IFN-γ Acts Directly on Activated CD4+ T Cells during Mycobacterial Infection to Promote Apoptosis by Inducing Components of the Intracellular Apoptosis Machinery and by Inducing Extracellular Proapoptotic Signals

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IFN-γ Acts Directly on Activated CD4+ T Cells during Mycobacterial Infection to Promote Apoptosis by Inducing Components of the Intracellular Apoptosis Machinery and by Inducing Extracellular Proapoptotic Signals

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Despite many studies, the regulation of CD4+ T cell apoptosis during the shutdown of immune responses is not fully understood. We have investigated the molecular mechanisms of IFN-γ in regulating apoptosis of CD4+ T cells during bacillus Calmette-Guérin (BCG) infection of mice. Our data provide new insight into the regulation of CD4+ T cell apoptosis by IFN-γ. As CD4+ T cells responded to BCG infection, there was a coordinated increase in IFN-γ production by effector CD4+ T cells and a coordinated IFN-γ-dependent up-regulation of many diverse apoptosis-pathway genes in effector CD4+ T cells. Unexpectedly, IFN-γ up-regulated transcripts and protein expression of Bcl-2, Bax, Bim, Bid, Apaf-1, and caspase-9 in activated CD4+ T cells—components of the apoptosis machinery that are involved in promoting mitochondrial damage-mediated apoptosis. Wild-type, but not IFN-γ knockout, CD4+ T cells underwent apoptosis that was associated with damaged mitochondrial membranes. IFN-γ also up-regulated expression of cell-extrinsic signals of apoptosis, including TRAIL, DR5, and TNFR1. Cell-extrinsic apoptosis signals from TNF-α, TRAIL, and NO were capable of damaging the mitochondrial membranes in activated CD4+ T cells. Moreover, activated CD4+ T cells from BCG-infected DR5, TNFR1, and inducible NO synthase knockout mice had impaired caspase-9 activity, suggesting impaired mitochondria-pathway apoptosis. We propose that IFN-γ promotes apoptosis of CD4+ T cells during BCG infection as follows: 1) by sensitizing CD4+ T cells to apoptosis by inducing intracellular apoptosis molecules and 2) by inducing cell-extrinsic apoptosis signals that kill CD4+ effector T cells. The Journal of Immunology, 2007, 179: 939–949.

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cells respond to infection by becoming activated proliferating effector cells. After the expansion of a large population of effector cells, the majority of the cells die by apoptosis. Apoptosis promotes homeostasis in the T cell compartment and eliminates potentially pathogenic effector T cells. A variety of molecules have been implicated in this process, yet the overall regulation of apoptosis in effector T cells is not understood. The regulation of apoptosis in T cells has been recently reviewed (1–3).

The TNF family of death receptors has been well-studied in apoptosis of activated T cells. However, there are many conflicting studies on the roles of TNF-related death receptors in apoptosis of activated T cells. Many studies have shown that mice with a deficiency of Fas, mice lacking both Fas and TNF-α, and mice lacking TNFR1 have impaired apoptosis or deletion of activated peripheral T cells (4–10). Many other studies have reported normal apoptosis or deletion of activated T cells in mice with a deficiency of Fas, mice with deficiencies of both TNFR1 and Fas, and even in mice with triple deficiencies of TNFR1, Fas, and TNFR2 (11–15). These studies highlight a controversy about whether death receptors such as Fas and TNFR1 play essential roles during apoptosis of activated T cells.

Recently, there has been increased emphasis on the Bcl-2-regulated pathway during T cell apoptosis (1, 16, 17). This pathway of apoptosis is initiated by damage to the mitochondrial membranes. Damage to the mitochondria is mediated by several pro-apoptotic members of the Bcl-2 family including, Bax and Bak, called “multidomain” proapoptotic proteins (18). Mitochondrial damage allows cytochrome c to escape from the mitochondria into the cytosol, to combine with Apaf-1, and to activate caspase-9 (19). Caspase-9 then initiates a death program in the cells by cleaving the executioner caspase-3.

The Bcl-2-regulated pathway is called the “intrinsic” pathway of apoptosis because it is triggered by damage to the mitochondria within the T cells. In contrast, death receptor-mediated apoptosis is called the “extrinsic” pathway because the signals for apoptosis originate from outside of the cells. The TNF-related death receptor-associated pathways and the Bcl-2-related mitochondria-mediated pathways of apoptosis are currently considered to be distinct and independently regulated during T cell apoptosis (1, 2, 16). The intracellular balance between prosurvival Bcl-2 proteins and the proapoptotic molecules Bak and Bax regulates mitochondrial membrane permeability and cellular apoptosis (20). Bim is also a proapoptotic Bcl-2 family member. In Bim knockout KO mice, 3

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3 Abbreviations used in this paper: KO, knockout; BCG, bacillus Calmette-Guérin; iNOS, inducible NO synthase; WT, wild type; DR5, death receptor 5; ROS, reactive oxygen species; 7-AAD, 7-aminoactinomycin D; TCM, T cell medium; MnTBAP, manganese (III) tetrakis 4-benzoic acid; SNAP, S-nitroso-N-acetyl-penicillamine; Δψm, mitochondrial transmembrane potential.
impaired apoptotic deletion prolongs the survival of responding T cells during several immune responses (12, 21–23). However, little is known about how Bim, Bax, Bcl-2, and Bak are induced or regulated during T cell apoptosis (12). It has been proposed that the Bcl-2-regulated pathway of apoptosis in T cells is triggered by withdrawal of growth factors after an acute infection is cleared (1). However, because some infections are prolonged (e.g., mycobacteria), this model of Bcl-2-pathway regulation may not apply to prolonged infections, where Ags persist.

