Transient Inhibition of Interleukin 4 Signaling by T Cell Receptor Ligation

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Abstract

Interleukin (IL)-4 and IL-12 together with T cell receptor (TCR) engagement are crucial for the differentiation of $CD4^+$ T cells into T helper (Th)2 or Th1 cells, respectively. Although IL-4 receptors (IL-4Rs) but not IL-12Rs are expressed on naive $CD4^+$ T cells, IL-4 has no apparent advantage over IL-12 in driving naive T cell differentiation when the cells are primed with both IL-4 and IL-12 in vitro. It was found that IL-4–induced phosphorylation of Janus kinases 1 and 3, IL-4Rα, signal transducer and activator of transcription 6, and insulin receptor substrate 2 was strikingly but transiently inhibited by TCR ligation both in conventional and TCR transgenic T cells. TCR engagement also blocked the expression of an IL-4–inducible gene. Signals induced by other cytokines, including IL-2, IL-6, and interferon α, but not by insulin-like growth factor 1, were also blocked by TCR engagement. The capacity of various inhibitors to reverse TCR-mediated inhibition of IL-4 signaling suggested that activation of the Ras–mitogen-activated protein kinase pathway and of the calcineurin pathway contribute to desensitizing IL-4R. IL-4 responsiveness returned at about the time (~12 h) that IL-12–mediated signaling was first observed. Thus, through different mechanisms, neither IL-4R nor IL-12R has any clear advantage in polarizing cells; rather, the availability of cytokine is probably the limiting factor in this process.

Key words: cytokine signal transduction • T cell activation and differentiation • cross-talk • calcineurin • mitogen-activated protein kinase

Introduction

IL-4 plays a central role in regulating the behavior of hematopoietic cells. It controls the specificity of Ig class switching in B cells and Th2 polarization in T cells (1). IL-4 can act as a costimulant of lymphocyte growth and can prevent apoptosis (2, 3). These functions of IL-4 are achieved through IL-4R–mediated signal transduction followed by specific gene expression (4).

IL-4 heterodimerizes the IL-4Rα chain and the IL-2Rγ chain (γc)[1]; γc is also shared by cytokine receptors for IL-2, IL-7, IL-9, and IL-15 (5). The nonreceptor tyrosine kinases Janus kinase (Jak)1 and Jak3, constitutively associated with IL-4Rα and γc, respectively (6–8), become activated after IL-4 binding to its receptor and phosphorylate some or all of the conserved tyrosines of IL-4Rα. These phosphotyrosines and the immediately surrounding amino acids within IL-4Rα provide docking sites for the phosphotyrosine binding (PTB) domain proteins insulin receptor substrate (IRS)-1/2 (9–11), Shc (12), and IL-4R interacting protein (FRIP) (13), and for signal transducer and activator of transcription (Stat)6 (4, 14).

Two major IL-4–signaling pathways are mediated by the PTB domain proteins and Stat6, respectively (15, 16). The former is mainly important for cell proliferation and cell survival (17). The genes that are regulated by IL-4 through IRS-2 and other PTB domain adapters are unknown, whereas Stat6 is directly responsible for inducing expression of a set of IL-4–inducible genes (18–21). Although the

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[1]*Abbreviations used in this paper:* γc, IL-2Rγ chain; CsA, cyclosporin A; ERK, extracellular signal–regulated kinase; IGF, insulin-like growth factor; IRS, insulin receptor substrate; Jak, Janus kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NFAT, nuclear factor of activated T cells; PCC, pigeon cytochrome c; PKC, protein kinase C; PTB, phosphotyrosine binding; SOCS, suppressor of cytokine signaling; Stat, signal transducer and activator of transcription.

role of IRS-2 and other PTB domain proteins during Th2 cell differentiation is unclear, Stat6 plays a critical role in driving this process (22–25).

Ligation of TCRs by antigen–MHC complexes on APCs triggers T cell activation (26). A second signal, mediated by the interaction of CD80/86 and CD28 or other molecules, is required for full activation (27). TCR ligation induces two major signal transduction pathways, the protein kinase C (PKC)–mitogen-activated protein kinase (MAPK) pathway and the calcium-dependent calcineurin-mediated pathway (28).

The differentiation of naive T cells in the Th1 or Th2 direction requires both TCR-mediated and cytokine (IL-12 or IL-4)-mediated signals. It is known that, in the presence of TCR-mediated signals, IL-4 activates Stat6, leading to the development of a Th2 response (22–25), whereas IL-12 activates Stat4 and causes development of a Th1 response (29–31). How the signals from the TCR and the cytokine receptors are integrated is not well understood.

IL-4–mediated activation of Stat6 may antagonize the capacity of IL-12 to drive Th1 differentiation (32, 33). One aspect of this inhibition involves downregulating IL-12Rβ2 expression (33, 34). Interestingly, IL-4 can induce strong signals in naive T cells whereas IL-12 fails to do so until its receptor is induced by TCR engagement for certain period of time. Despite this, IL-4 has no apparent advantage over IL-12 in driving T cell differentiation when both of them are present. To attempt to understand this, the early signaling events induced by IL-4 or IL-12 during T cell activation were studied. It was found that although IL-4 induced strong signals in naive T cells, this signal was strikingly but transiently inhibited by TCR engagement. Responsiveness returned after 12 h of TCR ligation at a time when the activated cells also respond to IL-12.

Materials and Methods

Mice and T Cell Purification. 8–12-wk-old BALB/c mice, B10.A mice, and TCR transgenic 5CC7/Rag2$^{-/-}$ mice were obtained from the Frederick Cancer Research and Development Center (Frederick, MD). Spleen T cells were purified through mouse T cell enrichment columns (R&D Systems). Naive CD4$^+$ T cells were prepared as follows: lymph node cells were depleted of CD8$^+$ cells, B220$^+$ cells, and IA^{d+} cells by negative selection using FITC-labeled anti-CD8, anti-B220, and anti-IA^d (BD PharMingen) plus anti–fluorescein-conjugated magnetic beads (PerSeptive Biosystems). The purified CD4$^+$ T cells were then centrifuged on a discontinuous 50, 60, and 70% Percoll gradient. Cells with a density of >60% were collected and used for direct stimulation or priming. The purity of CD4$^+$ T cells after purification is usually ~98%. Of these, 90% are CD44^{low} and CD62L^{high}. For the signaling experiments using soluble anti-CD3 or antigen, lymph node and spleen cells from BALB/c mice or lymph node cells from 5CC7/Rag2$^{-/-}$ mice and T cell–depleted spleen cells from B10.A mice were pooled and stimulated with soluble anti-CD3 or pigeon cytochrome C (PCC) peptide for 6 h. CD4$^+$ T cells were purified by positive selection (through an automatic MACS machine) using anti–mouse CD4 (L3T4) MicroBeads (Miltenyi Biotec). Purified CD4$^+$ T cells were then stimulated by IL-4 (1,000 U/ml) for 20 min and total cell lysates were made. T cell–depleted APCs were prepared by incubating spleen cells with anti-Thy1.2 and rabbit complement (Cedarlane Laboratories Limited) at 37°C for 45 min, and then irradiated at 3,000 rads.

