immune responses both in trans and in cis (Malek, 2008). To further understand the role of such soluble factors, we analyzed culture supernatants of TCR- and CD28-stimulated T cells. We found that culture supernatants from activated T cells not only contained IL-2 and TNF-α, but surprisingly also contained large amounts of a secreted form of membrane γc proteins (Figure 1A). Although shedding is a classical mechanism of producing soluble forms of membrane proteins (Heaney and Golde, 1996), this was not the explanation for γc protein secretion, as shown by the fact that inhibition of membrane metalloproteases by TAPI2 treatment to prevent membrane protein shedding failed to prevent expression of soluble γc (Figure 1A). Instead, we found that activated T cells upregulated expression of a novel γc mRNA species that encoded a soluble form of γc (sγc) rather than the conventional membrane form of γc (Figures S1A–S1C available online).

The gene encoding γc (Il2rg) is composed of eight exons, and the entire transmembrane (TM) domain is encoded in exon 6 (Cao et al., 1993). The new γc isoform was generated by alternative RNA splicing that excluded exon 6 to encode a γc protein product with the full-length γc extracellular domain (ED) but without the TM and intracellular domain (ID) (Figures S1A and S1B). Mechanistically, an open reading frame shift at the splice junction of exon 5 to exon 7 created a new 9 amino acid epitope (CLQFPPSRI) (Figure 1C), and used these antibodies to probe serum sγc-specific antibodies (open histogram) versus control antibody staining (shaded histogram) is shown for γc surface (left) and mRNA expression (right). Cell surface γc expression (open histogram) versus control antibody staining (shaded histogram) is shown for γc protein secreting at low levels in resting T and B cells and were highly expressed in immature thymocytes and NK cells as well as in activated T cells (Figure S1D). Interestingly, we also identified alternatively spliced γc transcripts in human T cells, indicating an evolutionarily conserved mechanism of soluble γc expression and regulation (Figure S1E).

To examine whether sγc is expressed in vivo, we tested serum for sγc proteins. C57BL/6 wild-type (WT) and γc-deficient (Il2rg−/−) sera were immunoprecipitated with total γc-specific antibodies and then probed with γc extracellular domain-specific antibodies (α-γc-ED). As shown in Figure 1B, sγc proteins were detected in WT but not in Il2rg−/− serum. To determine whether serum sγc is a product of alternative splicing, we generated antibodies specific for the 9 residue C-terminal neoepitope, CLQFPPSRI (Figure 1C), and used these antibodies to probe anti-γc serum immunoprecipitates. Of note, the 9 residue neoepitope is not present in the mouse proteome (NCBI Blastp analysis, data not shown), and it is unique to the alternatively spliced sγc product. Immunoblot analysis showed immunoreactivity of CLQFPPSRI-specific antibodies with WT but not Il2rg−/− serum (Figure 1C). To further examine whether shedding contributes to sγc expression in vivo, we generated mice that were unable to
produce alternatively spliced sγc. We achieved this by generating a human CD2 mini-cassette-driven membrane γc transgene (γcTg) and then breeding the transgene into il2rg−/− mice to generate il2rg−/−γcTg mice (referred to as mγcTg mice). In mγcTg mice, all γc proteins are expressed from a prespliced, transgenic γc cDNA that encodes only the full-length membrane γc and cannot undergo splicing to produce the splice isoform. mγcTg T cells expressed large amounts of membrane γc mRNA and full-length γc protein, but no alternatively spliced sγc transcripts (Figures 1D). Strikingly, serum from such mγcTg mice lacked circulating sγc proteins, revealing that virtually all serum sγc is generated by alternative splicing, with little or no contribution from shedding (Figure 1E). Thus, mγcTg mice are sγc deficient, and serum sγc is the product of a posttranscriptional γc splice isoform.

Serum sγc Expression Is Upregulated upon Immune Activation In Vivo

Having identified sγc produced by activated T cells in vitro, we wished to know whether T cell activation would increase sγc expression in vivo. To this end, we assessed serum sγc amounts in BALB/c mice injected with CD3 antibodies (α-CD3) to induce acute polyclonal T cell stimulation in vivo. In fact, serum sγc titers were increased overnight after CD3 antibody injection concomitant with T cell activation even as total T cell numbers were not increased (Figures 2A, S2A, and S2B).

