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Regulation of IFN- γ Signaling Is Essential for the Cytotoxic Activity of CD8⁺ T Cells¹

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Abstract

Previous studies have demonstrated that, as naive murine CD4⁺ cells differentiate into Th1 cells, they lose expression of the second chain of IFN- γ R (IFN- γ R2). Hence, the IFN- γ -producing subset of Th cells is unresponsive to IFN- γ . Analysis of IFN- γ -producing CD8⁺ T cells demonstrates that, like Th1 cells, these cells do not express IFN- γ R2. To define the importance of IFN- γ signaling for the development of functional CD8⁺ T cells, mice either lacking IFN- γ R2 or overexpressing this protein were examined. While CD8⁺ T cell development and function appear normal in IFN- γ R2^{-/-} mice, CD8⁺ T cell function in IFN- γ R2 transgenic is altered. IFN- γ R2 transgenic CD8⁺ T cells are unable to lyse target cells in vitro. However, these cells produce Fas ligand, perforin, and granzyme B, the effector molecules required for killing. Interestingly, TG CD8⁺ T cells proliferate normally and produce cytokines, such as IFN- γ in response to antigenic stimulation. Therefore, although IFN- γ signaling is not required for the generation of normal cytotoxic T cells, constitutive IFN- γ signaling can selectively impair the cytotoxic function of CD8⁺ T cells.

Effector CD8⁺ T cells, also known as CTLs, are important mediators of immune responses against certain pathogens, transformed cells, and foreign organ grafts (1, 2). These T cells perform their functions by elaborating a number of distinct effector mechanisms. CD8⁺ T cells produce cytokines such as TNF- α and IFN- γ . TNF- α is a proinflammatory cytokine that functions to inhibit viral gene expression and replication and can initiate apoptotic signaling (3). IFN- γ has been shown to activate effector cells such as macrophages and neutrophils, regulate CD4⁺ Th cell differentiation, as well as metabolically suppress infected or transformed cells (4). CD8⁺ T cells can also directly attack and induce cytolysis of their

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target cells by two distinct pathways, both of which are activated in response to signaling through the TCR (5). One of these pathways involves the release of soluble cytotoxic factors such as the pore-forming molecule perforin and the granzymes, which are stored within cytoplasmic granules (6, 7). These molecules are released shortly following TCR triggering and lead to the perforation of the membrane and the activation of caspases in target cells, resulting in their eventual lysis. Following activation and de novo protein synthesis, CD8⁺ T cells also up-regulate the expression of Fas ligand (FasL)³ (CD95 ligand) on their cell surface that, through interaction with Fas (CD95) on the target cell, triggers the apoptotic pathway (3, 7). In addition to these two pathways, 24–48 h postactivation CD8⁺ T cells begin producing TNF- α , which can also lead to killing of target cells (8).

The differentiation of naive Th cells into mature effector cells is regulated by a number of factors, including the strength of the activating stimulus (peptide affinity, Ag dose, and level of costimulation), the route of immunization, and the cytokines present in their immediate microenvironment (9-11). Like Th cells, the activation and maturation of naive CD8⁺ T cells into CTLs require TCR signaling (7, 12). The quality of the TCR signal can also regulate the effector function of the CTL (13, 14). For example, it has been shown that different epitopes of a particular viral Ag can lead to functionally heterogeneous effector CD8⁺ T cells (15, 16).

Extensive work has demonstrated that cytokines present at the time of TCR triggering are critical in regulating the course of Th differentiation, leading to the generation of distinct, mature effector Th subsets, such as Th1 and Th2 cells (17, 18). Moreover, during their differentiation, Th1 and Th2 cells acquire differential responsiveness to cytokines. For instance, while Th2 cells can signal through the IFN- γ R, Th1 cells are unresponsive to IFN- γ because they do not express the second chain of its receptor (IFN- γ R2) (19-21). Transgenic (TG) overexpression of this protein, and the resultant responsiveness to IFN- γ in Th1 cells, profoundly impairs the effector function of these cells, indicating that the regulation of responsiveness to this cytokine is critical for normal Th1-dependent immunity (22).

The role that cytokines play in the thymic development, activation of CD8⁺ T cells, and the acquisition of mature CTL phenotypes is less clear. The regulation of responsiveness to cytokines by CD8⁺ T cells is also virtually unexplored. IFN- γ -producing CD8⁺ T cells, like Th1 cells, may potentially regulate their ability to signal through IFN- γ R during their differentiation. It is also possible that IFN- γ signaling participates in, or affects, certain stages in the development of the mature effector phenotype in CD8⁺ T cells. To explore this possibility, IFN- γ signaling in CD8⁺ T cells was investigated.

In this study, IFN- γ -producing CD8⁺ T cells are shown to be incapable of signaling through IFN- γ R because they lack expression of IFN- γ R2, an integral component of the IFN- γ R complex. As a result, these cells are insensitive to the biologic functions of this cytokine, such as up-regulation of class I MHC molecules. In TG mice, in which IFN- γ R2 expression

³Abbreviations used in this paper: FasL, Fas ligand; GAS, γ -activated site; IRF, IFN-regulatory factor; SOCS, suppressors of cytokine signaling; TG, transgenic; WT, wild type.

is dysregulated, CD8⁺ T cells are responsive to IFN- γ . In response to antigenic stimulation, these T cells are activated, and proliferate and produce cytokines normally. However, these cells are functionally impaired, as they are unable to lyse allogeneic target cells. In contrast, CTL function in CD8⁺ T cells isolated from IFN- γ R2^{-/-} mice, which lack this receptor chain, is normal. These findings suggest that the regulation of responsiveness to IFN- γ in CD8⁺ T cells somehow participates in their maturation into CTLs. Therefore, in addition to the quality of the TCR signal, cytokines may regulate the acquisition of mature effector functions by CD8⁺ T cells.

Materials and Methods

Mice

IFN- γ R2 TG mice were originally described by Tau et al. (22). TG mice were bred to the C57BL/6 genetic background for at least 10 generations. All TG mice used were heterozygous for the IFN- γ R2 transgene. IFN- γ R2 TG litters were screened by PCR using the following primers: 5'-GCACGTGGTTAAGCTCTCG (located in the CD2 promoter) and 5'-TGTCTCTGTGATGTCCGTACA. Appropriate controls (sex-matched littermates or age- and sex-matched mice) were used where normal or wild-type mice are indicated. IFN- γ R2-deficient mice were originally generated and described by Lu et al. (23). These mice were bred into the 129 genetic background for at least 10 generations. Where normal or wild-type mice are indicated, age- and sex-matched pure bred 129 mice were used (The Jackson Laboratory, Bar Harbor, ME). BALB/c mice were purchased from Jackson Laboratory. All mice were 4–6 wk of age at the start of a given experiment. All animal experiments conformed to Columbia University's Institutional Animal Care and Use Protocols.

Tumor cell lines and tissue culture

Unless otherwise noted, cells were grown in complete RPMI containing 10 μ g/ml penicillin-streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-ME, 1 mM sodium pyruvate, 10 mM HEPES, and 10% FBS. IL-2 media is complete RPMI containing IL-2 (20 U/ml). The EL-4 and S49.1 tumor cell lines were purchased from the American Type Culture Collection (Manassas, VA).