Cell Culture and Priming. Purified T cells or naive CD4$^+$ T cells were treated with plate-bound anti-CD3 (2C11, 3 μg/ml; Harlan) and anti-CD28 (3 μg/ml; Harlan) in complete RPMI 1640 for various times. In some experiments, anti–IL-4 (11B11) or inhibitors were added to the culture during TCR engagement. At the end of TCR treatment, cells were washed and incubated with IL-4 (1,000 U/ml) for 20 min. MAPK kinase (MEK) inhibitor U0126 was purchased from Promega. PMA, ionomycin, PKC inhibitor (GF109203X), calcineurin inhibitors (cyclosporin A [CsA] and FK506), and protein synthesis inhibitor (cycloheximide) were purchased from Calbiochem. To study in vitro priming for cytokine expression, 10^6 naive CD4$^+$ BALB/c T cells were cocultured with 10^7 irradiated T cell–depleted spleen cells in the presence of anti-CD3 (3 μg/ml), anti-CD28 (3 μg/ml), IL-2 (10 U/ml), and different combinations of anti–IL-4 (11B11, 10 μg/mL), IL-4 (1,000 U/ml), anti–IL-12 (10 μg/ml), and IL-12 (10 ng/ml) for 20 h. Cells were then washed and stimulated with anti-CD3, anti-CD28, IL-2, IL-4, and IL-12 for an additional 3 d. After incubation in IL-2 for an additional 3 d, cells were restimulated with plate-bound anti-CD3 and anti-CD28 for 6 h; monensin (2 μM) was present during the last 4 h. Cells were harvested for intracellular staining for IFN-γ and IL-4 expression.

Immunoprecipitation and Western Blotting. Cytokine-treated cells (10^7 per sample) were harvested and lysed with 1 ml lysis buffer (50 mM Hepes, pH 7.0, 0.5% NP-40, 5 mM EDTA, 50 mM sodium chloride, 10 mM sodium pyrophosphate, and 50 mM sodium fluoride), freshly supplemented with inhibitors (1 mM sodium orthovanadate, 1 mM PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin), on ice for 20 min. After centrifugation at 12,000 rpm for 15 min, supernatants were incubated with 2–3 μg antibody for 1 h on ice and precipitated with protein G–agarose (Pierce Chemical Co.) at 4°C overnight on a rocker. The complexes were then washed three times with lysis buffer and eluted with 2× SDS-PAGE loading buffer. The eluted samples were separated in 8 or 12% premade acrylamide gels (Novex) and transferred onto Immobilon-P membranes (Millipore). Membranes were then probed with specific antibodies followed by horseradish peroxidase–labeled secondary antibodies (Amersham Pharmacia Biotech) and visualized with Supersignal West Dura extended duration substrate (Pierce Chemical Co.). For some experiments, the probed membranes were stripped with stripping buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8, and 100 mM 2-ME) at 60°C for 30 min and then reprobed with a second antibody. Anti-Jak1 and anti-Jak3 antiserum for immunoprecipitation were purchased from Upstate Biotechnology (cat. nos. 06-272 and 06-342). Anti-Jak1 for Western blot was purchased from Santa Cruz Biotechnology, Inc. (cat. no. sc-277) and monoclonal anti-Jak3 for Western blot was from Upstate Biotechnology (cat. no. 05-406). Anti-Stat6, anti-Stat4, anti-Stat3, and anti-Stat5b were purchased from R&D Systems. Anti–phosphorylated tyrosine 4G10 and anti–IRS-2 were from Upstate Biotechnology. Anti–IL-4Rα (M2) for immunoprecipitation was a gift of Immunex Corporation (Seattle, WA). Anti–IL-4Rα, anti–p-ERK, and anti–ERK-1 for Western blot were purchased from Santa Cruz Biotechnology, Inc.

Flow Cytometry Analysis and Intracellular Staining. For IL-4Rα chain or γc chain staining, lymph node cells from 5CC7/Rag2$^{-/-}$ mice were cocultured with T cell–depleted spleen cells in the presence of 0 or 1 μM PCC for 6 h, and stained with either anti–IL-4R (M1; Immunex), anti-γc (18361D; BD PharMingen), or

rat isotype control for 30 min in FACS® buffer (PBS with 3% FCS). The cells were then washed three times in FACS® buffer and incubated with biotin-labeled anti–rat IgG. After further washing, they were stained with streptavidin-PE and FITC-labeled anti-CD4. For intracellular staining, cells were restimulated with plate-bound anti-CD3 and anti-CD28 for 6 h in the presence of monensin (2 μM) during the last 4 h. Harvested samples were fixed with 4% formaldehyde, washed, and permeabilized in 0.5% saponin/1% BSA in PBS before staining with anti–IL-4–PE and anti–IFN-γ–FITC (BD PharMingen). Samples were analyzed on a FACScan™ (Becton Dickinson).

Semiquantitative Reverse Transcription PCR. Purified naive $CD4^+$ T cells were stimulated with IL-4 (1,000 U/ml) in the absence or presence of plate-bound anti-CD3/anti-CD28 for 4 h. Total RNA was isolated using TRIzol (GIBCO BRL) and first strand cDNAs were made using the SuperScript™ Preamplification System (GIBCO BRL) according to the manufacturer's protocol. Semiquantitative PCR was carried out in a GeneAmp PCR system 9700 (PerkinElmer) using Platinum PCR SuperMix (GIBCO BRL) by a serial dilution of the cDNA templates. The primers for IL-4Rα were AATGTGACCTACAAGGAACC and ATGGCCACCAAGGGACTGCG. The primers for β-actin were GATGACGATATCGCTGCGCTG and TACGACCAGAGGCATACAGG.

