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Ligand-Independent Down-Regulation of IFN- γ Receptor 1 Following TCR Engagement¹

Heidi Skrenta,^{2*} Yang Yang,[†] Sidney Pestka,^{*} and C. Garrison Fathman^{3†}

Activated T lymphocytes modulate the level of many molecules on their cell surface, including cytokine receptors. This regulation of cytokine receptor expression affects the ability of T cells to respond to cytokines and thus influences the outcome of an immune response. The receptor for IFN- γ , a proinflammatory cytokine, consists of two copies of a ligand binding chain (IFN- γ R1) as well as two copies of a second chain (IFN- γ R2) required for signal transduction. The expression of IFN- γ R2 is down-regulated at the mRNA level on CD4⁺ T cells when they differentiate into the Th1, but not the Th2, phenotype. This down-regulation has been demonstrated to depend on the ligand, IFN- γ , which is produced by Th1 but not Th2 T cells. The regulation of the cell-surface expression of IFN- γ receptors during primary T cell activation has not been reported. Naive and differentiated T lymphocytes express IFN- γ R1 at the mRNA level and as a cell-surface protein. In this study, we present evidence that cell-surface expression of IFN- γ R1 is transiently down-regulated on the surface of naive CD4⁺ T cells shortly after TCR engagement. Furthermore, this down-regulation is not mediated by the ligand, IFN- γ , but results from TCR engagement and can be inhibited by cyclosporin A. *The Journal of Immunology*, 2000, 164: 3506–3511.

Activated T cells both produce and respond to a number of cytokines that direct the adaptive immune response. Some of these cytokines, including TNF and IFN- γ , lead to a cell-mediated immune response whereas others, such as IL-4, IL-5, and IL-13, lead to humoral immunity. Still others, such as IL-2, act as growth factors for T cells. The ability of a cell to respond to a particular cytokine depends on the expression of the receptor for that cytokine on the cell surface. Cytokine receptors are composed of one or more membrane-spanning components (1). These components may be constitutively expressed or induced by activation signals and are subject to regulation by the cytokines themselves. Cytokines that lead to cell-mediated immunity and those that lead to humoral immunity can antagonize each other (2). Regulation of subunit expression occurs at both the mRNA and protein level. For example, the high-affinity receptor for IL-2 is composed of three chains, α , β , and γ . Although the mRNA for the γ -chain is constitutively expressed in resting T cells, the protein is only found on the surface of activated T cells. The mRNA and protein for the IL-2 receptor α - and β -chains are expressed only in activated cells (3). Thus, Ag-specific T cells can proliferate in response to stimulation, but nonspecific T cells in the area, lacking IL-2 receptors, cannot respond. Because the γ -chain of the IL-2 receptor is also a component of the IL-4, IL-7, IL-9, and IL-15 receptors, resting T cells are also unresponsive to these cytokines.

The ligand binding chain of the IL-4 receptor is up-regulated on the surface of T cells in response to TCR stimulation, but the mRNA levels are not increased. However, activation of T cells in the presence of IL-4 increases receptor expression at both the protein and mRNA levels (4). The β 1 and β 2 subunits of the IL-12 receptor are also up regulated on the surface of T cells after activation. The up-regulation of IL-12R β 2 is enhanced by IFN- γ and diminished by IL-4 (5–8). In contrast to the receptor subunits for growth and differentiation promoting cytokines, including IL-2, IL-4, and IL-12, the ligand binding chain of the IFN- γ receptor is constitutively expressed and can be detected on resting T cells.

The receptor for IFN- γ is composed of two distinct membrane-spanning subunits (reviewed in Refs. 9 and 10). The IFN- γ R1 chain consists of an extracellular domain that binds the IFN- γ homodimer, and an intracellular domain that contains binding sites for the JAK1 intracellular protein tyrosine kinase and the STAT1 transcription factor. The IFN- γ R2 chain is not necessary for ligand binding, but does increase the affinity of the associated heterodimeric receptor complex. However, the IFN- γ R2 chain is required for signal transduction. Its intracellular domain contains a binding site for the JAK2 protein kinase. It has recently been reported that IFN- γ R2 is not expressed on Th1 cells, one of the major cell types producing IFN- γ (11–14), nor on human peripheral blood T cells after primary activation (15), thus rendering these cells unresponsive to this cytokine. Th2 cells express both chains of the IFN- γ receptor and do not grow in the presence of IFN- γ (16). In fact, high levels of expression of both receptor chains on human T lymphocytes can lead to apoptosis following ligand binding (17). Because naive T cells express both chains of the receptor, IFN- γ produced upon cellular activation could inhibit proliferation or induce cell death. To determine why naive T cells, following activation, were not inhibited or driven to apoptosis, we performed a kinetic analysis of IFN- γ receptor expression on activated naive murine T cells and found that IFN- γ R1, the ligand binding chain, was down-regulated on the surface of T cells in response to signaling through the TCR. Interestingly, unlike the down-regulation of IFN- γ R2 on Th1 cells, down-regulation of IFN- γ R1 was not mediated by ligand binding but instead depended upon signaling through the TCR, did not require costimulation