Cytokines and Abs

The mAbs 145-2C11 (anti-CD3; unconjugated, FITC), GK1.5 (anti-CD4; unconjugated), RM4-4 (anti-CD4; biotin, PE, FITC), 53-6.7 (anti-CD8; biotin, PE, FITC), M1/70 (anti-CD11b/Mac-1 α ; unconjugated), RA3-6B2 (anti-CD45R/B220; unconjugated), 57.51 (anti-CD28; unconjugated), 74D (anti-CD25; biotin), H1.2F3 (anti-CD69; FITC), MFL3 (anti-CD95L/FasL; biotin), AF6-88.4 (anti-H-2K^b; FITC), and XMG1.2 (anti-IFN- γ ; PE, FITC) were purchased from BD PharMingen (San Diego, CA). GR-20 (anti-IFN- γ R1; FITC) and RA3-6B2 (anti-CD45R/B220; biotin) were a gift of M. Nawijn. Streptavidin-conjugated fluochromes (FITC, PE, and CyChrome) were purchased from BD PharMingen. Human rIL-2 was provided by the National Cancer Institute Biological Research Branch (Frederick, MD). Recombinant murine IFN- γ and human IFN- α A/D were purchased from Genzyme (Cambridge, MA).

CD8⁺ T cell purification

CD8⁺ T cells were purified by negative selection similarly to previously described CD4⁺ T cell purification (22). Briefly, single cell suspensions from lymph nodes and/or spleens containing no RBCs were first incubated with rat anti-mouse mAbs against B cells (anti-B220/CD45R), monocytes (anti-CD11b), and CD4⁺ T cells (anti-CD4) at 20 μ g/ml each, washed, and then incubated with anti-rat IgG Dynabeads (Dyna, Lake Success, NY). Ab-coated cells were removed using a magnetic concentrator (Dyna). The purity of CD8⁺ T cells was at least 80%, as determined by flow cytometry.

CD8⁺ T cell lines and clones

Allo-specific CD8⁺ T cells were derived based on published protocol (24). Accordingly, mice were primed by i.p. injection of $2-4 \times 10^7$ irradiated (17 Gy; ⁶⁰Co Gammacell), allogeneic (BALB/c-derived) splenocytes (25). Two weeks later, CD8⁺ cells were purified from spleens and mesenteric lymph nodes, as described above. To generate cell lines, CD8⁺ T cells ($1-3 \times 10^6$ /well in a 24-well plate) were stimulated with 7×10^6 APCs (irradiated BALB/c splenocytes)/well. Cultures were expanded in IL-2-containing media 24–48 h after stimulation. CD8⁺ T cells were cultured in IL-2 media for 10 days following stimulation, at which point they were restimulated with APCs, as above. Cultures were maintained by serial 9- to 14-day cycles of stimulation with APCs and expansion in IL-2 media. Allo-specific CD8⁺ T cell clones were generated as follows: 96 multiples of 8, 40, 200, and 1000 CD8⁺ T cells purified from allo-primed mice (as above) were aliquoted into U-bottom microtiter plates in 100 μ l complete RPMI. Cells were stimulated with 5×10^4 allogeneic APCs/well. Every 2–4 days, a portion of the supernatant was aspirated and replaced with IL-2 media. Cells were restimulated at days 10 and 20 by adding 5×10^4 APCs/well. Only clones from plates in which fewer than 30% of the wells contained live cells were expanded and propagated (as above) and used in experiments.

Analysis of Stat1 activation

Stat1 activation was detected by EMSA, as previously described (26, 27). Briefly, cells were treated with either IFN- α (15 ng/ml) or IFN- γ (10 ng/ml) for 30 min, and whole cell protein extracts were prepared (28). The extracts were incubated with a radiolabeled probe derived from the IFN-regulatory factor-1 (IRF-1) γ -activated site (GAS) element (5'-GATCGATTTCCCGAAAT). The shift in electromobility imparted by the binding of activated STATs to the probe was detected by resolving the complexes on a polyacrylamide gel and visualized by autoradiography.

RNA isolation and Northern analysis

Total RNA was prepared by using QiaShredder, followed by RNeasy Mini Kit, according to the instructions of the manufacturer (Qiagen, Valencia, CA). RNA (10 μ g) from each sample was fractionated on a formaldehyde agarose gel and transferred to a nylon membrane, which was then hybridized with a radiolabeled probe and visualized by autoradiography, all according to standard protocol (29). cDNA probes were radiolabeled with [α -³²P]dCTP and [α -³²P]dATP using the Readyprime II random prime labeling system (Amersham Pharmacia Biotech, Piscataway, NJ). The IRF-1 cDNA was a kind gift of C. Schindler (30). The IFN-

γ R2 cDNA was previously described (20, 31). RT-PCR using total RNA isolated from wild-type (WT) CTLs was performed with primers 5'-CTCCACGTGCTT TCACCAA and 5'-GGAAAATAGTACAGAGAGGCA for granzyme B, and 5'-TGCTACACTGCCACTCGGTCA and 5'-TTGGCTACCTTG GAGTGGGAG for perforin cDNA, according to previously described primers and conditions (32). The suppressors of cytokine signaling-1 (SOCS)-1 and SOCS-3 cDNAs were previously described (33).

Intracytoplasmic cytokine analysis and flow cytometry

All Ab staining, unless otherwise indicated, was performed in PBS containing 1% BSA and 0.1% sodium azide based on standard protocol. Following staining, cells were fixed in PBS containing 2% paraformaldehyde and analyzed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Data analysis was performed using CellQuest software (BD Biosciences). Intracellular cytokine staining was performed based on recommended manufacturer's protocol (BD PharMingen). In brief, 10^6 freshly purified or day 4–9 CD8⁺ T cells in IL-2 media were either stimulated with 0.5 μ g/ml PMA (Sigma-Aldrich, St. Louis, MO) and 5 μ g/ml ionomycin (Sigma-Aldrich) or left unstimulated for 2–4 h at 37°C. At this point, brefeldin A (Sigma-Aldrich) was added to 10 μ g/ml for an additional 2 h. Alternatively, 3×10^5 freshly purified or day 5 CD8⁺ T cells were either stimulated with 7×10^5 APCs, or left unstimulated for 2 h, at which point brefeldin A was added for an additional 10 h. Cells were then stained for cell surface markers, fixed in 2% paraformaldehyde, permeabilized with 0.5% saponin (Sigma-Aldrich), and stained for intracytoplasmic IFN- γ or with an isotype-matched Ab as a control.

Cytotoxicity assays

Cytotoxicity assays were performed based on standard protocol (34). In brief, $1-2 \times 10^6$ target cells (S49 or EL-4) were labeled with 0.1–0.2 mCi⁵¹Cr, washed three times, and plated in 96-well microtiter plates at 5×10^3 /well in 100 μ l complete RPMI with or without 5 μ g/ml Con A (Amersham Pharmacia Biotech). Effector cells (CD8⁺ T cell lines or clones 4–7 days poststimulation) were added in triplicate and in equal volume of media at the indicated ratios. In one set of experiments, CD8⁺ T cells freshly purified from primed mice (as above) were used as effectors 7 days following in vitro stimulation. In place of effectors, media alone (spontaneous lysis) or 1% Triton X (maximum lysis) was added to control wells. After incubation for 4 h at 37°C, 100 μ l of culture supernatant was removed from each well and counted for radioactivity. Values for percentage of specific lysis were calculated as follows:

$$\% \text{ specific lysis} = \frac{(\text{test sample value}) - (\text{spontaneous release value})}{(\text{maximum release value}) - (\text{spontaneous release value})} \times 100$$

Net Con A-induced lysis was determined by calculating the difference in percent specific lysis values at each E:T ratio between the Con A-treated and untreated assays.