Next, we wished to analyze serum sγc titer in mice with chronically activated T cells. Ctla4−/− mice contain chronically activated T cells that mediate a lethal lymphoproliferative autoimmune disease (Waterhouse et al., 1995). We found the amount of serum sγc to be larger in Ctla4−/− mice than in WT mice (Figure 2B). Importantly, elevated sγc titer was dependent on the presence of activated T cells and not on Ctla4 deficiency itself, because sγc expression was not elevated in nonautoimmune Ctla4−/−Cd28−/− mice that lack activated T cells and consequently are disease free (Figure 2B; Tai et al., 2007). To further assess sγc expression during in vivo immune activation, we also analyzed autoimmune il2−/− and CD25-deficient (il2ra−/−) mice. Both mouse strains have defective CD4+ regulatory T cell generation that results in massive in vivo T cell activation and lethal autoimmunity (Sadlack et al., 1993; Willerford et al., 1995). We found that both il2−/− and il2ra−/− mice expressed increased amounts of serum sγc, confirming that sγc expression was elevated during in vivo immune activation (Figure 2C). Collectively, our results demonstrate that serum sγc protein levels are increased during T cell activation in vivo.

Generation of sγc Transgenic Mice

To understand the effect of increased sγc expression, we generated transgenic mice overexpressing sγc in T cells (sγcTg). In sγcTg mice, serum sγc levels were substantially increased (Figures 2D and 2E), but T cell numbers and phenotypes were unchanged from WT mice (Figures 2F, S2C, and S2D). To assess whether in vivo sγc upregulation had any effect on T cells, we stimulated sγcTg CD4+ T cells with PMA/ionomycin and examined their ex vivo cytokine expression profiles. γc cytokines play an instructive role in CD4 effector T cell differentiation (Zhu et al., 2010), and we wished to know how in vivo exposure to increased sγc would affect T cell function. Interestingly, sγcTg CD4+ T cells contained higher proportions of IFN-γ producers than did WT CD4+ T cells (Figure 2G). Expression of the Th2 cytokine IL-4, however, was not affected (Figure S2E). Notably, sγcTg CD4+ T cells contained a significantly larger population of IL-17-producing cells (p < 0.001), suggesting that increased sγc level promoted a Th1- and Th17-cell-prone proinflammatory environment (Figure 2G), but this could also reflect an increased presence of memory cells. Importantly, such settings were not created by defects in FoxP3+ CD4+ Treg cells as shown by the fact that both development and function of Treg cells were unaffected in sγcTg mice (Figures S2F and S2G).

sγc Is Proinflammatory and Exacerbates EAE Pathology

Because IL-17-producing Th17 cells are potent inducers of cell-mediated autoimmunity (Harrington et al., 2005; Park et al., 2005), we wished to know whether sγc would promote autoimmunity. Experimental autoimmunity encephalomyelitis (EAE) is a cell-mediated neuroimmunological disease that is largely mediated by Th17 cells and serves as an experimental model for human multiple sclerosis (Governor, 2009). Injection of MOG peptides results in the activation and brain infiltration of autoreactive lymphocytes, which, in our hands, induced paralysis that peaked around day 5 after onset of the disease. Strikingly, when assessing serum sγc expression in EAE-induced mice, we found increased sγc levels in diseased mice (Figure 3A). To understand whether increased sγc expression contributed to or was the consequence of EAE pathology, we assessed disease progression and found sγcTg mice to be significantly more susceptible to EAE than control mice (p < 0.001) (Figure 3B). In fact, in every parameter examined, sγcTg mice displayed a more pronounced phenotype than did WT mice. Specifically, we found in sγcTg mice that EAE severity worsened, disease progression accelerated, and disease recovery was substantially delayed (Figure 3C). Moreover, we found increased numbers of spinal-cord-infiltrating CD4+ T cells, which correlated with dramatically increased percentages and numbers of Th1 and Th17 cells and IL-17, IFN-γ double-producing CD4+ T cells (Figures 3D and 3E). Taken together, these results indicate that sγc enhances inflammatory immune responses and increases differentiation of pathogenic Th17 CD4+ effector cells in vivo.