Results

TCR Ligation Transiently Downregulates IL-4 Signaling. Stat6 and IRS-2, two major IL-4R signaling molecules (4, 15, 16), are promptly tyrosine phosphorylated in response to IL-4 treatment of BALB/c splenic T (Fig. 1, A and B) and $CD4^+$ lymph node T (Fig. 1, C and D) cells. However, if the cells were stimulated with plate-bound anti-CD3 plus anti-CD28 for 6 h, phosphorylation of both Stat6 and IRS-2 is dramatically inhibited; no change in the amounts of Stat6 or IRS-2 was observed, although IRS-2 levels are at the border of detectability. Interestingly, IL-4–induced phosphorylation of Stat6 was no longer inhibited after 22 h of TCR engagement (Fig. 1 A); IRS-2 phosphorylation was barely detectable at this time. Inhibition of Stat6 phosphorylation was observed within 1 h of TCR ligation and reached its maximum at 4 h. IRS-2 phosphorylation appeared even more sensitive to inhibition by TCR ligation, as it was almost fully blocked within 1 h of TCR engagement (Fig. 1 B).

Inhibition was observed when concentrations of anti-CD3 as low as 0.08 μg/ml were used to coat culture plates, and was almost complete at a coating concentration of 0.4 μg/ml (Fig. 1 C). If anti-CD28 was omitted, inhibition was less dramatic although still substantial, particularly for IRS-2. Anti-CD28 by itself caused no inhibition of IL-4–mediated phosphorylation of Stat6 or IRS-2 (Fig. 1 D).

Inhibition of IL-4–mediated phosphorylation was observed with a broad range of IL-4 concentrations from 100 U/ml (~50 pg/ml) to 10,000 U/ml (~5 ng/ml). Such phosphorylation was inhibited even if IL-4 was continuously present during the period of TCR ligation (data not shown).

Phosphorylation of Stat6 and IRS-2 by IL-4 depends on proximal events, including the tyrosine phosphorylation of

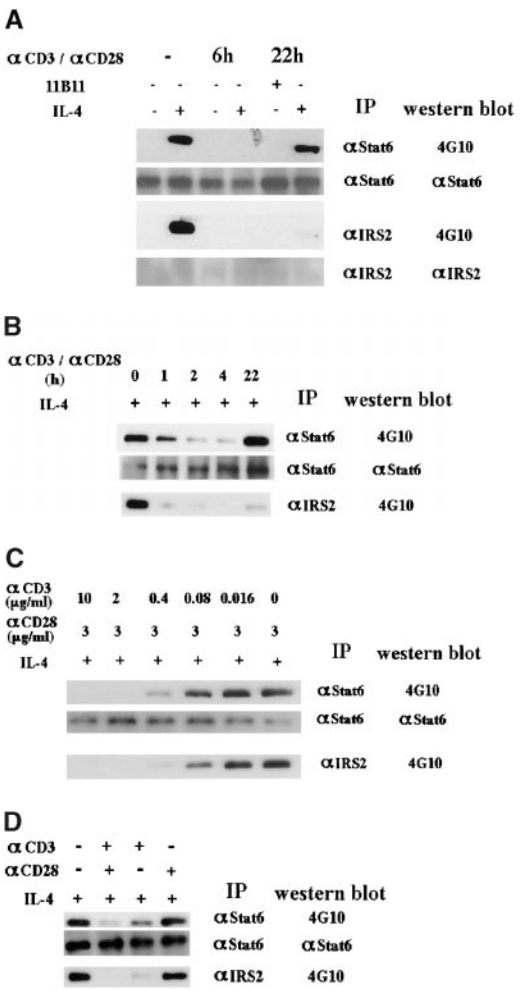

Figure 1. TCR ligation transiently downregulates IL-4–induced tyrosine phosphorylation of Stat6 and IRS-2 in naive T cells. BALB/c splenic T cells (A and B) and lymph node $CD4^+$ T cells (C and D) were either stimulated with immobilized anti-CD3 and/or anti-CD28 for various times, or not stimulated. Unless otherwise specified, cells were treated with 3 μg/ml anti-CD3 and/or 3 μg/ml anti-CD28 for 4 h. At the end of the culture period, some cell samples were challenged with IL-4 (1,000 U/ml) for 20 min. The cells were then lysed, and the lysates were immunoprecipitated with either anti-Stat6 or anti–IRS-2. SDS-PAGE was performed and immunoblots with the antiphosphotyrosine antibody 4G10 were carried out. Some filters were stripped and reprobed with anti-Stat6 antibody to check the amount of protein.

Jak1, Jak3, and IL-4Rα (4, 6–8). The IL-4–stimulated phosphorylation of these molecules was also inhibited by 6 h of TCR ligation (Fig. 2). As with Stat6, the amount of these molecules was not diminished by receptor engagement. In T cells that had been stimulated with plate-bound anti-CD3 and anti-CD28 for 22 h, the phosphorylation of Jak1, Jak3, and IL-4Rα was similar to or even greater than in resting T cells. Indeed, amounts of Jak3 and IL-4Rα, but not Jak1, were enhanced after 22 h of TCR ligation. Thus, TCR ligation inhibited essentially all early IL-4–mediated signaling events. The simplest explanation would be that the TCR signal inhibited the capacity of the Jak1 and/or Jak3 to respond to IL-4–mediated heterodimerization of

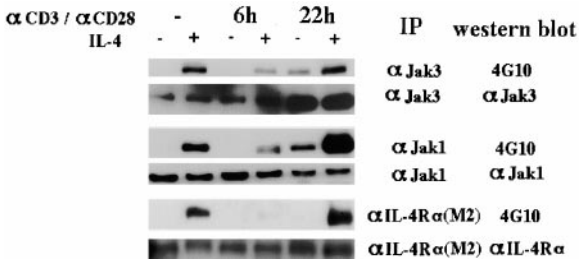

Figure 2. IL-4–induced tyrosine phosphorylation of Jak3, Jak1, and IL-4Rα is also transiently affected by TCR ligation. Lymph node $CD4^+$ T cells were stimulated by plate-bound anti-CD3/anti-CD28 for 0, 6, or 22 h in the presence of anti–IL-4 (11B11). They were then washed and challenged with IL-4 (1,000 U/ml) for 10 min. Lysates were immunoprecipitated and immunoblotted, and the blots were then stripped and reprobed with anti-Jak3, Jak1, and IL-4Rα.

IL-4Rα and γc. Mimicking TCR ligation with PMA plus ionomycin had the same or an even stronger effect on IL-4 signaling; this effect was also transient (data not shown).

TCR Ligation Also Inhibits Signaling Events Induced by Other Cytokines but Not by Insulin-like Growth Factor 1. It has been reported that in primed T cells, TCR engagement inhibits IL-2–mediated phosphorylation of Stat5 (35). To determine if that was true for resting T cells and if TCR ligation blocked signaling by other cytokines, we tested responses to IL-2, IL-6, and IFN-α in freshly prepared $CD4^+$ T cells from BALB/c lymph nodes. 4 h of TCR ligation strikingly inhibited phosphorylation of key substrates (Stat5b, Stat3, and Jak1, respectively) in response to each of these cytokines; responses had returned by 22 h of TCR stimulation (Fig. 3). By contrast, IRS-2 phosphorylation induced by insulin-like growth factor (IGF)-1 was not inhibited by TCR ligation. This is particularly striking in view of the exquisite sensitivity of the IL-4R's induction of IRS-2 phosphorylation to inhibition by TCR ligation.