Cell division cycle profile analysis using CFSE

CD8⁺ cells (freshly purified clones or lines, described elsewhere) were labeled with CFSE (Molecular Probes, Eugene, OR) in a modification of a previously described technique (22, 35). Briefly, cells were washed with serum-free RPMI. Cells (10⁷ cells/ml) were then labeled with 10 μM CFSE in serum-free RPMI at 37°C for 10 min, and CFSE was then neutralized with complete RPMI. CFSE-labeled CD8⁺ T cells were cultured either on anti-CD3 mAb + anti-CD28 mAb-coated plates (5 μg/ml each Ab in PBS overnight at 4°C) or stimulated with APCs as above. Initial CFSE-labeling efficiency and the fluorescein intensities at the end of each experiment were detected using a FACScan flow cytometer. Histogram overlays and peak distribution analyses were performed using CellQuest software.

Results

CD8⁺ T cells do not express IFN-γR2 mRNA and are unresponsive to IFN-γ

It has previously been demonstrated that IFN-γ-producing CD4⁺ T cells (Th1) are unresponsive to IFN-γ because they do not express IFN-γR2 (19-21). Other IFN-γ-producing lymphocytes, such as CD8⁺ T cells, may, like Th1 cells, modulate their ability to respond to this cytokine during their differentiation. To test this possibility, allo-specific CD8⁺ T lines and clones were generated. The ability of these cell lines to respond to IFNs was assessed.

Signaling by IFNs leads to the phosphorylation of STAT monomers, allowing the formation of homo- and heterodimers that can bind specific elements in the promoters of IFN-responsive genes and ultimately regulate their expression (36). Type II IFN (IFN-γ) stimulates the phosphorylation of Stat1 and the formation of Stat1 homodimers, which bind GAS elements in the promoters of IFN-γ-responsive genes. The type I IFNs (IFN-α/β) stimulate the phosphorylation of Stat1 and Stat2 and activate the expression of genes whose promoters contain either GAS elements (which bind Stat1 homodimers) or IFN-stimulated response elements (which bind a complex of Stat1, Stat2, and IRF-9).

To determine whether CD8⁺ T cells are responsive to IFN-γ, protein extracts from IFN-γ- and IFN-α-treated cells were examined for GAS-binding activity (activated Stat1 complexes) by EMSA. Activated Stat1 complexes were detected in extracts prepared from control EL-4 cells treated with either IFN-γ or IFN-α (Fig. 1A). Activated Stat1 complexes were also detected in CD8⁺ T cells cultured with IFN-α, but strikingly, CD8⁺ T cells cultured with IFN-γ were unable to activate Stat1 (Fig. 1A). This suggests that one or more components of the IFN-γ signaling pathway are either absent, defective, or inhibited in CD8⁺ T cells. The observation that Stat1 activation is detected following treatment with IFN-α indicates that Stat1, as well as Janus kinase 1 are present and functional in CD8⁺ T cells. To specifically identify the signaling defect in CD8⁺ T cells, the integrity of the IFN-γR complex was examined. Similarly to Th1 cells (20, 21), IFN-γR1 protein was detected on the surface of CD8⁺ T cells using the GR-20 Ab (Fig. 1B). However, mRNA encoding the IFN-γR2 chain was not detected in these cells (Fig. 1C).

Downstream of Stat1 activation, IFNs induce the expression of many genes, including the class I MHC genes and *irf-1* (37). As expected, treatment of control T cells with either IFN- α or IFN- γ resulted in increased levels of cell surface H-2K^b (Fig. 1D). In CD8⁺ T cells, however, while IFN- α had an inductive effect, IFN- γ had no apparent effect on cell surface H-2K^b levels (Fig. 1D). Furthermore, IFN- γ was unable to induce *irf-1* gene expression in CD8⁺ T cells (Fig. 1E). Together, these data suggest that CD8⁺ T cells are unable to respond to IFN- γ because they may lack IFN- γ R2, the signal-transducing component of the IFN- γ R (38).

CD8⁺ T cells from IFN- γ R2^{-/-} mice develop and function normally

Because CD8⁺ T cells do not appear to express IFN- γ R2, it may be that this receptor, and perhaps IFN- γ responsiveness in general, are dispensable for the development, differentiation, and the function of CD8⁺ T cells. To examine this possibility, CD8⁺ T cells isolated from mice that are unable to respond to IFN- γ were analyzed. Prior studies have shown that CD8⁺ T cells from mice deficient in IFN- γ R1 can elaborate normal lytic and proliferative memory responses against virally infected targets (39). IFN- γ R1-deficient mice are also resistant to most viral infections, suggesting that IFN- γ signaling may not be required for the development and function of CD8⁺ T cells. To directly examine the requirement for IFN- γ R2 in the CD8⁺ T cell system, CD8⁺ T cells were isolated from IFN- γ R2-deficient mice and were analyzed. Prior studies have demonstrated normal numbers of CD8⁺ T cells in the lymphoid organs of IFN- γ R2^{-/-} mice (23), which is similar to findings in other IFN- γ -insensitive or IFN- γ -deficient systems. IFN- γ R2^{-/-} allo-specific CD8⁺ T cells proliferated and produced normal levels of IFN- γ in response to a number of activating stimuli, such as phorbol ester + calcium ionophore or allogeneic APCs (Fig. 2A and data not shown). Furthermore, these cells exhibited equivalent levels of specific allogeneic target lysis as compared with allo-specific CD8⁺ T cells derived from WT littermate controls (Fig. 2B). IFN- γ R2^{-/-} CD8⁺ T cells therefore appear indistinguishable from their WT counterparts despite their inability to transduce the IFN- γ signal at all points during their development and maturation. These data suggest that IFN- γ signaling is not essential either for the development of CD8⁺ T cells or for their function.

TG expression of IFN- γ R2 endows CD8⁺ T cells with IFN- γ responsiveness

The experiments above suggest that CD8⁺ T cells lack expression of IFN- γ R2 and are insensitive to IFN- γ . To examine whether these two findings are directly linked, CD8⁺ T cells were isolated from IFN- γ R2 TG mice, and their ability to signal through IFN- γ R was analyzed. In these mice, TG expression of IFN- γ R2 is driven by the CD2 promoter/enhancer. This transgene mRNA, which had been previously shown to be expressed in CD4⁺ T cells (22), is also detected in CD8⁺ T cells isolated from IFN- γ R2 TG mice (Fig. 3A). In addition, IFN- γ R2 TG CD8⁺ T cells are able to activate Stat1 in response to treatment with IFN- γ , in contrast to WT CD8⁺ T cells (Fig. 3B). This indicates overexpression of IFN- γ R2 is sufficient to restore IFN- γ signaling in CD8⁺ T cells, and suggests that IFN- γ R2 deficiency may be the sole locus of the defect in IFN- γ signaling seen in WT CD8⁺ T cells.