sγc Impairs IL-2 Signaling

Having observed that sγc expression exacerbated T-cell-mediated autoimmune diseases in vivo, we wished to determine the effect of soluble γc proteins on the generation of inflammatory Th17 cells. Th17 cell differentiation is negatively regulated by IL-2 signaling (Laurence et al., 2007) and is increased by inhibition of IL-2 signaling (Chen et al., 2011; Pandiyan et al., 2007). Therefore, we wished to know whether increased Th17 cell generation in sγcTg mice was the result of diminished IL-2 signaling. To this end, we produced sγc as recombinant proteins (rsγc) (Figure S3). Importantly, we found that rsγc proteins were expressed as disulfide-linked homodimers (Figure 4A), and that the same was true for endogenously produced sγc proteins in serum (Figure S4A). In fact, alternative splicing of γc in both humans and mice creates a C-terminal cysteine residue that promotes dimerization of sγc proteins (Figures S1A and S1E). Therefore, unlike membrane γc proteins, sγc proteins exist as dimers.
Because γc has no affinity for IL-2 (Minami et al., 1993), soluble γc would not diminish IL-2 signaling by binding free IL-2. Thus, we wished to know whether sγc might diminish IL-2 signaling by binding to IL-2 receptor proteins on the cell surface. To test this idea, we performed surface plasmon resonance (SPR) studies with purified rsγc on immobilized IL-2Rβ extracellular domain (ED) proteins. Strikingly, SPR analysis showed that sγc proteins directly bound to IL-2Rβ-ED and that dimeric sγc proteins bound much more strongly (Kd of 16.6 ± 5.5 μM) than monomeric sγc (Kd of 695 ± 76 μM) (Figure 4B). Of note, we consider that the theoretical affinity of sγc binding measured by in vitro SPR study reflects only the minimum potential affinity of sγc for IL-2Rβ and that sγc would bind to actual cell surface IL-2Rβ with much higher affinity under physiological conditions.
To further assess whether s\(\gamma c\) directly binds to T cells, we utilized flow cytometry. To do so, we examined TCR\(\beta^+\) mature thymocytes of mice with conditional deletion of \(\gamma c\) in DP thymocytes (\(Il2rg^{fl/flE8IIICre}\)) because they lack surface \(\gamma c\) expression (Figure S4B; McCaughtry et al., 2012). Notably, incubation of \(Il2rg^{fl/flE8IIICre}\) thymocytes with s\(\gamma c\) proteins revealed direct binding of s\(\gamma c\) to the surface, which we visualized by staining with \(\gamma c\)-specific antibodies (Figure 4C) and which was still detectable at 0.06 \(\mu g\) (~10 nM) (Figure 4D). More importantly, addition of IL-2 significantly increased \((p < 0.0001)\) s\(\gamma c\) binding to \(Il2rg^{fl/flE8IIICre}\) cells (Figures 4C and 4D), indicating that s\(\gamma c\) affinity to IL-2R\(\beta\) is increased by IL-2. In sum, s\(\gamma c\) proteins, especially dimeric s\(\gamma c\) proteins, directly bind to surface IL-2R\(\beta\) chains, especially in the presence of IL-2.

To directly test whether s\(\gamma c\) binding to IL-2R\(\beta\) suppressed IL-2 signaling in T cells, we assessed IL-2-induced STAT5 and
AKT phosphorylation in the presence or absence of rsγc (Malek, 2008). We found that rsγc effectively suppressed downstream IL-2 signaling (Figure 4E) and that rsγc-induced impairment of IL-2 signaling reduced IL-2’s biological effects on T cells. In mice, naïve CD8+ T cells respond to IL-2 by vigorous proliferation (Cho et al., 2010). We confirmed such IL-2-induced proliferation in purified CD8+ T cells and found that rsγc suppressed IL-2-driven proliferation in the same cells (Figure 4F), demonstrating a direct inhibitory role of γc in IL-2 signaling. Thus, by binding to IL-2RIβ proteins on the cell surface, γc prevents association with membrane γc proteins to impair IL-2 signaling (Figure S4C).
Soluble γc Receptor Controls Cytokine Signaling

$sy_c$ Promotes Th17 Cell Differentiation by Suppressing IL-2 Signaling

To assess the IL-2 inhibitory effect of $sy_c$ on Th17 cell differentiation, we analyzed Th17 cell generation by using in vitro cultures in the presence of $rsy_c$. As previously described (Laurence et al., 2007), recombinant IL-2 potently suppressed, whereas antagonizing IL-2 antibodies substantially enhanced, Th17 cell differentiation (Figures 5A and 5B). Importantly, $sy_c$ treatment or $sy_c$ overexpression had the same effect as IL-2 antibodies as it increased both the proportion of IL-17-producing cells and overall IL-17 expression (Figures 5B and 5C). In fact, coincubation of $rsy_c$ and antagonizing IL-2 antibodies did not have additive effects, indicating that the Th17-cell-promoting mechanisms of $rsy_c$ and IL-2 antibodies were redundant (Figure 5D).

To further demonstrate that the $sy_c$ effect on Th17 cells was due to its inhibition of IL-2 signaling, we analyzed $sy_c$ effects on IL-2-deficient conditions. Naive CD4+ T cells were stimulated under Th17-cell-polarizing conditions to assess intracellular IL-17A and IFN-γ expression. Where indicated, $rsy_c$ were added at the beginning of the differentiation culture. Contour plots are representative of four independent experiments (left). Bar graph shows percent increase of IL-17A-producing CD4+ T cells by $rsy_c$ treatment compared to vehicle control. Error bars represent mean and SEM.