The Induction of an IL-4–responsive Gene Is Also Inhibited by TCR Engagement. Although very few genes are known whose expression levels are rapidly upregulated by IL-4 in naive $CD4^+$ T cells, one that does show this property is the IL-4Rα chain itself (36, 37). The induction of IL-4Rα mRNA expression has been shown to depend on Stat6 activation. Upon IL-4 stimulation for 4 h, naive $CD4^+$ T cells express approximately fivefold more IL-4Rα chain mRNA than cells not stimulated by IL-4 (Fig. 4). By contrast, if the cells were stimulated with IL-4 in the presence of anti-CD3 plus anti-CD28 for 4 h, IL-4Rα chain mRNA upregulation was completely inhibited, showing that TCR-mediated inhibition of IL-4 signaling is functional.

In other experiments, we showed that the TCR ligation–mediated inhibition of IL-4Rα mRNA upregulation was only transient: after TCR ligation for 20 h, IL-4 regained the ability to induce IL-4Rα chain mRNA (data not shown).

Soluble Anti-CD3 or Antigen Also Inhibits IL-4–mediated Signaling. To determine whether more physiologic modes of TCR engagement also resulted in transient inhibitions of IL-4R–mediated signaling, we tested the effect of soluble anti-CD3 or of antigen in the presence of APCs.

Lymph node cells from BALB/c mice were stimulated with or without soluble anti-CD3 plus anti-CD28 for 6 h, and BALB/c spleen cells were used as APCs. Cultured $CD4^+$ T cells were purified by positive selection on a MACS (Miltenyi Biotec). The purified T cells were then stimulated with IL-4 for 20 min. IRS-2 and Stat6 phosphorylation was inhibited, and Stat6 expression levels were unaffected (Fig. 5 A).

Lymph node cells from 5CC7/$Rag2^{-/-}$ mice were stimulated with PCC peptide in the presence of spleen cells from B10.A mice as APCs. IRS-2 and Stat6 phosphorylation in response to IL-4 was inhibited by stimulation with 1 or 100 μM peptide for a period of 6 h (Fig. 5 B). Although 100 μM PCC may have modestly diminished Stat6 expression, 1 μM had no effect. The regulation of cell surface markers CD25, CD69, and CD62L by PCC stimulation was also checked. Both CD25 and CD69 were upregulated, whereas CD62L was downregulated equally by 1 or 100 μM PCC stimulation for 6 h as expected, indicating normal cell activation (data not shown). Jak3 and IL-4Rα chain phosphorylation were also checked in these two experiments. Both were inhibited (data not shown), suggesting that TCR engagement inhibits IL-4 signaling through the same mecha-

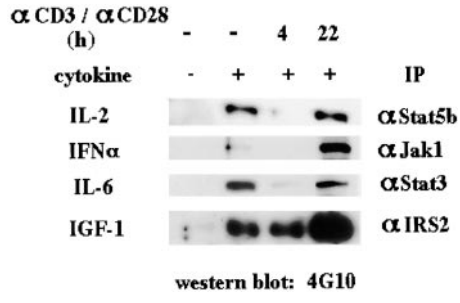

Figure 3. TCR ligation affects signaling by IL-2, IL-6, and IFN-α but not IGF-1. BALB/c lymph node $CD4^+$ T cells were treated with immobilized anti-CD3/anti-CD28 for 0, 4, or 22 h. They were then challenged with IL-2 (500 U/ml), IFN-α (10 ng/ml), IL-6 (10 ng/ml), or IGF-1 (100 ng/ml) for 20 min. Lysates were immunoprecipitated and immunoblotted.

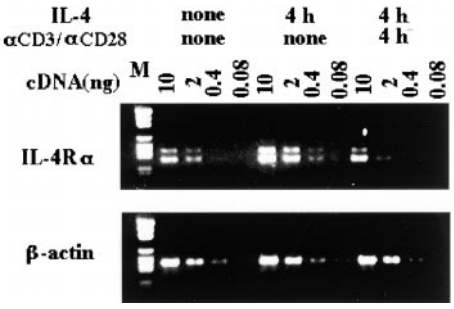

Figure 4. IL-4 upregulation of IL-4Rα mRNA is blocked by TCR ligation. BALB/c lymph node $CD4^+$ T cells were treated with or without IL-4 in the presence or absence of anti-CD3/anti-CD28 for 4 h. At the end of the culture period, total RNA was prepared and reverse transcription PCR for IL-4Rα and β-actin was performed on dilutions of cDNA. M, marker lane.

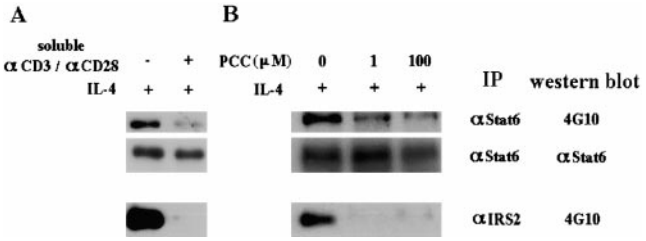

Figure 5. Both soluble anti-CD3/anti-CD28 and antigen treatment of T cells inhibit IL-4 signaling. BALB/c lymph node cells and spleen cells (A) or 5CC7/Rag2$^{-/-}$ lymph node cells and T cell–depleted spleen cells from B10.A mice (B) were pooled. Cultures were stimulated with soluble anti-CD3 (3 μg/ml) plus anti-CD28 (3 μg/ml) (A) or PCC peptide (1 or 100 μM; B) for 6 h. CD4$^+$ T cells were purified by positive selection by an automatic MACS machine. Purified CD4$^+$ T cells were then stimulated with IL-4 (1,000 U/ml) for 20 min and total cell lysates were prepared. Lysates were immunoprecipitated and immunoblotted.