The altered IFN- γ signaling potential observed in TG CD8⁺ T cells suggests that their biologic responses to IFN- γ may differ from those of WT CD8⁺ T cells. As expected, TG CD8⁺ T cells up-regulate levels of cell surface class I MHC in response to treatment with IFN- α , like WT CD8⁺ T cells (Fig. 3C). However, unlike their WT counterparts, IFN- γ R2 TG CD8⁺ T cells can increase cell surface expression of class I MHC molecules in response to IFN- γ (Fig. 3C). Interestingly, the basal level of class I MHC molecules is higher in TG than WT CD8⁺ T cells, which may be the reason for the more modest effect of IFNs on the up-regulation of these molecules in TG cells (Fig. 3C). Nevertheless, the up-regulation of class I MHC molecules in response to IFN- γ in TG cells is both significant and reproducible.

As previously reported, IFN- γ R2 TG mice appear healthy and thrive when housed in a conventional facility (nonbarrier), and have normal proportions of CD8⁺ T cells in their spleens and lymph nodes (22). Furthermore, FACS analysis of thymocytes (CD4 vs CD8 vs CD3, and CD4 vs CD8 vs CD69) has revealed no differences between WT and TG mice. TG CD8⁺ T cells proliferate and can be propagated in vitro similarly to their WT counterparts. However, because IFN- γ has been reported to have antiproliferative effects (4), it is possible that TG CD8⁺ T cells have a proliferative disadvantage secondary to autocrine effects of this cytokine. To examine the effects of IFN- γ signaling on the mitotic profiles of CD8⁺ T cells, CD8⁺ T cells were purified from WT and TG mice and were labeled with CFSE, an amine-reactive, membrane-permeant, fluorescein-based dye that does not interfere with the biologic function of CD8⁺ T cells (40). The decay of the CFSE label was followed under a number of stimulation and proliferative conditions. WT and TG CD8⁺ T cells proliferate equally well when stimulated with either anti-CD3 plus anti-CD28 mAbs (Fig. 3E and Table I) or allo-APCs (data not shown). These experiments were also performed using standard [³H]thymidine incorporation techniques, which yielded similar results (data not shown). Together, these data demonstrate that the IFN- γ R2 transgene uniquely endows CD8⁺ T cells with IFN- γ responsiveness, but does not appear to affect their in vivo development or in vitro proliferation.

Altered gene expression in IFN- γ R2 TG CD8⁺ T cells

IFN- γ has been shown to regulate the expression of over 200 genes, including transcription factors, such as IRF family members, *c-fos*, and *c-jun*, and signaling molecules such as SOCS family members, *csk*, *lyn*, and *lck* (37, 41-43). Stat1 is constitutively activated in TG CD8⁺ cells (Fig. 3B), most likely as a result of autocrine effects of IFN- γ . Furthermore, the basal levels of cell surface class I MHC molecules are elevated on IFN- γ R2 TG CD8⁺ T cells. It is likely that autocrine effects of IFN- γ (Fig. 3B) induce the higher baseline of cell surface MHC molecules (Fig. 3C). To examine whether the IFN- γ R2 transgene affects the expression of other IFN- γ -regulated genes, mRNA was isolated from activated CD8⁺ T cell lines derived from WT and TG mice. The mRNA levels of *irf-1* and, more modestly, *SOCS-1* and *SOCS-3*, were found to be elevated in TG cells (Fig. 3D). This suggests that the pattern of expression of these, and potentially other, IFN- γ -regulated genes is altered in IFN- γ R2 TG CD8⁺ T cells.

IFN- γ signaling does not affect CD8⁺ T cell cytokine and proliferative memory responses *ex vivo*

To examine whether the signals transduced through IFN- γ R affect CD8⁺ T cell responses *in vivo*, Ag-specific memory responses, such as proliferation and cytokine production, were examined in IFN- γ R2 TG mice. CD8⁺ T cells were purified from primed mice, labeled with CFSE, and stimulated with allogeneic APCs. Both the fraction of CD8⁺ T that are alloreactive in the primed mice, and the proportion of these alloreactive cells that are IFN- γ producers, were determined by flow cytometry. Approximately 0.1% of isolated CD8⁺ T cells from mice primed with syngeneic APCs produced IFN- γ in response to *ex vivo* allogeneic stimulation (Fig. 4A). In contrast, at least 6-fold greater numbers of allo-primed WT and TG CD8⁺ T cells produced IFN- γ following *ex vivo* stimulation with allogeneic APCs (Fig. 4A). These results corroborate the *in vitro* data showing no difference in IFN- γ production between WT and TG cells (see below).

Three days following allostimulation, the distribution of the CFSE label in the population was analyzed by flow cytometry to determine the proportion of allo-responding CD8⁺ T cells. Both allo-primed WT and TG mice had comparable proportions of alloresponding CD8⁺ T cells (51.8% and 56.6%, respectively), which were consistently higher than the proportion of alloresponders from mice primed with syngeneic cells (35%) (Fig. 4B). Moreover, WT and TG alloresponder populations exhibited similar rates of CFSE decay (Fig. 4B, and data not shown). Therefore, in response to *ex vivo* antigenic challenge, primed TG mice generate equivalent proportions of CD8⁺ responders that proliferate and produce IFN- γ similarly to their WT counterparts.

IFN- γ signaling specifically impairs the effector killing function of CD8⁺ T cells

Although CD8⁺ T cells are resistant to IFN- γ , and signaling through IFN- γ R appears to be dispensable for the normal development and function of these cells, IFN- γ R2 TG CD8⁺ T cells appear to proliferate normally (Figs. 1–3). This raises the question of why CD8⁺ T cells become unresponsive to IFN- γ . It is possible that unresponsiveness to IFN- γ is not a mere phenomenon, but is an essential feature of CD8⁺ T cells intimately associated with the some element of their function. To test this possibility, two effector functions of CD8⁺ T cells, cytokine production and target cell lysis, were examined *in vitro*. Allo-specific CD8⁺ T cells derived from WT and IFN- γ R2 TG mice were assayed for their ability to lyse ⁵¹Cr-labeled syngeneic (S49) and allogeneic (EL-4) tumor cell lines. WT CD8⁺ T cells specifically lysed allogeneic targets efficiently, but could not lyse syngeneic target cells (Fig. 5B). Strikingly, IFN- γ R2 TG cells were unable to lyse allogeneic or syngeneic target cells (Fig. 5B). This defect in killing was confirmed multiple times with both CD8⁺ T cell lines and clones, as well as with freshly isolated CD8⁺ T cells from primed WT and TG mice (Fig. 5A). In contrast, both WT and TG CD8⁺ T cells produced equivalent amounts of IFN- γ in response to a number of stimuli, including plate-bound anti-CD3 + anti-CD28 mAbs, allo-APCs, and PMA + ionomycin, as assayed by both intracytoplasmic staining and ELISA (Fig. 5C and data not shown). Together, these data suggest that IFN- γ responsiveness selectively alters the target cell lysis effector function of TG CD8⁺ T cells, but not their ability to produce IFN- γ .

IFN- γ signaling does not affect the ability of CD8⁺ T cells to produce effector molecules

Allo-specific IFN- γ R2 TG CD8⁺ T cells are unable to lyse allogeneic target cells, but are capable of normal proliferation and IFN- γ production. To identify the mechanism responsible for the observed defect in cytotoxicity, the ability of TG cells to produce effector molecules involved in killing was examined. WT and TG CD8⁺ T cells express equivalent levels of both perforin- and granzyme B-encoding mRNA at baseline, and these mRNA species are similarly up-regulated following treatment with Con A in WT and TG CD8⁺ T cells (Fig. 6A). Furthermore, cell surface levels of FasL are up-regulated in both WT and TG CD8⁺ T cells in response to stimulation with either allo-APCs or polyclonal stimuli (Fig. 6B and data not shown). The extent of increase in cell surface levels of FasL in TG cells (median fluorescence intensity rising from 4.5 to 11.4 upon stimulation) is consistently more modest than that seen in WT cells (5.3 rising to 16.3). These data suggest that the effector molecules required for the cytotoxic function of CTLs are expressed by TG CD8⁺ T.