Figure 5. $sy_c$ Promotes Th17 Cell Differentiation of CD4+ LN T Cells

(A) $sy_c$ promotes IL-17A expression. Sorted naive CD4+ T cells were stimulated under Th17-cell-polarizing conditions and assessed for intracellular IL-17A and IFN-γ expression. Where indicated, recombinant human IL-2 (rhIL-2), neutralizing mouse IL-2-specific antibodies (α-IL-2), recombinant $sy_c$ (4 μg/ml $rsy_c$), or vehicle (Veh) were added at the beginning of the culture. Data are representative of ten independent experiments.

(B) Summary of IL-17A+CD4+ T cell differentiation under modified Th17-cell-polarizing conditions. Each symbol represents an individual experiment under the indicated condition as described above.

(C) IL-17A secretion in WT and $sy_c$-Tg CD4+ T cells. Culture supernatants were collected at day 4 of Th0 or Th17 cell differentiation cultures. ELISA data are representative for five independent experiments.

(D) Redundant effect of $rsy_c$ and mIL-2-specific antibodies on Th17 cell differentiation. Naive CD4+ T cells were stimulated under Th17-cell-polarizing conditions in the presence of $rsy_c$ or mIL-2-specific antibodies. Each symbol represents an individual mouse.

(E) $sy_c$ effect on IL-17A expression under IL-2-deficient conditions. Naive CD4+ T cells were stimulated under Th17-cell-polarizing conditions to assess intracellular IL-17A and IFN-γ expression. Where indicated, $rsy_c$ were added at the beginning of the differentiation culture. Contour plots are representative of four independent experiments (left). Bar graph shows percent increase of IL-17A-producing CD4+ T cells by $rsy_c$ treatment compared to vehicle control. Error bars represent mean and SEM.
results were observed in Il2Rα−/−sγcTg CD4+ T cells where sγc transgene expression failed to enhance Th17 cell differentiation compared to Il2Rα−/− cells (Figure 5E). Altogether, these results document that sγc promotes Th17 cell differentiation by specifically impairing IL-2 signaling.

**sγc Deficiency Ameliorates Th17-Cell-Mediated Inflammatory Autoimmune Diseases**

Because sγc exacerbated IL-17 expression and inflammation, we wished to know whether the absence of sγc would dampen inflammatory responses that otherwise occurred in WT mice. To this end, we assessed EAE induction in mγcTg mice that express only transgenic membrane γc receptors and lack endogenous sγc proteins (Figure 1B). Strikingly, EAE disease progression in mγcTg mice was dramatically reduced compared to WT mice, with delayed disease onset and lower clinical scores (Figure 6A). Analysis of spinal-cord- and brain-infiltrating T cells further utilized the CD4+CD45RBhi T cell transfer model of inflammatory responses as demonstrated by increased Th17 cell differentiation and dramatically reduced the severity of inflammatory autoimmune disease, resulting in markedly improved disease outcomes. Thus, this study identifies sγc as a potential therapeutic target for inflammatory autoimmune diseases.

To further demonstrate sγc binding to surface IL-7Rα proteins, we incubated Il2RαE8IIICre thymocytes with rsγc proteins and assessed rsγc binding to thymocyte subpopulations. TCRβhi mature thymocytes express high levels of IL-7Rα and strongly bound sγc, whereas DP thymocytes do not express surface IL-7Rα and did not bind sγc (Figure 7C). To directly show that recombinant sγc specifically bound to IL-7Rα proteins, we examined γc-deficient TCRβhi thymocytes that were additionally deficient in IL-7Rα (McCaughtry et al., 2012). Indeed, IL-7Rα deficiency substantially reduced sγc binding to sγc-deficient TCRβhi thymocytes (Figure 7D), documenting that sγc directly binds and interacts with surface IL-7Rα proteins.

IL-7 is critical for thymopoiesis and impaired IL-7 signaling results in reduced thymus cellularity (von Freeden-Jeffry et al., 1995). Notably, increased sγc expression in sγcTg mice resulted in decreased total thymocyte numbers (Figure 7E), CD25 is reduced percentages and numbers of naive T cells in sγcTg mice (Figures 6B and 6C). Importantly, the severity of inflammatory reactions was markedly reduced in mγcTg mice despite the presence of normal numbers and appearances of peripheral T cells (Figures SSA and SSB).