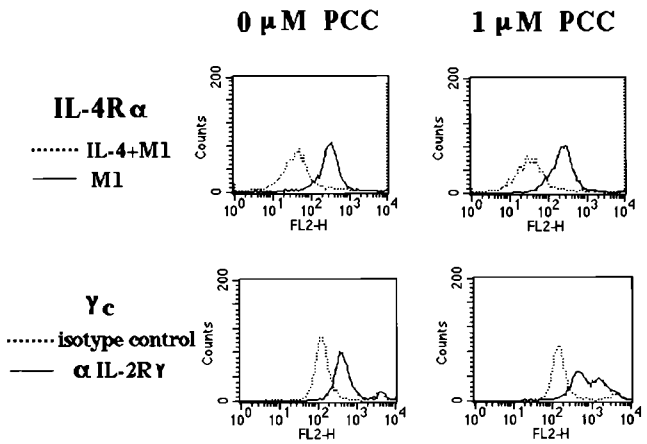

Figure 6. IL-4Rα is little affected and γc is upregulated by TCR ligation. Lymph node cells from 5CC7/Rag2$^{-/-}$ mice were cocultured with T cell–depleted spleen cells from B10.A mice in the presence or absence of 1 μM PCC for 6 h. Cells were then preincubated with nothing or IL-4 (1 μg/ml) for 30 min on ice. They were then incubated with anti–IL-4R (M1) (top panels) or with anti–IL-2Rγ using a rat Ig of the same isotype as a control (bottom panels). This was followed by biotin-labeled anti–rat IgG and streptavidin-PE. FITC-labeled anti-CD4 was added in the last step and used to gate for CD4$^+$ cells.

nism whether plate-bound anti-CD3 or a peptide–MHC complex on a living cell was used for stimulation. These results imply that inhibition of IL-4 signaling by TCR engagement is a feature of physiologic T cell activation.

Mechanism of Inhibition of IL-4 Signaling by TCR Ligation. Although IL-4Rα expression was little affected by TCR ligation as judged by Western blot experiments, it is still possible that IL-4R expression on the cell surface was diminished. To exclude this possibility, we measured IL-4R surface expression on CD4$^+$ T cells by flow cytometry. As the level of IL-4Rα expression on naive CD4$^+$ T cells is low (~400 receptors/cell [38]), we used a three-step staining method for detection. Because of the potential of increasing the staining background when anti-CD3 was used, we measured IL-4Rα and γc chain expression on naive 5CC7/Rag2$^{-/-}$ cells that had been incubated with T cell–depleted APCs from B10.A mice but with or without PCC peptide for 6 h. Neither IL-4Rα nor γc (Fig. 6) expression was significantly diminished by PCC stimulation. Indeed, a subset of cells showed increased surface expression of γc as a result of stimulation with PCC. These results indicate that the inhibitory effects of TCR ligation are not mediated by downregulating the expression of IL-4R subunits on the cell surface.

As IFN-γ is known to antagonize IL-4 effects (39) and, in some systems, to block IL-4–mediated signaling as a result of suppressor of cytokine signaling (SOCS)1 induction (40), we tested supernatants of lymph node CD4 T cells stimulated for 6 h with plate-bound anti-CD3 plus anti-CD28 for inhibitory activity. Such supernatants failed to inhibit IL-4–mediated phosphorylation of either Stat6 or IRS-2 (data no shown). In addition, when freshly purified naive CD4$^+$ T cells were exposed to recombinant IFN-γ directly or after 6 h of TCR engagement, we failed to detect Stat1 phosphorylation (data not shown). When T cells from IFN-γ$^{-/-}$ mice were studied, the inhibition of IL-4 signaling by TCR ligation was still observed (data not shown). Thus, it is unlikely that IFN-γ plays a role in inhibiting IL-4 signaling in T cells stimulated through their TCR for short periods of time.

To determine whether inhibition of IL-4–mediated signals by TCR engagement required new protein synthesis, cycloheximide was added to cultures during TCR engagement. Without new protein synthesis, IRS-2 phosphorylation was still inhibited as a result of TCR ligation, and Stat6 phosphorylation was only partially rescued (Fig. 7 A). However, the interpretation of this experiment is made difficult by the rather striking effects of cycloheximide treatment on the level of IL-4–mediated phosphorylation of IRS-2 in T cells that had not been treated with anti-CD3/anti-CD28.

TCR ligation may induce some inhibitory factors that enhance the inhibition of Stat6 phosphorylation. The obvious candidates for such a role are members of the SOCS family (41–43). However, when SOCS1, SOCS3, and cytokine-inducible SH2 protein 1 (CIS1) mRNA expression was checked after 4 h of TCR ligation, there was no significant change in the expression of these molecules (data not shown). Our results indicate that at least some of the inhibitory effects of TCR ligation on IL-4 signaling are independent of new protein synthesis.

It has been shown that activation of MEK and extracellular signal–regulated kinase (ERK) blocks IL-6–induced phosphorylation of Stat3, independent of new protein synthesis and without altering IL-6R expression (44). We studied the role of the PKC-MAPK pathway triggered by TCR activation on IL-4–mediated signaling by the addition of a specific MEK inhibitor. The addition of U0126 (Promega), a potent MEK inhibitor, during the period of TCR ligation completely rescued the capacity of the cells to phosphorylate Stat6 and IRS-2 in response to IL-4 (Fig. 7 B). The PKC inhibitor GF109203X (45) partially restored both IRS-2 and Stat6 phosphorylation in response to IL-4. This strongly suggests that the induction of the