The defect in killing may potentially be caused by an inability of IFN- γ -responsive CD8⁺ T cells to either normally recognize, or be properly activated by, allogeneic targets. To address this question, the effect of allogeneic stimulation on TG CD8⁺ T cell activation was examined. Following incubation with allo-APCs or stimulation with anti-CD3, TG cells can up-regulate expression of the activation markers CD69 and CD25 (IL-2R α) equally well as their WT counterparts, suggesting that these cells recognize and are normally activated by allogeneic stimuli (Fig. 6B and data not shown). These data, together with the observation that TG CD8⁺ T cells proliferate normally (Fig. 3E and Table I), argue that IFN- γ signaling does not affect the ability of CD8⁺ T cells to be specifically activated through TCR-MHC contact, or through polyclonal TCR triggering.

It appears that TCR contact with physiologically appropriate MHC molecules results in the activation of IFN- γ R2 TG CD8⁺ T cells and induction of cytokine production. Although TCR triggering in WT cells results in activation of their cytotoxic pathways, this type of stimulus may be insufficient to induce the exocytosis of cytotoxic granules in TG cells. It is possible that a particularly strong activating stimulus, such as Con A, may be able to induce exocytosis in TG CD8⁺ T cells, resulting in target cell lysis. Con A aggregates cell surface glycoproteins, thereby supernormally activating T cells independently of allo-MHC molecules (44). The addition of Con A to a standard killing assay (redirected killing) enhanced the cytotoxicity of both WT and TG CD8⁺ T cells (Fig. 6C). TG cells did not achieve the same absolute levels of S49 target cell lysis as did their WT counterparts in this redirected killing assay (Fig. 6C). However, the relative increase in cytotoxicity at each E:T ratio was equivalent among all WT and TG CD8⁺ T cell lines (Table II). Moreover, redirected killing using the syngeneic cell line EL-4 as a target resulted in equivalent increases in both relative and absolute values of lysis among all effector cell lines tested (Fig. 6D).

Together, these data suggest that IFN- γ R2 TG CD8⁺ T cells produce the effector molecules that are required for killing, but are, nevertheless, unable to lyse their targets under normal conditions. The killing defect in TG cells can be circumvented through the addition of Con A, a strong activating stimulus. This suggests that signaling through the IFN- γ R causes a

subtle, selective defect in CD8⁺ T cell activation that affects the elaboration of cytotoxicity rather than the production of the components required for this effector function.

Discussion

It is established that cytokines play a pivotal role in the development of mature, effector Th phenotypes (9). Previous studies have demonstrated that Th cells acquire differential responsiveness to cytokines during their differentiation (19-21). For instance, Th1 cells, the IFN- γ -producing CD4⁺ T cell subset, do not respond to IFN- γ because they lack expression of IFN- γ R2 (20, 21). The present study investigates cytokine responsiveness in CD8⁺ T cells and its potential effects on the acquisition of a mature CTL phenotype. The experiments in this study demonstrate that, like their CD4⁺ counterparts, IFN- γ -producing CD8⁺ T cells express IFN- γ R1, but not IFN- γ R2, and are unable to transduce the IFN- γ signal, or respond to the biologic effects of this cytokine. There is no effective Ab to IFN- γ R2, and standard binding assays may not be sufficiently sensitive to determine whether IFN- γ R2 is present on the surface of CD8⁺ T cells, because this chain is not required for binding IFN- γ (45). Therefore, our data demonstrating that CD8⁺ T cells lack IFN- γ R2 mRNA, are able to activate Stat1 in response to treatment with IFN- α but not with IFN- γ , and that TG expression of IFN- γ R2 confers IFN- γ responsiveness to these cells, is the best available set of experiments to demonstrate this causal relationship. In support of this, our original report describing the lack of IFN- γ signaling in Th1 cells demonstrated that transfection of cDNA encoding IFN- γ R2 into Th1 clones allows them to respond to IFN- γ (20).

Experiments using CD8⁺ T cells derived from IFN- γ R2^{-/-} mice demonstrate that IFN- γ R is not required for the development of a mature CTL phenotype. Furthermore, TG overexpression of IFN- γ R2 not only restores IFN- γ responsiveness in CD8⁺ T cells, but results in CTLs with altered function. This establishes an interesting paradigm, that IFN- γ -producing T cells down-modulate expression of IFN- γ R2 and consequently acquire an IFN- γ -unresponsive phenotype. Moreover, unresponsiveness to IFN- γ appears to be essential for the development of the normal effector phenotypes of these cells.

Much is known about how IFN- γ and other cytokines affect CD4⁺ Th subset differentiation and function. However, the current understanding of the role of cytokines in the development, differentiation, and function of CD8⁺ T cells is still rudimentary. The finding that CD8⁺ T cells are insensitive to IFN- γ suggests that the modulation of responsiveness to this cytokine by these cells may be an event integral to their development. Remarkably, forced responsiveness to IFN- γ in CD8⁺ T cells appears to specifically impair their ability to kill, but leaves their other functions intact. It is interesting that the function of both CD4⁺ and CD8⁺ T cells is impaired in IFN- γ R2 TG mice, but the effects of transgene expression on these cells are qualitatively different. TG Th1 cells elaborate defective IFN- γ -dependent responses, whereas production of this cytokine by TG CD8⁺ T cells is intact (22). It may be that these two cell types have innate differences in their "hard wiring" for IFN- γ production, as suggested by the dependence of Th1, but not CD8⁺ T cells on Stat4 for production of this cytokine (46).

A number of groups have described CTLs whose function is altered, with a phenotype similar to that of IFN- γ R2 TG CD8⁺ T cells. In these studies, CTLs stimulated by superantigen (47), subdominant viral epitopes (16), or altered peptides (altered peptide ligand phenomenon) (48, 49), and naturally occurring virus-specific CTLs (15, 50) appear to elaborate defective killing mechanisms, but can be activated to proliferate and produce IFN- γ normally. It seems that, depending on the nature of the TCR signal, CTLs that appear to be normally activated can be selectively unable to elaborate one or more effector mechanisms. One group has shown that CTLs that cannot generate an appropriate Ca²⁺ signal in response to TCR triggering are unable to kill through the perforin/granzyme pathway (51-53). Degranulation can be induced by treatment of these cells with phorbol ester plus calcium ionophore, which circumvents the TCR to induce a large spike in intracellular Ca²⁺.