To further assess the ameliorative effect of sγc deficiency, we utilized the CD4+CD45RBhi T cell transfer model of inflammatory bowel disease (IBD) (Powie et al., 1993). Adoptive transfer of naive T cells into immunodeficient Rag2−/− mice results in severe autoimmune inflammatory colitis that can be monitored by body weight loss and increased IBID clinical scores (Table S1). Notably, we found that Rag2−/− host mice injected with mγcTg CD4+ T cells had markedly diminished IBD, whereas Rag2−/− mice injected with WT CD4+ T cells suffered from significant body weight loss, destruction of colon tissues, and rapid deterioration of health (Figures 6D, 6E, S5C, and S5D). Importantly, adoptively transferred mγcTg CD4+ T cells were still activated in vivo and produced large amounts of IFN-γ, but it was specifically the expression of proinflammatory IL-17 that was dramatically diminished in these cells (Figure 6F). Thus, sγc-deficient T cells were unable to induce severe autoimmune IBD because of reduced Th17 cell generation. Conversely, T cell expression or overexpression of sγc increased the severity of inflammatory disease (Figure S5D). These results reveal that sγc exacerbates IBD, particularly in vivo inflammatory immune responses and contributes to the severity of in vivo autoimmune pathology.

**sγc Overexpression Impairs T Cell Development and Naïve T Cell Homeostasis**

Although sγc directly binds to LN T cells (Figure 7A), resting T cells do not express high levels of IL-2Rβ. These data suggested that sγc also bound to receptors for cytokines other than IL-2. Specifically, we considered that sγc might bind to IL-7 receptors because they are highly expressed on all resting T cells. SPR analysis showed that this was indeed the case. We found that sγc proteins directly interacted with mouse IL-7Rα-ED proteins and that dimeric sγc showed a dramatic increase of IL-7Rα-ED binding activity (Kd = 3.9 μM) over monomeric sγc proteins (Kd = 50.8 μM) (Figure 7B and Table S2).

**DISCUSSION**

Here we report a regulatory mechanism of γc signaling that tunes T cell responsiveness to IL-2 and IL-7 and potentially to other γc cytokines as well. This regulatory pathway was based on post-transcriptional generation and secretion of the γc extracellular domain that directly bound to γc cytokine proprietary receptors such as IL-2Rβ and IL-7Rα, especially in the presence of cytokines, and interfered with cytokine signaling. Importantly, secreted γc proteins required disulfide-linked homo-dimerization for high-affinity binding and inhibition of cytokine signaling. During steady-state conditions, γc interfered primarily with IL-7-dependent processes, resulting in impaired thymopoiesis and naive T cell survival. During T cell activation, sγc inhibited IL-2 signaling and promoted the development of proinflammatory Th17 cell responses as demonstrated by increased Th17 cell differentiation in vitro and by exacerbation of EAE disease in sγc transgenic mice in vivo. Conversely, in vivo sγc deficiency suppressed Th17 cell differentiation and dramatically reduced the severity of inflammatory autoimmune disease, resulting in markedly improved disease outcomes. Thus, this study identifies sγc as a potential therapeutic target for inflammatory autoimmune diseases.
Figure 6. sγc Deficiency Is Protective against Inflammatory Autoimmune Diseases

(A) EAE induction in WT and mγcTg mice. EAE clinical scores are summary of two independent EAE experiments, each with four mice per group.

(B) Th1 and Th17 cell subset analysis of spinal-cord- and brain-infiltrating CD4⁺ T cells in EAE-induced WT and mγcTg mice. Contour plots show representative IL-17A, IFN-γ profiles from four mice per group.

(C) Spinal-cord- and brain-infiltrating CD4⁺ T cell numbers in EAE-induced mice. On day 15 after immunization, IFN-γ- and/or IL-17A-producing CD4⁺ T cells were enumerated from spinal cords and brains of WT and mγcTg mice. Data are representative of four mice per group.

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Soluble γC Receptor Controls Cytokine Signaling

Proteins as endogenous immunomodulators that exacerbate in vivo inflammatory diseases by directly binding to cytokine receptors and dampening T cell responses to γC cytokines.

Cytokine signaling in T cells is tightly controlled at many levels. Expression of cytosolic inhibitory molecules, such as SOCS, limits the magnitude and duration of cytokine signaling (Yoshimura et al., 2007). Upregulation of surface cytokine receptor expression, on the other hand, positively affects cytokine signaling. Secretion of γC proteins now adds another layer of regulatory complexity because γC expression is upregulated upon T cell activation but, unlike the upregulation of other IL-2 receptor chains that enhance IL-2 signaling, upregulation of γC suppresses IL-2 signaling. Moreover, soluble γC is unique in its regulatory mechanism because it suppressed IL-2 signaling without binding to the IL-2 molecule itself. This is in contrast to other chains of the IL-2 receptor complex that regulate IL-2 responsiveness by direct interaction with IL-2 (Minami et al., 1993). Because IL-2 signaling requires membrane γC association with IL-2Rβ, γC binding to IL-2Rβ proteins probably blocks IL-2 signaling by preventing recruitment of membrane γC proteins and assembly of signaling-competent IL-2 receptor/membrane γC complexes.