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through CD28, and was inhibited by cyclosporin A (CsA).⁴ Furthermore, the decrease in surface expression of the IFN- γ R1 protein was not accompanied by a decrease in mRNA levels.

Materials and Methods

Mice

BALB/c mice and IFN- $\gamma^{-/-}$ mice on the BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME). The 3A9 mice, expressing the TCR for a peptide derived from hen egg lysozyme (HEL_{46–61}) as a transgene, were obtained from Mark Davis (Stanford University). IFN- γ R2 $^{-/-}$ mice were recently constructed (18). Mice of either sex between 8 and 20 wk of age were used in this study.

Cell culture

Single-cell suspensions were prepared from murine lymph nodes and/or spleens. In some cases, CD4⁺ cells were purified by positive selection with an Ab conjugated to magnetic beads (Miltenyi Biotec, Auburn, CA). Cells were grown in 96-well plates at a concentration of 5×10^5 cells per well (for unpurified cells) or 1×10^5 cells per well (for purified CD4⁺ cells) in RPMI supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 1:100 nonessential amino acids, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, and 10% FBS at 37°C and 6% CO₂. Where indicated, cells were treated with IFN- γ (Genzyme, Cambridge, MA) or activated with soluble rat anti-mouse CD3 (PharMingen, San Diego, CA), soluble hamster anti-mouse CD3 (purified from 145-2C11 hybridomas), latex beads coated with anti-CD3 and/or anti-CD28 (PharMingen), PMA and ionomycin (Calbiochem, San Diego, CA), or, in the case of the 3A9 mice, the peptide HEL_{46–61} (obtained from Mark Davis). For experiments with inhibitors, cells were incubated for 30 min with the indicated amounts of CsA or PD 98059 (Calbiochem) before activation.

Measurement of cell-surface IFN- γ R1

At the indicated times, cells were harvested and double stained with a PE-labeled Ab to CD4 (Caltag, Burlingame, CA) and a biotinylated Ab to IFN- γ R1 (PharMingen) followed by FITC-labeled streptavidin (Caltag). The cells were analyzed on a FACS (FACScan; Becton Dickinson, San Diego, CA). Dead cells were gated out by staining with propidium iodide (Sigma, St. Louis, MO). Because the Ab to IFN- γ R1 cannot bind to the receptor subunit when IFN- γ is present, cells from wild-type mice were washed for 5 min on ice with dilute acid (0.05 M acetic acid, 0.15 M NaCl, pH 2.9) before staining with this Ab.

Quantitation of IFN- γ R1 message

Purified CD4⁺ cells were cultured in 96-well plates with or without latex beads coated with anti-CD3 and anti-CD28 for the indicated times. The cells were then harvested and total RNA was isolated from each sample using the RNeasy kit (Qiagen, Santa Clarita, CA). The message in 0.5 μ g RNA per sample was analyzed by RNase protection with the MCR-3 probe (PharMingen) by following the protocol supplied with the probe. Alternatively, the cells were collected and resuspended in Direct Protect lysis buffer (Ambion, Austin, TX), and the lysate from 5×10^5 cells per sample was analyzed by RNase protection as described above.

Proliferation assay

Unpurified lymphocytes and splenocytes or purified CD4⁺ cells were cultured in 96-well plates with the indicated treatment for ~48 h, at which time RPMI 1640 containing [³H]thymidine was added to each well (1 μ Ci/well, sp. act., 25 Ci/mmol). The cells were incubated another 18 h and then harvested on a Tomtec Mach 2-56 plate harvester (Orange, CT), and incorporated thymidine was measured on a Wallac 1205 Betaplate liquid scintillation counter (Urku, Finland).