Like previously described CTLs with altered function, IFN- γ R2 TG CD8⁺ T cells are activated, produce IFN- γ , and up-regulate FasL in response to antigenic stimulation. Although the extent of FasL up-regulation is more modest on TG than on WT CD8⁺ T cells, it is unlikely that this is the locus of the defect in killing in IFN- γ R2 TG CD8⁺ T cells, because our cytotoxicity assay system does not measure FasL-mediated killing, which typically requires a longer assay time (54, 55). Furthermore, S49 cells, the allogeneic targets used in the killing assay, may be resistant to FasL-mediated killing (56). Remarkably, TG cells are unable to kill via the exocytotic pathway, although they produce the effector molecules required for this function. In contrast, in the presence of Con A, TG cells are able to lyse allogeneic and syngeneic targets. One effect of Con A is to promote cell-cell contact, which raises the possibility that TG CTLs may be unable to form the effector-target cell contacts that are required for killing (57). This potential dysfunction could be due to altered expression of adhesion molecules in these cells. However, since the typical net effect of IFN- γ is the up-regulation of cell surface levels of adhesion molecules, such as selectins (e.g., CD62L and CD62E) and integrins (e.g., CD49a-f and CD11a-c) on a host of cell lines, TG CTLs are probably able to bind with target cells (37). Con A can also induce multiple intracellular events, including protein tyrosine kinase activation, leading to increase in tyrosine-phosphorylated proteins, phosphatidylinositol 3-kinase activation, activation of pathways downstream of G proteins, rise of intracellular Ca²⁺, and the nuclear translocation of NF-ATp (58-62). These events correlate with the commonly accepted role of Con A as a polyclonal activator of T cells. It is likely that this lectin promotes killing by TG cells by triggering intracellular pathways in these cells that are not activated, or are differently activated, by TCR-MHC contact alone. Since many pathways activated by Con A are normally downstream of the TCR, these findings raise the possibility that the quality of the TCR signal in IFN- γ R2 TG CTLs is altered.

IFN- γ is a pleiotropic cytokine that is known to regulate the expression of a vast array of genes, and consequently a host of cellular and immunological processes (37). In IFN- γ R2 TG CD8⁺ T cells, Stat1 is constitutively active, and baseline levels of IFN- γ -inducible proteins are elevated. We believe this occurs because of autocrine effects of IFN- γ rather than an artifact of overexpression of IFN- γ R2 because we have previously reported that Stat1 is inactive in TG Th2 cell clones, which by definition do not produce IFN- γ (22). This suggests that the constitutive activation of the IFN- γ pathway seen in TG CD8⁺ T cells is

ligand dependent. Furthermore, altered pattern of gene expression in CD8⁺ T cells derived from IFN- γ R2 TG mice can potentially create an environment that is incompatible with the acquisition of killing function or the generation of an appropriate TCR signal, leading to a selective defect in perforin/granzyme-mediated cytotoxicity in these cells. One potential mechanism for this defect may involve the SOCS-1 and SOCS-3 proteins, whose expression is known to be induced by IFN- γ , and is up-regulated in IFN- γ R2 CD8⁺ T cells (42). These SOCS proteins may affect signaling by cytokines that are important in the function of CD8⁺ T cells. Both SOCS-1 and SOCS-3 have been implicated in the attenuation of the IFN- α signal (63-65). CTLs from mice that are deficient in the IFN- α β R were unable to elaborate Ag-specific killing functions, but were able to produce IFN- γ (66, 67). SOCS-3 has also been shown to inhibit Janus kinase 2 activation, and therefore, the activation of Stat5 by GM-CSF and perhaps by IL-5 (68, 69). Defects in IL-5 and GM-CSF have been shown to affect killing by CD8⁺ T cells (70, 71). Therefore, through the induction of SOCS expression, IFN- γ may potentially initiate an inhibitory cross-talk with the IFN- α , IL-5, and/or GM-CSF pathways, all of which have been implicated in CTL cytotoxicity. Beyond their role as suppressors of cytokine signaling, these members of the SOCS family have been implicated in inhibiting the activation of signaling pathways downstream of the TCR (72) (A. Banerjee, personal communication). In addition to the SOCS, there may be other IFN- γ -inducible factors that mediate this inhibitory cross-talk between the IFN- γ R and TCR pathways, or that regulate pathways linking T cell activation to granule exocytosis. These factors may also be dysregulated in IFN- γ R2 TG CD8⁺ T cells, leading to the observed phenotype.

This study has demonstrated that, during their development or differentiation, CD8⁺ T cells down-modulate expression of IFN- γ R2, thereby extinguishing their ability to respond to IFN- γ . This event appears to be crucial for the acquisition of a fully functional, mature, CTL phenotype, since signaling through IFN- γ R blocks the ability to utilize the perforin/granzyme cytotoxic pathway by CD8⁺ T cells that are stimulated by their cognate Ag. However, it seems that IFN- γ signaling is not required for either the thymic development of CD8⁺ T cells or their differentiation into mature CTLs. What, then, is the biological importance of the control of IFN- γ R2 expression? This phenomenon may be a self-protective mechanism invoked by CD8⁺ T cells to avoid the potential deleterious effects of IFN- γ . Therefore, down-modulation of IFN- γ R2 would occur automatically, either concomitantly with their acquisition of the capacity to produce IFN- γ , following the initial triggering of signaling through IFN- γ R, or in response to another signal delivered during their differentiation. On the other hand, the modulation of IFN- γ R2 expression could potentially be used as a self-regulatory switch by CD8⁺ T cells. Continued IFN- γ R2 expression in naive CD8⁺ T, which may, in fact, be normally responsive to IFN- γ , would delay their maturation or prevent it entirely. These cells would not be able to acquire a mature cytotoxic phenotype until down-modulation of IFN- γ R2 expression is induced. Furthermore, by modulating IFN- γ R2 expression, mature CTLs may be able to actively regulate their capacity to kill using the perforin/granzyme pathway. These scenarios represent an IFN- γ -mediated mechanism by which the immune system can self-limit its responses, thereby controlling the supply of new CTLs or modulating the function of existing CTLs. Whether the IFN- γ responsiveness phenotype becomes fixed or remains

flexible, the regulation of IFN- γ R2 expression is an integral event in CD8⁺ T cell development and mature phenotype acquisition. IFN- γ -producing T cells, and potentially all IFN- γ -producing cells, may need to extinguish their ability to respond to IFN- γ by down-regulating the expression of IFN- γ R2 to develop into mature effector cells.

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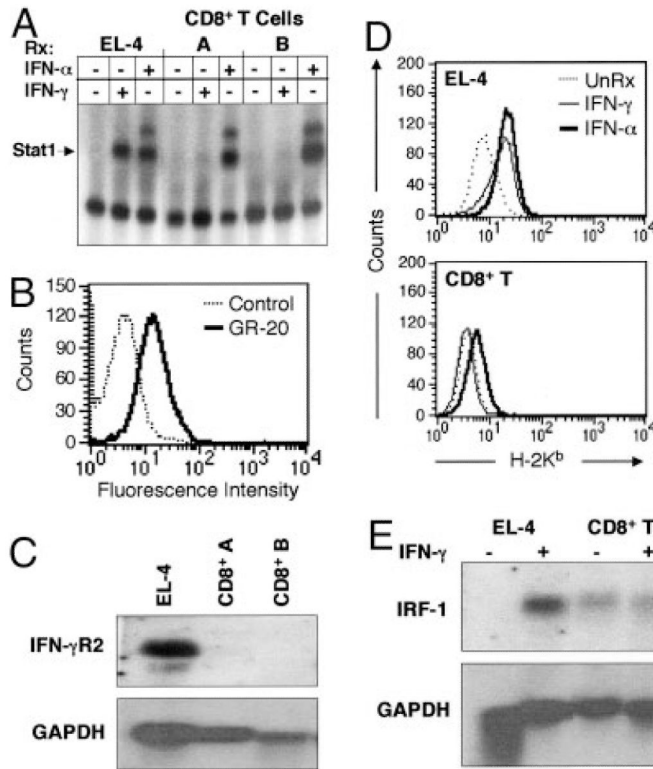


FIGURE 1.