The secreted γC protein is structurally identical to the membrane γC receptor, except for absent transmembrane and intracellular domains. Because ligand binding is mediated by the extracellular domain, it was initially unclear how soluble γC could compete with membrane γC for binding to proprietary receptor proteins. The mechanism was partly revealed by SPR analysis, which showed that dimeric γC proteins possessed >40-fold increased affinity of binding to IL-2Rβ than membrane γC proteins, which are monomers and do not display any meaningful affinity to IL-2Rβ (Rickert et al., 2004). As a result, γC proteins effectively outcompete membrane γC protein for binding to proprietary cytokine receptors IL-2Rβ and IL-7Rα. Therefore, upon dimerization, γC proteins acquire binding specificities that make them distinct from monomeric γC and membrane γC proteins. In this regard, we think it is not a coincidence that alternative splicing of γC pre-mRNA creates a new C-terminal cysteine residue that promotes disulfide linkage and dimerization of γC proteins. Indeed, we found such de novo C-terminal cysteine residues in both mouse and human γC proteins, indicating that dimerization of γC proteins is an evolutionarily conserved mechanism of regulating cytokine signaling.

Soluble cytokine receptors have been observed in previous studies (Baran et al., 1988; Mortier et al., 2004; Nielsen et al., 1998). Expression of soluble cytokine receptors had been associated with inflammation, autoimmunity, and cancer (Heaney and Golde, 1996), but it was uncertain whether their expression was the cause or the consequence of immune activation. In theory, soluble cytokine receptors might either potentiate or antagonize cytokine signaling (Heaney and Golde, 1996). Soluble IL-6Rα, for example, directly binds IL-6 and potently enhances IL-6 signaling (Rose-John, 2012), whereas soluble IL-15Rα sequesters IL-15 and prevents it from binding to membrane IL-15 receptors (Mortier et al., 2004). γC proteins, however, do not show significant binding affinity to free cytokines, so it was not immediately obvious what role soluble γC would play in cytokine signaling. By using recombinant γC proteins and γC transgenic mice, we have demonstrated that γC antagonizes IL-2 and IL-7 signaling and increases inflammatory immune responses.

Because T cell activation induces γC production and γC in turn induces inflammatory cytokine expression, γC production is a mechanism to reinforce proinflammatory immune responses downstream of T cell activation. In fact, γC expression would limit IL-2 responses and promote effector T cell differentiation. Thus, our present results provide an explanation for earlier reports on soluble γC expression in which a significant correlation was found in Crohn’s disease patients between disease activity and serum γC levels (Nielsen et al., 1998). According to our present findings, increased soluble γC expression would dampen γC cytokine signaling, which would enhance inflammatory cell activation to worsen the disease outcome. In agreement with our perspective, soluble γC is highly expressed under inflammatory conditions in both humans and mice. Soluble γC was abundantly expressed in synovial fluid of patients with rheumatoid arthritis and also highly induced in mice challenged with L. major (Meissner et al., 2001; Nishio et al., 2001). We also found that EAE disease outcome and in vivo inflammatory responses were exacerbated in sγC-Tg mice. Collectively, these data indicate a role for sγC as an active inducer of pathogenic T cells and inflammation.

Because γC promotes inflammation, it is interesting to speculate whether neutralization of serum γC expression would ameliorate inflammatory disease and dampen destructive T cell responses. The striking reduction in disease severity of EAE or IBDe-induced mγC-Tg mice lacking γC observed here suggests that depletion of γC might be an effective therapy in patients suffering from inflammatory immune diseases. In this regard, the 9 aa C-terminal neoeptipe CLQFPPSR (and the 7 aa neoeptipe RCPEFP in humans) of γC provides an excellent target for intervention with neutralizing antibodies. These peptide epitopes are generated only upon alternative splicing of γC pre-mRNA exon 5 to exon 7, and they are found only in γC and not in membrane γC proteins. In contrast to conventional γC-specific antibodies, C-terminal γC-specific antibodies will neutralize only alternatively spliced γC proteins, making this epitope an attractive target to clear γC from circulation without affecting membrane γC receptor function.