Measurement of IL-2 production

Supernatants were collected at the indicated times from cultures of purified CD4⁺ lymphocytes, and IL-2 levels were assayed by a capture ELISA protocol as follows. Supernatants were incubated in 96-well plates coated with anti IL-2 (PharMingen) and detected with a biotin-conjugated IL-2 Ab (PharMingen) followed by streptavidin-europium (Wallac). Absorbance was measured on a Wallac Victor model 1420 multilabel counter, and units of IL-2 were calculated by comparison with a standard curve generated with recombinant murine IL-2 (Genzyme, Cambridge, MA).

MHC induction assay

Purified CD4⁺ cells from BALB/c IFN- $\gamma^{-/-}$ mice were rested or activated with anti-CD3/anti-CD28-coated beads for 24 h, after which time they were harvested and treated with the indicated amount of IFN- γ (Genzyme) for 10 min. The reaction was stopped by washing the cells with ice-cold PBS, and the cells were spun down, resuspended in RPMI 1640, and cultured in 96-well plates for 48 h. The cells were then harvested, stained with a FITC-conjugated MHC class I Ab (PharMingen), and analyzed on a FACScan.

Results and Discussion

Kinetic expression of IFN- γ R1 on the surface of activated CD4⁺ lymphocytes

To determine whether primary activation of naive T lymphocytes affected cell-surface expression of the IFN- γ receptor, T cells were isolated from lymph nodes and/or spleens of 3A9 mice and stimulated *in vitro* in the presence of APCs with either soluble anti-CD3, HEL peptide, or PMA plus ionomycin, and receptor expression was monitored by flow cytometry. IFN- γ R1 was down-regulated on the surface of CD4⁺ T cells within 5 h when cultured with unpurified lymphocytes and splenocytes, with the lowest levels detected at around 16 h (Fig. 1A). The down-modulation of IFN- γ R1 on activated cells was transient. By 48 h, surface levels of IFN- γ R1 returned to values similar to baseline levels in resting cells (Fig. 1A). To show that the down-regulation of surface IFN- γ R1 was specific, CD4 levels were monitored concurrently and did not diminish (Fig. 1B). Instead, the surface CD4 levels began to increase after 24 h of activation with either anti CD3 or the HEL peptide, as described previously (19). Signaling through the IFN- γ R2 chain was not required for the down-regulation of IFN- γ R1 as demonstrated by the fact that surface levels of IFN- γ R1 were diminished on activated CD4⁺ cells from mice lacking the IFN- γ R2 chain (Fig. 1C) as well as mice expressing IFN- γ R2 (Fig. 1A). These data demonstrated that the ligand binding chain of the receptor (IFN- γ R1) was down-regulated shortly after activation independently of the second chain (IFN- γ R2). Previous reports have suggested that the two receptor chains are not tightly preassociated in the membrane before ligand binding (20, 21). Moreover, the promoter regions of the two genes for the receptor chains are dissimilar, all of which supports the idea that the two chains of the IFN- γ receptor may be differentially regulated during T cell activation (10, 22, 23).

To determine whether the decrease in surface IFN- γ R1 protein was due to a corresponding decrease in message level, RNA from CD4⁺ cells was assayed by RNase protection as described in *Materials and Methods*. Three assays with either equal cell numbers or equal RNA amounts showed no significant difference in the IFN- γ R1 message level between resting and activated cells over 48 h (data not shown). This result indicated that the regulation of surface IFN- γ R1 is controlled at the protein level, perhaps by internalization or shedding of the receptor.

Ligand independence of surface IFN- γ R1 down-modulation

Activated T cells can produce IFN- γ , and the binding of this cytokine to its receptor triggers the internalization of the ligand-receptor complex in many cell types (24–28). Therefore, it was possible that the down-regulation of IFN- γ R1 observed on activated T cells was due to ligand-induced internalization. To address this question, T cells were isolated from the lymph nodes and spleens of mice lacking the gene for IFN- γ (IFN- $\gamma^{-/-}$). These cells were stimulated with soluble anti-CD3. After harvesting at the indicated times, T cell surface levels of IFN- γ R1 were measured by flow cytometry. IFN- γ R1 surface levels were decreased on activated T

⁴ Abbreviations used in this paper: CsA, cyclosporin A; HEL, hen egg lysozyme; MAPK, mitogen-activated protein kinase.