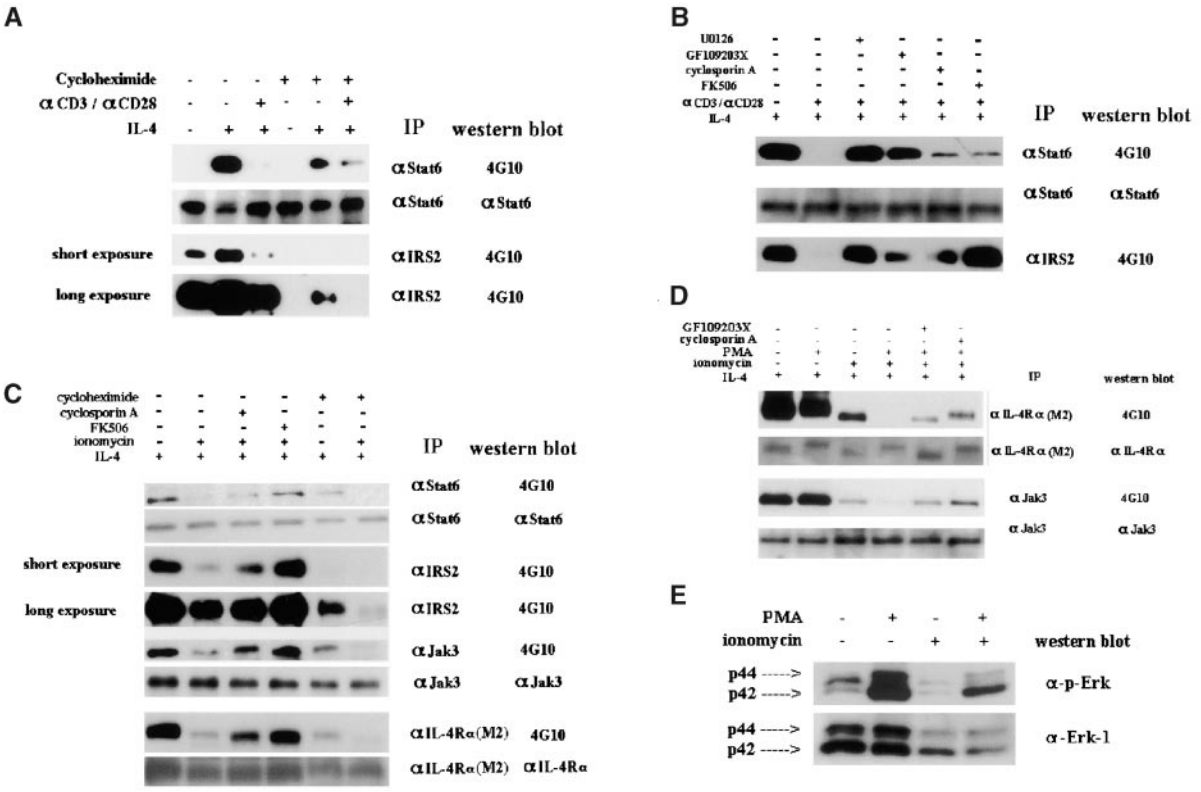

Figure 7. The mechanism of desensitizing IL-4R by TCR engagement. Lymph node $CD4^+$ T cells from BALB/c mice were pretreated with or without cycloheximide (10 μg /ml), the MEK inhibitor U0126 (100 μM), the PKC inhibitor GF109203X (1 μM), or the calcineurin inhibitors CsA (500 ng/ml) and FK506 (0.1 μM) for 20 min. The cells were treated with or without anti-CD3/anti-CD28 (A and B) or with or without PMA (10 ng/ml) and/or ionomycin (1 μM; C, D, and E) for 4 h. They were then challenged with or without IL-4 for 20 min. Lysates were immunoprecipitated and immunoblotted (A–D). Total lysates were loaded onto SDS-PAGE and immunoblotted with anti–p-ERK. The blot was stripped and reprobed with anti–ERK-1 (E).

PKC-MAPK pathway in response to TCR ligation is involved in inhibition of IL-4 signaling.

To determine whether the calcineurin-mediated pathway contributes to inhibition, two calcineurin inhibitors, CsA and FK506, were used. Interestingly, these inhibitors rescued IRS-2 phosphorylation but had limited effects on Stat6 phosphorylation (Fig. 7 B).

As PMA and the calcium ionophore, ionomycin, can mimic to some extent the activation of the PKC-MAPK and calcineurin pathways elicited by TCR ligation, we tested the ability of these agents, individually or together, to inhibit IL-4 signaling. Ionomycin caused striking but incomplete inhibition of IL-4–induced phosphorylation of IL-4Rα, Jak3, Stat6, and IRS-2 (Fig. 7 C). FK506 and CsA restored IL-4–mediated phosphorylation in ionomycin-treated cells. Ionomycin-mediated inhibition of IL-4 signaling was not reversed by cycloheximide, suggesting that calcineurin's effect may not require nuclear factor of activated T cells (NFAT)-driven transcription and protein synthesis.

PMA by itself had only a modest effect on IL-4Rα phosphorylation, and none on Jak3 phosphorylation (Fig. 7 D). However, adding PMA to ionomycin resulted in complete inhibition of both IL-4Rα and Jak3 phosphorylation. The PKC inhibitor reversed the capacity of PMA to enhance the inhibitory activity of ionomycin. These results suggest that both the PKC-MAPK and the calcineurin pathways are important for desensitizing the IL-4R.

PMA dramatically activated MAPK (Fig. 7 E), as shown by the appearance of phosphorylated ERK-1 and ERK-2. As PMA alone had little effect on IL-4 signaling, this suggests that activation of the MAPK pathway by itself is not sufficient to desensitize IL-4R. Ionomycin alone was able to inhibit IL-4 signaling but did not activate MAPK, and this inhibition was not rescued by the MEK inhibitor (data not shown), suggesting that the calcineurin pathway may play a more important role in desensitizing IL-4R when sustained high intracellular calcium concentrations are achieved. However, the striking reversal by the MEK inhibitor of IL-4R desensitization induced in response to TCR ligation implies that the magnitude of the calcineurin effect induced by the TCR is insufficient for independent inhibition. That is, with TCR ligation, neither pathway alone is capable of inhibiting IL-4 signaling; both are required for full suppression.

As IL-4 signaling was only transiently inhibited by TCR engagement and MAPK plays an important role in this inhibition, we measured the degree of MAPK activity at different times after TCR engagement by immunoblotting with anti–phospho-MAPK. Phospho-MAPK was detect-

able at 1 h after TCR ligation, peaked at 4 h, and decreased at 20 h (Fig. 8 A). Furthermore, as the cells had regained the ability to respond to cytokines at 20 h, part of the MAPK activity seen at that time may be elicited by cytokines rather than TCR engagement. Thus, MAPK activity correlates temporally with TCR-mediated inhibition of IL-4 signaling.

Although the return of IL-4 signaling at 20 h might be explained by a diminution in the magnitude of TCR signaling, the cells may have changed their responsiveness to the inhibitory signal at that time. To explore this possibility, cells that had been activated by TCR ligation for 20 h were exposed to PMA plus ionomycin, a treatment that inhibits IL-4 signaling in naive T cells independent of TCR. These 20-h-activated T cells, unlike naive T cells, did not show inhibition of IL-4–induced Stat6 phosphorylation in response to PMA plus ionomycin (Fig. 8 B). These results indicate that the IL-4R became resistant to inhibition after 20 h of TCR engagement. Although the mechanism underlying resistance is unclear, the upregulation of IL-4R, Jak3, and Stat6 expression (Figs. 1, 2, and 8 B) may contribute to this resistance.