Discovery of the unique C-terminal γC epitope was enlightening because it helped us identify the molecular origin of γC. Specifically, the 9 aa C-terminal sequence is not found in membrane γC proteins so that its presence in serum directly demonstrated that γC proteins are products of alternative RNA splicing.

(D) Body weight change of IBDe-induced mice. Rag2<sup>−/−</sup> mice were injected with each 0.5 million WT or mγC-Tg CD4<sup>+</sup>CD45RB<sup>+</sup> T cells and monitored for change in body weights. Data show results from four mice per group.
(E) Colon histology from Rag2<sup>−/−</sup> host mice injected with naive CD4<sup>+</sup> T cells from the indicated donor mice at 6 weeks after T cell transfer.
(F) Th1 and Th17 cell subset analysis in CD4<sup>+</sup> LNC T cells from IBDe-induced WT and mγC-Tg mice (left). Data show results from three mice for each group (right). Error bars represent mean and SEM.
Soluble γc Receptor Controls Cytokine Signaling

A) CD4+ LN T cell surface γc staining

B) sγc(monomer)/IL-7Rα-ED

C) II2rg^E8^Cre thymocytes

D) sγc(dimer)/IL-7Rα-ED

E) Mice

F) Thymocyte numbers

G) Naive CD4+ T cells

(legend on next page)
Soluble cytokine receptors are usually produced by one of the following two major mechanisms; proteolytic cleavage and shedding of membrane cytokine receptors or alternative splicing of cytokine receptor pre-mRNA (Levine, 2004). TNF receptors are classical examples of cytokine receptors that are cleaved by transmembrane metalloproteinases and shed from cells (Hwang et al., 1993). Soluble IL-6 receptors, on the other hand, are usually generated by alternative splicing (Horuchi et al., 1994). Our data document that γc is exclusively produced by alternative splicing. It is conceivable that genetic variations might also contribute to increased γc expression. In fact, IL-7Rα gene polymorphisms can result in increased expression of soluble IL-7Rα (sIL7Rα) (Gregory et al., 2007), and sIL7Rα expression has been proposed as a significant risk factor for multiple sclerosis in humans (Harley, 2007; Lundström et al., 2013). Specifically, these SNPs led to exclusion of exon 6 during IL-7Rα pre-mRNA splicing and resulted in the deletion of the transmembrane domain to produce soluble IL-7Rα proteins (Goodwin et al., 1990; Gregory et al., 2007). It would be interesting to assess γc genes for genetic variations that increase exon 6 exclusion and to test whether such SNPs are associated with increased risk for MS or other autoimmune disease. Potentially, such γc genetic variations could predispose carriers to excessive inflammatory responses by increased levels of γc.

In conclusion, the present study has identified γc as an endogenous regulatory mechanism that dampens T cell signaling to γc cytokines and results in enhanced activity of Th17 cells. Enforced γc expression constrained T cell survival under homeostatic conditions and promoted a proinflammatory environment under immune activation. Reciprocally, in vivo γc deficiency suppressed inflammatory Th17 cell differentiation and effectively reduced the severity of Th17-cell-mediated autoimmune disease. Thus, γc represents a naturally occurring immunomodulator of T cells and introduces a target for intervention in inflammation and disease.

**EXPERIMENTAL PROCEDURES**

**Animals**

C57BL/6 (B6) mice and B6.Ly5.2 mice were obtained from the Jackson Laboratory, C574−/−, C574−/−−Cd28−/−, Il2−/−, Il2ra−/−, Rag2−/−, and Il2rg−/− mice were bred in our colony. IL2Rγk−/−EB6Cre and IL7raFl/flRgk−/−EB6Cre mice were kindly provided by A. Singer (NCI). The γc- and γc-transgenic constructs were generated by ligating a murine γc or γc cDNA into human CD2 (hCD2) enhancer-promoter-based vectors, respectively, and injected into fertilized B6 oocytes to generate γcTg and sγcTg mice. Animal experiments were approved by the National Cancer Institute Animal Care and Use Committee (ACUC), and all mice were cared for in accordance with US NIH guidelines.

**Surface Plasmon Resonance**

Purified mouse IL-2Rα ectodomain expressed from yeast was purchased from Mybiosource and used without further modification. IL-2Rα was amine-coupled to a CMS sensor chip after buffer exchange into PBS (pH 7.4) via a NAP-5 (GE Healthcare) column by similar methods described previously (McElroy et al., 2012). Concentration series of monomer and dimer forms of insulin-cell-derived rsγc were injected over immobilized IL-2Rα at a flow rate of 25 μl/min via HBS-EP (pH 7.4). Surface plasmon resonance (SPR) experiments were performed at 25°C with a Biacore 3000 instrument (GE Healthcare). SPR sensorsgrams were double-referenced and trimmed with BioEvaluation 4.1 (GE Healthcare). Dose-response curves (Rmax versus [rsγcmonomer or dimer]) were fit with Prism5.0 (GraphPad). Experiments were performed in triplicate.