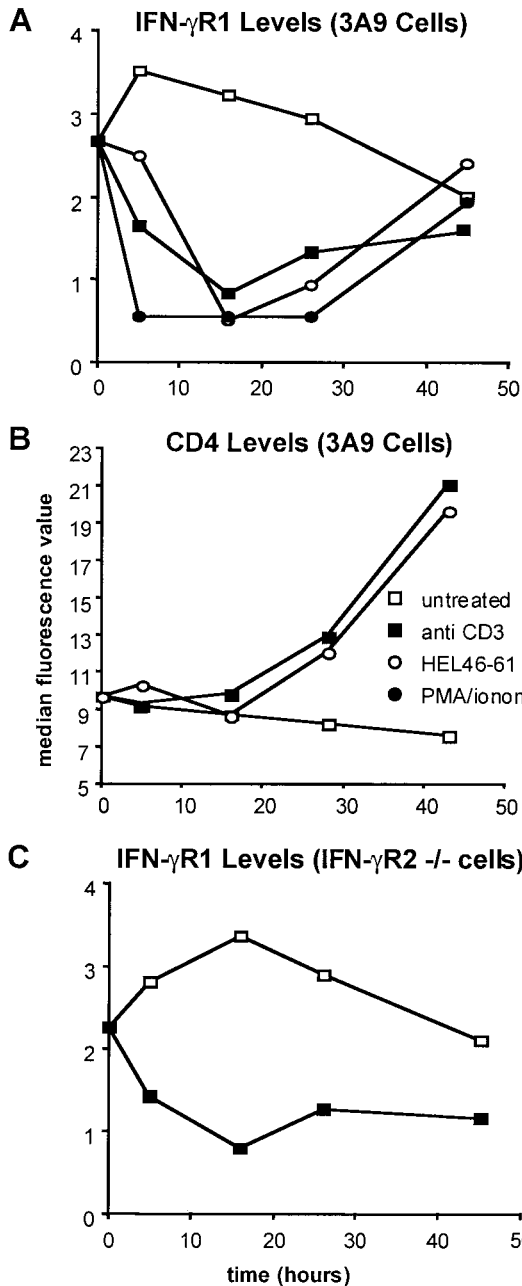


FIGURE 1. Kinetics of IFN- γ receptor expression on the surface of CD4⁺ T cells. Single-cell suspensions of lymphocytes and splenocytes from 3A9 mice (A) were cultured in RPMI 1640 as described in *Materials and Methods* with or without the addition of either 5 μ g/ml rat anti-mouse CD3, 0.1 μ M HEL₄₆₋₆₁, or 1 μ g/ml PMA plus 1 μ M ionomycin. At 0, 5, 16, 26, and 45 h, cells were harvested, washed with dilute acid to remove any IFN- γ , and stained with PE-labeled anti-CD4 plus biotinylated anti-IFN- γ R1 followed by streptavidin-FITC and analyzed by flow cytometry. Data are presented as graphs representing the median fluorescence value of IFN- γ R1 for the population of gated CD4⁺ cells for each particular time point and condition. Dead cells were gated out using propidium iodide. The median fluorescence value of CD4 is plotted over time (B) as a control. IFN- γ R1 levels were also measured on CD4⁺ cells from IFN- γ R2^{-/-} mice activated with 5 μ g/ml rat anti-mouse CD3 (C). These cells were stained as in A. Data are representative of at least three separate experiments.

cells from IFN- γ ^{-/-} mice as well as cells from wild-type mice (Fig. 2, A and B). Furthermore, T cells cultured in the presence of IFN- γ , without any additional stimuli, displayed no significant change in the surface expression of IFN- γ R1 when compared with