CD8⁺ T cells do not express IFN- γ R2 and are not responsive to IFN- γ . *A*, EMSA of protein extracts from untreated or IFN- γ or IFN- α -treated (Rx) CD8⁺ T cell clones or control cells using GAS site as probe. *B*, Flow cytometric analysis of IFN- γ R2 expression on CD8⁺ T cell clones with GR-20 Ab with isotype-matched control (73). *C*, Northern blot analysis of IFN- γ R2 mRNA expression using mRNA by CD8⁺ T cell clones and control, with GAPDH probe used as loading control. *D*, FACS analysis of class I MHC (H-2K^b) expression by CD8⁺ T cell clones and control cells (UnRx) treated with either IFN- α (15 ng/ml) or IFN- γ (10 ng/ml) for 72 h. *E*, Northern blot analysis of *irf-1* mRNA expression using mRNA isolated from CD8⁺ T cell clones and control cells either untreated or treated with IFN- γ for 12 h, with GAPDH probe used as loading control.

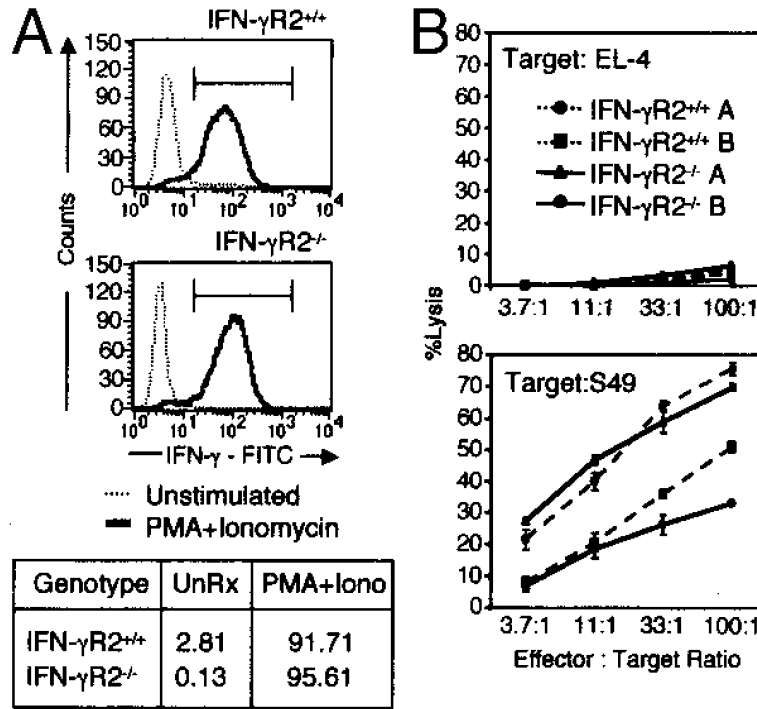


FIGURE 2. Signaling through IFN- γ R is not required for the normal cytokine production and killing by CD8⁺ T cells. *A*, IFN- γ production of allo-specific CD8⁺ T cell lines derived from WT and IFN- γ R2^{-/-} mice. Six days postallostimulation, untreated (---) or PMA + ionomycin (IONO)-treated (—) cells were stained for intracytoplasmic IFN- γ and analyzed by flow cytometry. Chart below indicates the percentages of events within the indicated IFN- γ positive gate. *B*, ⁵¹Cr release assay measuring the specific cytotoxicity of allo-specific IFN- γ R2^{-/-} (—) and WT (---) CD8⁺ T cell lines toward syngeneic (EL-4, *top*) or allogeneic (S49, *bottom*) tumor cell lines.

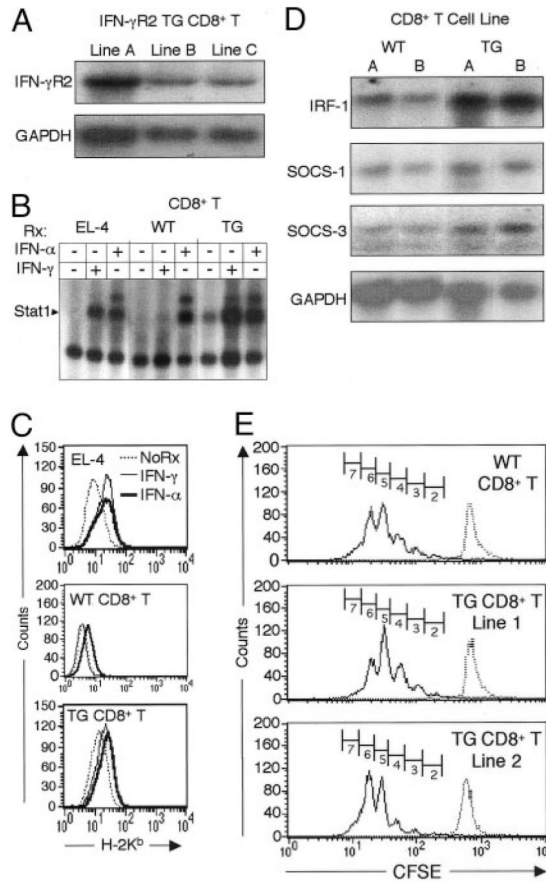


FIGURE 3.

TG expression of IFN- γ R2 restores responsiveness to IFN- γ in CD8⁺ T cells and does not affect their ability to proliferate, but alters their gene expression. *A*, Detection of IFN- γ R2 mRNA expression in IFN- γ R2 TG CD8⁺ T cell clones and control cells by Northern blot as in Fig. 1*C*. *B*, EMSA of protein extracts from IFN- γ and IFN- α -treated (Rx) IFN- γ R2 TG CD8⁺ T cell clones and control cells as in Fig. 1*a*. *C*, FACS analysis of class I MHC (H-2K^b) expression by IFN- α - or IFN- γ -treated IFN- γ R2 TG CD8⁺ T cell clones and control cells, as in Fig. 1*D*. *D*, NoRx, Untreated. *E*, mRNA was isolated from WT and TG CD8⁺ T cells (two cell lines each) 5 days following stimulation with allogeneic APCs. *IRF-1*, *SOCS-1*, *SOCS-3*, and *GAPDH* expression was visualized by Northern blotting. *E*, Analysis of the distribution of CFSE label by flow cytometry. Freshly isolated WT and CD8⁺ IFN- γ R2 TG CD8⁺ T cells from spleens and mesenteric lymph nodes were labeled with CFSE and stimulated with anti-CD3 + anti-CD28 Abs in the presence of IL-2. Cells were analyzed by FACS on day 3. Cell divisions undergone by CD8⁺ cells are represented by numbered gates.