**Detection of Soluble Cytokine and Receptor Levels**

Serum γc was detected in a sandwich ELISA with γc-specific polyclonal antibodies (R&D Systems) as capture antibodies and biotin-conjugated γc-specific monoclonal antibodies (4G3; BD) as detection antibodies. Recombinant γc protein was used as positive control. IL-2, IFN-γ, TNF-α, and IL-17A were analyzed by ELISA according to the manufacturer’s instructions (R&D Systems).

**In Vitro CD4+ T Helper Cell Differentiation**

Naïve CD4+ T cells were electronically sorted by gating on CD62L+CD44hiCD25− cells. Sorted cells were stimulated with plate-bound α-CD3 and α-CD28 and cultured under nonskewing Th0 cell conditions (medium alone) or were differentiated into Th17 cells with human TGF-β1 (5 ng/ml; Peprotech), mouse IL-6 (30 ng/ml; BD), anti-mouse IL-4 (10 μg/ml), and anti-mouse IFN-γ (10 μg/ml). Where indicated, human IL-2 (100 U/ml; NCRI) or anti-mouse IL-2 (10 μg/ml; Becton Dickinson) or mouse rsγc (4 μg/ml) was added to the cell culture.

**Cell Proliferation Assay**

Naïve CD8 responder T cells were labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester; Invitrogen) and incubated with human IL-2 (10 μg/ml; Peprotech) for 2 or 3 days.

**CD4+CD45RB+ T Cell Transfer-Induced Colitis**

CD4+ T cells were purified from WT, sγcTg, and γcTg mice with the EasySep mouse CD4+ T cell isolation kit (StemCell Technologies). CD4+CD45RB+ T cells were then electronically sorted by FACS. Rag2−/− mice were injected with 5 × 10^6 CD4+CD45RB+ T cells and monitored for 7 weeks for body weight change and clinical signs of colitis. Experimental mice showing clinical signs of severe colitis and a body weight loss of >20% were sacrificed according to the NCRI ACUC guidelines.

**Statistical Analysis**

Statistical differences were analyzed with Student’s two-tailed t test. Clinical score comparisons between two groups were performed with nonparametric tests.

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**Figure 7. γc Overexpression Impairs T Cell Development and Homeostasis**

(A) Surface γc chain staining on WT and sγcTg CD4+ T cells. Data are the summary of four independent experiments.

(B) γc binding affinities to IL-7Rα determined by surface plasmon resonance. Binding sensorgrams and Kd of monomeric (left) and dimeric (right) γc proteins to immobilized IL-7Rα proteins.

(C) Cell surface γc binding of IL2Rγk−/−EB6Cre thymocytes incubated with recombinant γc. IL2Rγk−/−EB6Cre thymocytes were incubated for 2 hr with 4 μg rsγc or Veh (BSA in PBS) and assessed with γc antibodies for surface γc staining. Histograms show surface γc staining on DP and TCRγδ thymocytes.

(D) Cell surface γc staining of IL2Rγk−/−EB6Cre and IL7raFl/flRgk−/−EB6Cre thymocytes incubated with rsγc. Surface γc staining was assessed as mean fluorescence intensity (MFI) in TCRγδ+ thymocytes. Data are summary of two independent experiments.

(E) Total thymocyte numbers in WT and sγcTg mice. Results are the summary of eight independent experiments.

(F) T cell apoptosis in WT and sγcTg LN T cells. Caspase-3 activity was determined in naïve CD4+ CD4+ or CD8+ LN T cells via CaspGLOW apoptosis kits. Histograms show caspase-3 activity in indicated cells (left). Bar graphs show summary of three independent experiments (right).

(G) Naïve and memory phenotype analysis of WT and sγcTg LN T cells. Contour plots show CD62L/CD44 and CD122/CD44 profiles and percentages of CD4+ and CD8+ LN T cells, respectively (left). Bar graph shows naïve CD4+ and CD8+ LN T cells numbers from WT and sγcTg mice (right). Data are the summary of ten independent experiments.

Error bars represent mean and SEM.
Mann-Whitney test. Curves of body weight change were fitted with nonlinear regression and group difference was examined by slope and intercept. p values of less than 0.05 were considered significant. **p < 0.01, ***p < 0.001, and NS (not significant) (Student’s two-tailed t-test).

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.04.020.

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