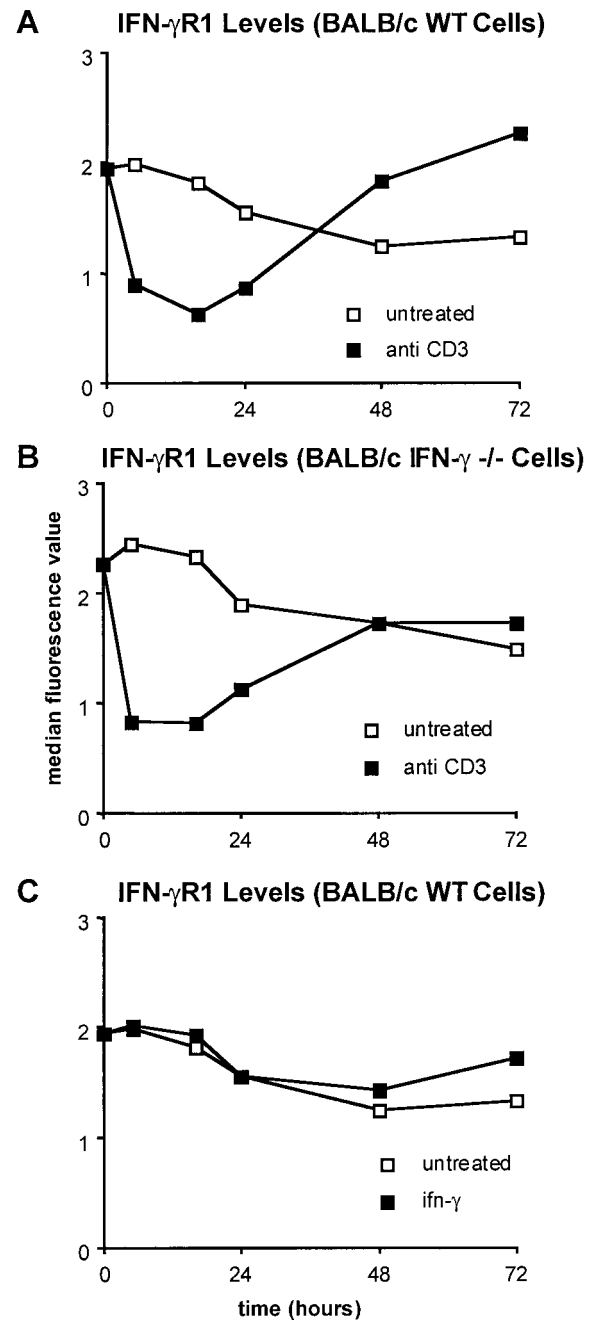


FIGURE 2. Down-regulation of IFN- γ R1 is not mediated by IFN- γ . Single-cell suspensions of lymphocytes and splenocytes from either BALB/c mice (A and C) or IFN- γ ^{-/-} mice on the BALB/c background (B) were cultured with media with or without 3.5 μ g/ml hamster anti-mouse CD3 (A and B) or 100 ng/ml IFN- γ (C) and then harvested, stained, and labeled for CD4 and IFN- γ R1 at 0, 5, 16, 24, 48, and 72 h as described in Fig. 1A. Data are representative of at least three different experiments.

untreated T cells (Fig. 2C). Additional evidence that IFN- γ signaling is not required for down-regulation of IFN- γ R1 is provided by the data with the mice lacking IFN- γ R2. Although these mice cannot signal in response to IFN- γ , the surface levels of the ligand binding chain of the receptor were decreased on their CD4⁺ cells after TCR-induced activation (Fig. 1B). Taken together, these results indicate that the down-regulation of IFN- γ R1 on the surface of activated T cells is not mediated by IFN- γ , either through binding or signaling.