Possible Biological Importance. As functional IL-4R but not IL-12R is expressed on naive CD4$^+$ T cells (33, 34, 36–38), one might anticipate that polarization to the Th2 pathway would be favored when naive CD4 cells are primed in the presence of both IL-4 and IL-12. However, as TCR ligation transiently desensitizes IL-4R, this might serve to redress the balance in priming for a cytokine-producing phenotype. We wished to determine the dynamics of acquisition of competence to respond to IL-12 on the part of naive CD4 T cells and of their reestablishment of responsiveness to IL-4 in T cells that had been stimulated through their TCR. Naive CD4$^+$ T cells were primed with anti-CD3 plus anti-CD28 in the presence of both IL-4 and IL-12 for various times, and Stat6 and Stat4 phosphorylation was checked. Although Stat6 phosphorylation was induced by IL-4 in naive CD4$^+$ T cells, TCR ligation resulted in its inhibition; Stat6 phosphorylation did not return to normal until 12 h after initiation of TCR ligation (Fig. 9). By contrast, IL-12 did not induce Stat4 phosphorylation in resting, naive T cells; however, by 8 h of T cell ligation, Stat4 phosphorylation was induced by IL-12, and the degree of IL-12–mediated Stat4 phosphorylation did not further increase over times up to 20 h. Thus, the transient inhibition of IL-4 signaling as a result of TCR ligation may have ablated any potential advantage IL-4 might have, and gave IL-12 a greater opportunity to drive naive CD4$^+$ T cells to polarize to Th1 cells.

For this observation to be of any significance in the polarization process, it would be important that the early cytokine environment have a substantial role to play in the decision of CD4 T cells to differentiate into Th1 or Th2 cells. To test this, naive CD4$^+$ T cells were primed in the presence of IL-4 or anti–IL-4 and IL-12 or anti–IL-12 during the initial 20 h of culture; all of the cells were then stimulated in the presence of IL-4 and IL-12 for an additional 3 d. After another 3 d in IL-2 medium, the cells were stimulated with immobilized anti-CD3 plus anti-CD28, and the proportion of IL-4 and of IFN-γ–producing cells was measured (Fig. 10). Priming for the initial 20 h in the presence of IL-4 and anti–IL-12 suppresses priming for IFN-γ compared with priming in the presence of anti–IL-4 and anti–IL-12, whereas priming in the presence of IL-12 and anti–IL-4 enhances priming for IFN-γ compared with priming with anti–IL-4 and anti–IL-12. Cells that were primed with both IL-4 and IL-12 were similar to cells primed with anti–IL-4 and anti–IL-12. In a sense, it could be said that IL-12 strikingly reverses the inhibitory effect of IL-4 on early priming for IFN-γ production. This striking effect of transient exposure to different combinations of IL-4 and IL-12 in the first 20 h of culture is even

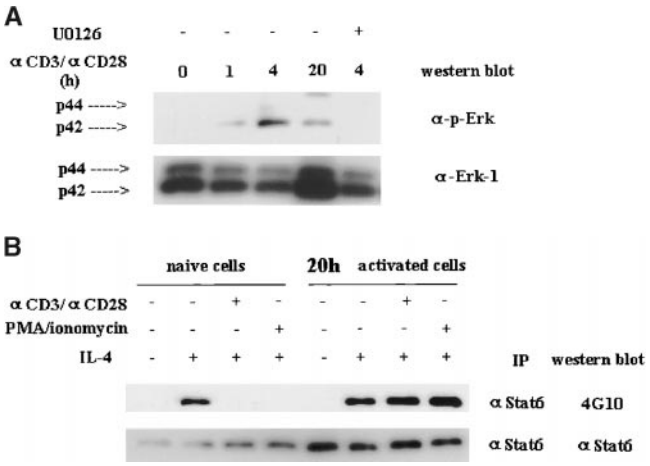

Figure 8. Possible mechanism of IL-4 signaling return. (A) A time course of MAPK activity induced by TCR ligation. Lymph node CD4$^+$ T cells from BALB/c mice were pretreated with or without the MEK inhibitor U0126 (100 μM) for 20 min. The cells were then treated with anti-CD3/anti-CD28 for 0, 1, 4, and 20 h. Total lysates were made and loaded to SDS-PAGE. Immunoblots with anti–p-ERK was carried out. The blot was then stripped and reprobed with anti–ERK-1. (B) Lymph node naive CD4$^+$ T cells were activated by anti-CD3/anti-CD28 in the presence of 11B11 for 20 h, and the cells were then washed and stimulated with fresh anti-CD3/anti-CD28 or PMA plus ionomycin for an additional 4 h. They were then challenged with or without IL-4 for 20 min. The freshly isolated naive CD4$^+$ T cells were treated in the same way as a control. Lysates were immunoprecipitated and immunoblotted. The blot was stripped and reprobed with anti-Stat6.

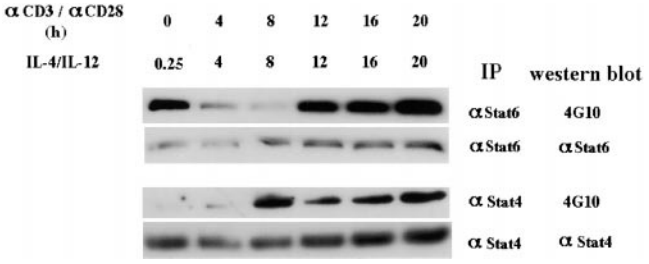

Figure 9. Dynamics of IL-4 signaling and IL-12 signaling in the early stage of T cell activation. CD4$^+$ lymph node T cells from BALB/c mice were treated with anti-CD3/anti-CD28 for 0, 4, 8, 12, 16, or 20 h in the presence of both IL-4 (1,000 U/ml) and IL-12 (10 ng/ml). Lysates were immunoprecipitated and immunoblotted.

more remarkable when one considers that the cells are insensitive to IL-4 for ~12 h of this period and to IL-12 for ~8 h. Thus, even a very brief exposure to a given cytokine environment early in the period of stimulation strikingly effects the polarization of cells despite the fact that they are cultured under identical conditions for the bulk of the priming period. These results strongly suggest that early cytokine-mediated signals received by CD4 T cells play an important role in their decision to polarize into the Th1 or Th2 phenotype. This emphasizes the potential importance of the TCR-mediated desensitization events that limit responsiveness of naive T cells to IL-4 and other type I and type II cytokines.

Discussion

In this study, it was shown that IL-4–mediated signals were strikingly but transiently inhibited by TCR engagement of naive T cells. This inhibition involved triggering of both the PKC-MAPK and calcineurin pathways. It has recently been reported by Ivashkiv and colleagues that the IL-2 signal was inhibited by TCR engagement in preactivated T cells and the inhibition was mediated by the PKC-MAPK pathway (35). We have also checked the IL-4 signal in activated cells and observed results quite similar to those described above for naive T cells (data not shown). In addition, we found that the IL-2 signal was also inhibited by TCR engagement in naive T cells. McMahon and colleagues have shown that sustained activation of the Raf–MEK-ERK pathway elicited cytokine unresponsiveness in T cells (46). These results are consistent in indicating that MAPK plays an important role in the cross-talk between TCR and cytokine signals. Our data further show that although MAPK activation is necessary, it is not sufficient to inhibit the IL-4 signal (Fig. 7, C and D).