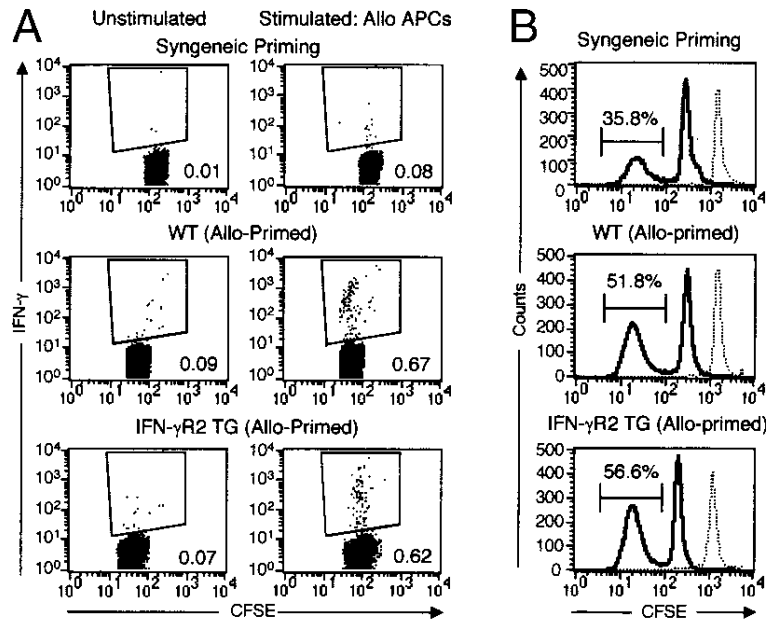


FIGURE 4. IFN- γ R2 TG mice elaborate normal CD8⁺ T cell proliferative and cytokine memory responses ex vivo. *A* and *B*, Ex vivo IFN- γ production and alloresponder frequency following syngeneic or allogeneic priming. WT and IFN- γ R2 TG mice were primed as indicated. At day 10, CD8⁺ T cells were purified, labeled with CFSE, and stimulated with allo-APCs or not. *A*, At 12 h, cells were stained for intracytoplasmic IFN- γ and analyzed by flow cytometry. Plots represent CD8-positive, CFSE^{bright} (non-APC) events. The values in each panel represent the percentages of IFN- γ -positive events within the indicated gates. *B*, On day 3, the CFSE label distribution of the allostimulated cells analyzed by flow cytometry. Plots represent CD8⁺, CFSE^{bright} (non-APC) events. CFSE intensity at day 0 (dashed curve) is overlaid with the CFSE distribution at day 3 (solid curve). Of two clusters of CFSE^{bright} cells in each histogram, the high intensity peak corresponds to the nonalloreactive cell population, whereas the low intensity peak represents the responder population that has undergone four to six cell division cycles. Values represent the percentages of CD8⁺ T cells responding to allogeneic stimulation within the indicated gates.

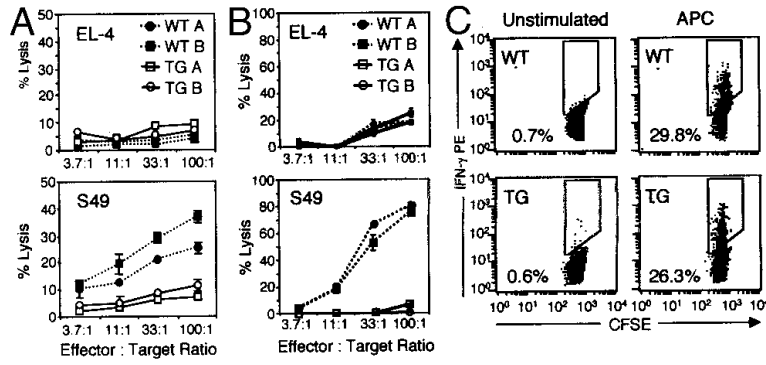


FIGURE 5.

IFN- γ signaling impairs CD8⁺ T cell cytotoxicity, but not cytokine production. *A* and *B*, ⁵¹Cr release assay measuring the specific cytotoxicity of allo-specific IFN- γ R2 TG (—) and WT (- - -) CD8⁺ T cell clones (*B*) or CD8⁺ T cells freshly isolated from allo-primed mice (*A*) toward syngeneic (EL-4, *top*) or allogeneic (S49, *bottom*) tumor cell lines. *C*, IFN- γ production of allo-specific CD8⁺ T cell lines derived from IFN- γ R2 mice. Six days postallostimulation, CFSE-labeled CD8⁺ T cells were stimulated with APCs or not. Twelve hours later, cells were stained for intracytoplasmic IFN- γ and analyzed by flow cytometry. Plots represent CFSE^{bright} (non-APC) events. Values represent the percentages of IFN- γ positive events within the indicated gates.

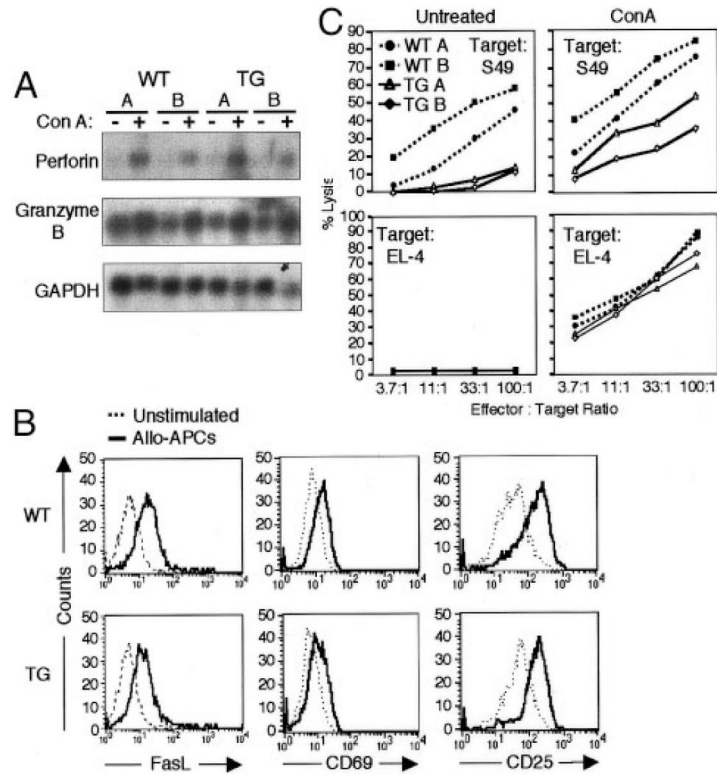


FIGURE 6. IFN- γ R2 TG CD8⁺ T cells are normally activated, produce effector killing molecules, and exhibit cytotoxic activity following chemical stimulus. *A*, Detection of *perforin*, *granzyme B*, and *GAPDH* mRNA expression in unstimulated (5 days postallostimulation) or Con A-stimulated (5 days) WT and IFN- γ R2 TG CD8⁺ T cell lines by Northern blot. *B*, Flow cytometric analysis of cell surface FasL, CD69, and CD25 expression on WT and IFN- γ R2 TG CD8⁺ T cell clones that were either stimulated with APCs for 12 h (—) or left unstimulated (---). Histogram represents CD8-positive events. *C*, Redirected killing assay measuring ⁵¹Cr release by labeled targets incubated with WT and IFN- γ R2 TG CD8⁺ T cell lines in the presence or absence of Con A.

Table I
Distribution of the CFSE label in CD8⁺ T cells

	No. of Cycles					
	2	3	4	5	6	7
WT	2.27	6.48	17.37	33.61	32.26	8.4
TG line 1	0.79	2.58	9.91	32.86	45.01	9.41
TG line 2	2.63	9.25	23.75	38.84	22.36	3.57

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Table II
Net Con A-mediated lysis of allogeneic targets (S49)

	3.7:1	11:1	33:1	100:1
WT A	18.5	28.2	31.1	30.2
WT B	21.2	19.4	23.9	26.8
TG A	12.4	30.0	32.2	40.6
TG B	8.2	18.5	21.2	24.1

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