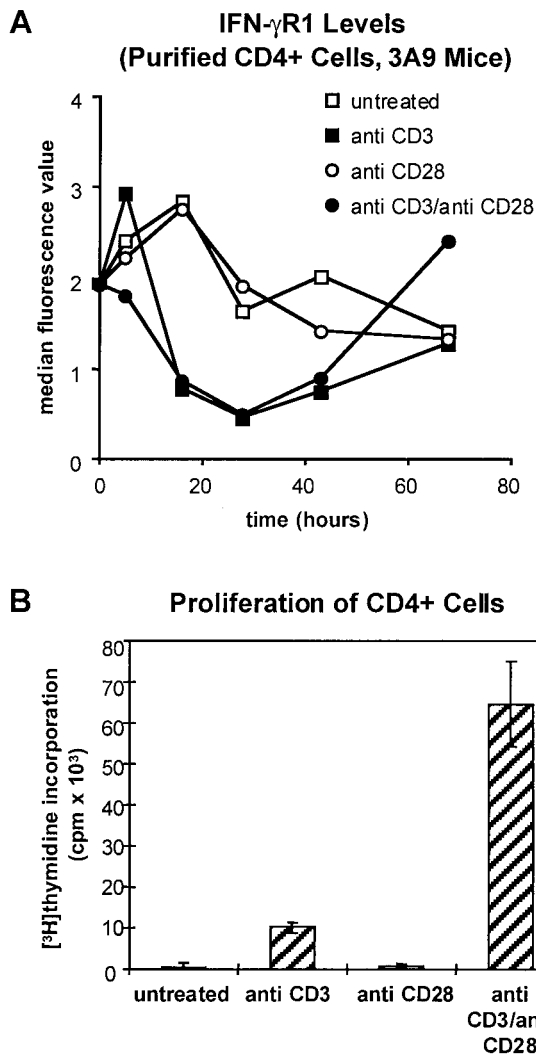


FIGURE 3. Signaling requirements for down-regulation of IFN- γ R1. CD4⁺ T cells were purified from a single-cell suspension of 3A9 lymphocytes using anti-CD4-coated magnetic beads. The cells were incubated with or without latex beads coated with Abs to either CD3, CD28, or both. Cells were stained for IFN- γ R1 and analyzed as described in Fig. 1A, and the median fluorescence values were plotted over time (A). Data are representative of three experiments. A proliferation assay was performed as described in *Materials and Methods* to confirm that only those cells receiving signals through CD3 and CD28 could proliferate (B). Each bar is an average of five replicate wells.

Extracellular signaling requirements for IFN- γ R1 down-regulation

Two signals are required for a T cell to become activated and proliferate. In addition to stimulation through the TCR by Ag or mAbs, the costimulatory molecule CD28 must be engaged by its ligand B7 (29, 30). In unpurified lymphocytes and splenocytes, APCs can provide the necessary costimulation. However, purified CD4⁺ T cells, in the absence of APC, require Abs to CD28 to generate the necessary second signal(s) for proliferation. To determine the extracellular signal requirements for the down-regulation of IFN- γ R1, CD4⁺ T cells were purified from murine lymph nodes and stimulated with latex beads coated with either anti-CD3, anti-CD28, or both Abs. Anti-CD3 alone (or in conjunction with anti-CD28) induced down-regulation of IFN- γ R1 in purified CD4⁺ cells, whereas anti-CD28 by itself had little or no effect (Fig. 3A). A proliferation assay was performed as a control to

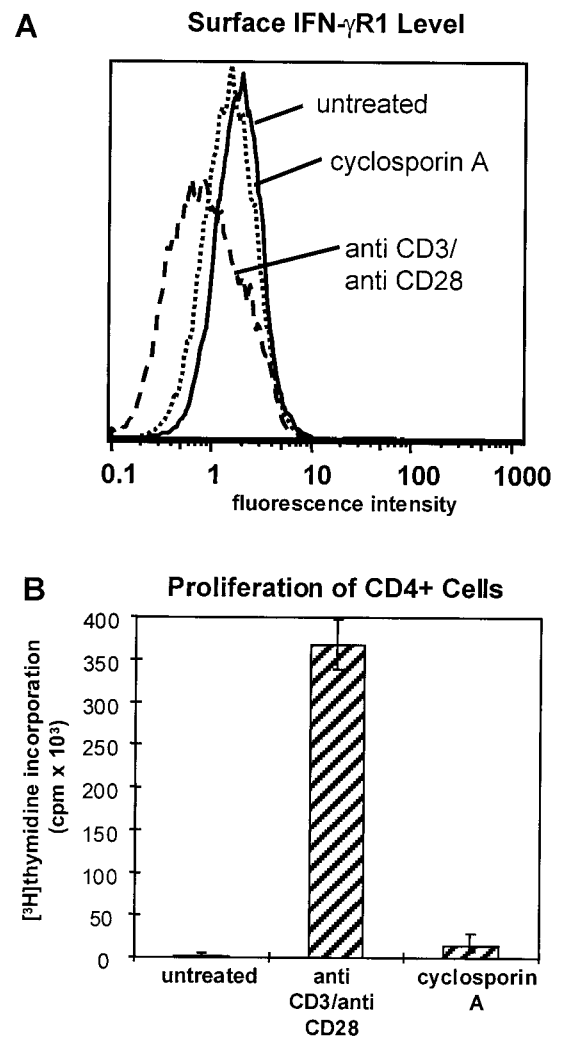


FIGURE 4. Surface IFN- γ R1 modulation is blocked by CsA. Purified CD4⁺ T cells from BALB/c IFN- γ ^{-/-} mice were incubated in media alone, activated with latex beads coated with anti-CD3 plus anti-CD28, or preincubated with 1 μ g/ml CsA, 30 min before addition of the beads. A shows the surface IFN- γ R1 levels on CD4⁺ cells 10 h after adding the anti-CD3/anti-CD28-coated beads, and B shows the corresponding proliferation assay. Each bar represents an average of six replicate wells.

ensure that the Ab-coated beads were functioning properly. The beads coated with both anti-CD3 and anti-CD28 induced significant T cell proliferation, whereas anti-CD3 alone caused only a minimal response and anti-CD28 alone had no effect (Fig. 3B). These results demonstrated that costimulation was not necessary for down-regulation of IFN- γ R1; instead, receptor modulation was dependent upon signaling through the TCR.