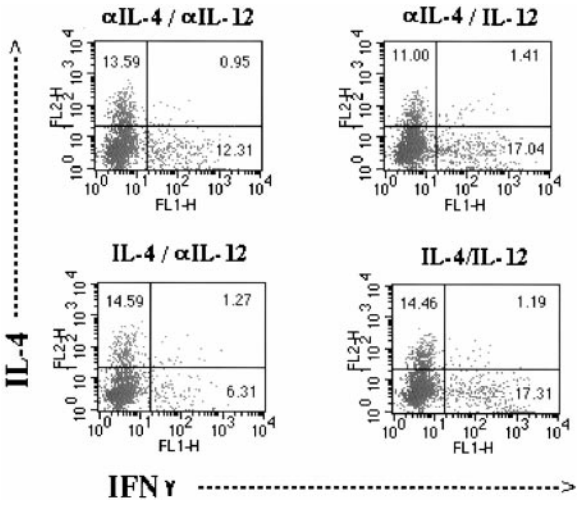

Figure 10. The cytokine environment early in the course of T cell activation affects Th1/Th2 polarization. 10^6 naive $CD4^+$ BALB/c T cells were cocultured with 10^7 irradiated T cell–depleted spleen cells in the presence of anti-CD3 (3 μg /ml), anti-CD28 (3 μg/ml), IL-2 (10 U/ml), and different combinations of anti–IL-4 (11B11, 10 μg/ml), IL-4 (1,000 U/ml), anti–IL-12 (10 μg/ml), and IL-12 (10 ng/ml) for 20 h. Cells were then washed and stimulated with anti-CD3, anti-CD28, IL-2, IL-4, and IL-12 for an additional 3 d. After incubation in IL-2 for an additional 3 d, cells were restimulated with plate-bound anti-CD3 and anti-CD28 for 6 h; monensin (2 μM) was present during the last 4 h. Cells were harvested and stained for intracellular IFN-γ and IL-4 expression. The data are representative of three independent experiments.

Our experiments reveal some new and potentially very critical points. One is that the cytokine signal returns after TCR ligation for a certain period of time. The mechanism through which sensitivity to IL-4 is regained is unclear. IL-4 itself did not contribute to the reacquisition of sensitivity, as the addition of anti–IL-4 antibody (11B11) during TCR engagement did not delay the return of sensitivity (Fig. 2). Downregulation of cell surface TCR and CD3 expression by TCR ligation could be an explanation for the return of sensitivity to IL-4 and other cytokines (35, 47). As MAPK plays a critical role in this inhibition, the return of the cytokine signal may simply be due to a decrease of MAPK activity (Fig. 8 A). Alternatively, TCR ligation may cause the expression of a new protein(s) that reverses the inhibition caused by the TCR signal, as the cells activated by TCR ligation for 20 h did not show the inhibition of IL-4 signal by PMA plus ionomycin treatment (Fig. 8 B). We observed that IL-4Rα, Jak3, and Stat6 expression levels were induced by TCR engagement; such increased expression may offer a partial explanation of returned sensitivity (Figs. 1, 2, and 8 B). Clarifying the mechanism of signal return will be very interesting and potentially very important. Indeed, if cells lose the capacity to overcome transient inhibition and thus the cytokine signal does not return, they may become anergic.

Another new finding in our experiments is that the calcineurin pathway can play a major role in inhibiting IL-4 signaling. Indeed, ionomycin treatment by itself inhibits phosphorylation of IL-4Rα, Jak3, Stat6, and IRS-2 phosphorylation. FK506 rescues this inhibition and the inhibition of IRS-2 phosphorylation mediated by TCR engagement. CsA also reverses inhibition, but it was less effective. Calcineurin plays a major role in regulating NFAT activity, which in turn is critical for the transcription of cytokine genes (48). Nonetheless, the finding that an inhibitor of protein synthesis failed to reverse the effects of ionomycin suggests that inhibition of calcineurin blocks IL-4 signaling through an NFAT-independent pathway.

IL-4Rα as well as γc are expressed on naive $CD4^+$ T cells, so such cells can immediately respond to IL-4 (36–38). By contrast, IL-12Rβ2 is not expressed on naive cells; such cells are unresponsive to IL-12. However, IL-12Rβ2 can be induced by TCR ligation (33, 34) and as these cells already express IL-12Rβ1, they gain responsiveness to IL-12. As IL-4 and IL-12 are the key cytokines in determining the commitment of naive T cells to the Th2 or Th1 phenotype, the differential expression and/or sensitivity of their receptors could be an important factor in determining the T cell phenotype polarization (1). This could be particularly important because IL-4 not only commits cells to become Th2 cells but also suppresses their capacity to develop into IFN-γ producers (1, 32).

Our data also demonstrated that the presence of IL-4 unopposed by IL-12 during the first 20 h of culture strikingly changed the ratio of IL-4 to IFN-γ producers that developed upon subsequent culture in the presence of IL-4 and IL-12 for 3 d (Fig. 10). This skewing in the Th1/Th2 ratio was conserved even after a second round of priming (data not shown). This indicates that the early signals received by naive T cells play a critical role in determining the cells' differentiation fate. Indeed, the IL-4R was desensitized for 12 h, so the cells received effective IL-4 signals for only 8 h during the first 20 h of T cell activation. As the cells do not respond to IL-12 during the first 8 h, conditions involving IL-4 and anti–IL-12 for the first 20 h of culture may mimic the situation that would pertain if the IL-4R had not been desensitized by TCR ligation, providing IL-4 with an 8-h advantage over IL-12. Thus, one potential reason for regulating the IL-4R to make it insensitive for a period of ~12 h after TCR ligation is to overcome the advantage IL-4 would have in the IL-4/IL-12 competition in view of the absence of functional IL-12R from resting T cells.

The opportunity to regulate the relative degree of responsiveness of the IL-4 and the IL-12R, one by controlling its desensitization and the other by controlling its induction, provides an additional mechanism through which the system can "tune" the degree of priming to the Th2 and Th1 phenotypes and thus the quality of T cell responses can be controlled. Such relatively fine quantitative adjustments may prove to have substantial real world significance. Almost certainly, in physiologic priming both inducers will be present to some degree, and the outcome will be determined both by their relative concentrations and by the relative degree of responsiveness of the cells to the two stimulants.

Balancing Th1/Th2 differentiation may not be the only or even the major consequence of transiently modulating cytokine signaling through TCR engagement, as the signaling of a broad range of cytokines is inhibited by TCR ligation. Indeed, as so many of the functions of cytokine receptors depend on costimulation through the TCR, analyzing the integration of TCR- and cytokine-mediated signal transduction will have substantial importance in efforts to develop and understand of the dynamics of cytokine-mediated processes.

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