Intracellular signaling requirements for IFN- γ R1 down-regulation

The down-regulation of IFN- γ R1 was not observed until at least 4 h after TCR engagement and therefore may depend upon the transcription and/or synthesis of one or more new proteins. NF-AT is one important transcription factor induced by TCR signaling (31). AP-1, activated via a mitogen-activated protein kinase (MAPK) pathway, also mediates transcription in response to TCR signaling (32). To study the possible involvement of transcription in the down-modulation of IFN- γ R1, the drug CsA, which blocks the nuclear translocation of NF-ATp (33), and PD 98059, an inhibitor of the MAPK kinase Mek-1 (34), were added to cultures of

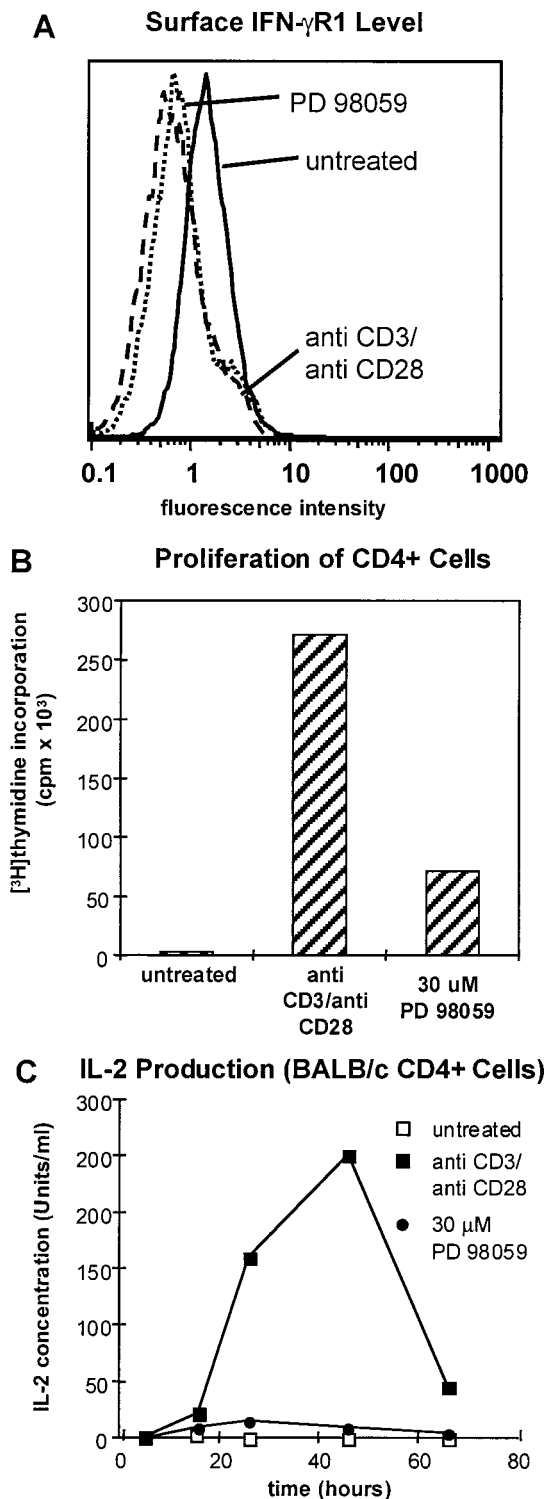


FIGURE 5. Surface IFN- γ receptor modulation is not inhibited by PD 98059. Purified CD4⁺ T cells from BALB/c mice were incubated in media alone, activated with latex beads coated with anti-CD3 plus anti-CD28, or preincubated with 30 μ M PD 98059, 30 min before addition of the beads. *A* shows the surface IFN- γ R1 levels on CD4⁺ cells 16 h after adding the anti-CD3/anti-CD28-coated beads, and *B* shows the corresponding proliferation assay. *C* shows a plot of IL-2 production over time, measured by ELISA as described in *Materials and Methods*.

CD4⁺ lymphocytes 30 min before stimulation with anti-CD3 and anti-CD28 and expression of IFN- γ R1 measured. CsA greatly diminished modulation of surface expression of IFN- γ R1 10 h after

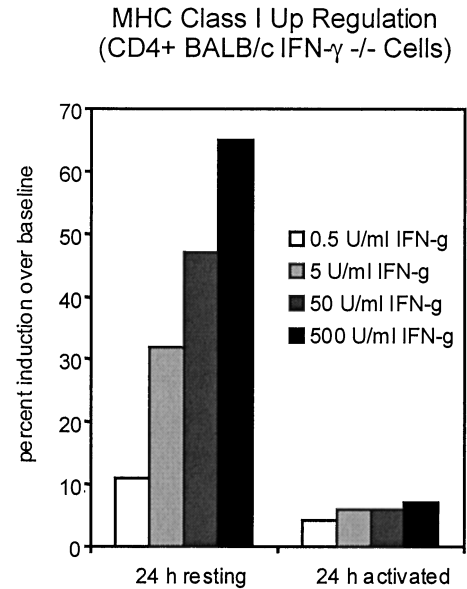


FIGURE 6. Recently activated T cells are less responsive to IFN- γ . Purified resting or activated CD4⁺ T cells were treated with IFN- γ , stained for MHC class I, and analyzed as described in *Materials and Methods*. BALB/c IFN- γ ^{-/-} mice were used in this experiment to ensure that there was no endogenous IFN- γ produced by the activated cells. The bars represent the percentage increase over the median fluorescence level in cells receiving no IFN- γ . Data are representative of three separate experiments in which baseline median fluorescence for MHC class I was around 5 in resting cells and 17 in activated cells.

activation, and strongly inhibited proliferation of the T cells (Fig. 4, *A* and *B*). In contrast, PD 98059 did not prevent anti-CD3-induced modulation of surface IFN- γ R1 (Fig. 5*A*). Proliferation of the T cells was inhibited by PD 98059, albeit not as effectively as by CsA (Figs. 4*B* and 5*B*). However, PD 98059 strongly inhibited the production of IL-2, suggesting functional blockage of AP-1 activity (Fig. 5*C*). These results suggested that the modulation of IFN- γ R1 may involve transcription of a gene regulated by NF-AT or that the signal for down-modulation lies along the pathway from TCR engagement to NF-AT translocation, and is not transduced through the MAPK pathway.

Recently activated T cells are less responsive to IFN- γ

IFN- γ mediates changes in the levels of >200 gene products via activation of the transcription factor STAT1, and among the most well characterized are those involved in Ag presentation (35). To determine whether the decrease in cell-surface IFN- γ R1 had any functional significance, the ability of IFN- γ to up-regulate MHC class I molecules on the surface of activated and resting T cells was compared at 24 h after stimulation. On resting CD4⁺ cells treated with 500 U/ml IFN- γ , surface expression of MHC class I molecules increased 65% over levels in cells receiving no IFN- γ . However, on activated cells treated with the same amount of IFN- γ , MHC class I surface expression increased only 7% over baseline levels, which is less than the 11% increase observed in resting cells treated with 0.5 U/ml IFN- γ (Fig. 6). Although these data showed a more robust unresponsiveness than one might expect from the level of reduction in surface levels of IFN- γ R1, they nevertheless suggest that the decrease in receptor number has biological relevance.

The activation of T cells up-regulates the expression of many cytokine receptors as described above. In contrast, Ag or mitogen activation of CD4⁺ T cells caused a rapid (within 4–6 h) down-

modulation of the cell-surface expression of IFN- γ R1. Although ligand binding-induced internalization of ligand-receptor complexes has long been characterized as a mechanism for surface receptor down-modulation (including IFN- γ R1 on many cell types, e.g., macrophages and fibroblasts), the down-modulation of IFN- γ R1 on activated CD4⁺ T cells was ligand independent. Thus, activation-induced, ligand-independent down-modulation of IFN- γ R1 on activated CD4⁺ T cells involves a new regulatory mechanism. Although the mechanism of this novel regulation remains to be determined, the transient down-regulation of IFN- γ R1 is significant and physiologically relevant. Because activated CD4⁺ T cells produce IFN- γ , which has inhibitory effects on cell proliferation (16) and may even cause apoptosis (17), down-regulation of the ligand binding chain of the IFN- γ receptor complex provides a mechanism to protect the activated T cells to permit a productive immune response. Down-regulation of the IFN- γ R2 chain, described in differentiated Th1 cells, may permanently shut down the response of these cells to IFN- γ . In addition, it would be counterproductive for activated T cells producing IFN- γ to bind IFN- γ in an autocrine fashion. Reducing surface expression of the ligand binding chain of the IFN- γ receptor during ligand secretion would allow the ligand to reach its proper target cells, including macrophages and other APCs.

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