IFN-γ is also involved in apoptosis and deletion of activated Ag-specific T cells. Mice lacking IFN-γ or the IFN-γ receptor exhibit impaired apoptosis or delayed contraction of activated Ag-specific T cells during many immune responses (24–29). Our previous work showed that IFN-γ was required for apoptosis of CD4+ T cells that were responding to bacillus Calmette-Guérin (BCG) infection. In vitro, we found a role for inducible NO synthase (iNOS) in apoptosis of activated CD4+ T cells during BCG infection (26). However, our recent work in CD4+ T cell-mediated CNS inflammation suggested that iNOS-independent pathways also contribute to apoptosis of CNS-infiltrating CD4+ T cells. Such pathways appeared to be dependent on IFN-γ (30). However, these other putative IFN-γ-dependent pathways of CD4+ T cell apoptosis have yet to be identified. To further understand the role of IFN-γ in apoptosis of CD4+ T cells, we used the BCG infection model to compare differential expression of apoptosis-pathway genes in wild-type (WT) activated CD4+ T cells with that of IFN-γ KO CD4+ T cells.

The data in this study provide novel evidence that IFN-γ induces many diverse apoptosis-related molecules in activated CD4+ T cells. An unexpected result was that IFN-γ coordinately induced the expression of Bcl-2, Bax, Bim, Bid, and Apaf-1 within activated CD4+ T cells. IFN-γ also coordinately induced the expression of several caspases, including caspase-9. All of these molecules are components of intracellular apoptosis pathways and promote the destruction of CD4+ T cells from within. IFN-γ also induced transscripts and proteins of extracellular signals of apoptosis in activated CD4+ T cells, including TRAIL, death receptor 5 (DR5), TNF-α, and TNFR1, in a coordinated manner during CD4+ T cell activation. Activated CD4+ T cells in WT, but not in IFN-γ KO, BCG-infected mice underwent apoptosis that was associated with damaged mitochondrial membranes. WT CD4+ T cells could be rescued from apoptosis by neutralizing intracellular signals during CD4+ T cell activation. Activated CD4+ T cells in WT, but not in IFN-γ KO, BCG-infected mice underwent apoptosis that was associated with damaged mitochondrial membranes. WT CD4+ T cells could be rescued from apoptosis by neutralizing intracellular reactive oxygen species (ROS). This indicates that IFN-γ is required for mitochondria-mediated apoptosis of T cells during mycobacterial infection. Also, several IFN-γ-inducible “cell-extrinsic” molecules were capable of damaging the mitochondrial membranes. Our results suggest that one major role for IFN-γ is to induce essential intracellular components of the apoptosis machinery in CD4+ T cells, thereby sensitizing the cells to proapoptotic signals during CD4+ T cell activation. A second role is for IFN-γ to induce the expression of several cell-extrinsic apoptosis signals that can damage the mitochondria and induce CD4+ T cell apoptosis. This study reveals previously unknown connections between many molecules that have been implicated in T cell apoptosis. Additionally, this study provides new information on how activated CD4+ T cells undergo apoptosis during a prolonged infection.

Materials and Methods

Mice

Mice were purchased from The Jackson Laboratory or bred at the Trudeau Institute: B6.129s7-Ifngtm1Lyd (IFN-γ KO), B6.129 Ifngtm1Apt (IFN-γ R KO), B6.129S6-Cybmtm1DsnJ (gp100-spec. KO), B6.129Nos2tm1Lau (iNOS KO mice), C57BL/6J (WT or B6 mice), and Tnfrsf1aintm1Mak (TNFR1). DR5 KO mice, backcrossed ≥5 generations onto the C57BL/6J background, were a gift from Dr. T. Mak (University of Toronto, Ontario, Canada). All other mice were backcrossed ≥10 generations onto the C57BL/6J background. Experiments with mice were approved by the Trudeau Institute Institutional Animal Care and Use Committee.

Bacteria and infection

Mycobacterium bovis BCG (Pasteur) was grown from seed stocks derived from the Trudeau Institute Mycobacterial Collection (TM1011). Live BCG was stored as a frozen suspension. Mice were infected i.v. with 0.2 ml of BCG (2 × 10^6 CFU).

Flow cytometry

Mononuclear cells were prepared from the spleens and were stained with Abs, collected on a FACSCalibur instrument (BD Biosciences) and analyzed using FlowJo software. The following conjugated Abs were obtained from BD Pharmingen or eBioscience: anti-CD4-allophycocyanin (RM4-5), anti-CD4-allophycocyanin-Cy7 (GK1.5), anti-CD44-PE (IM7), anti-CD45RB-PE (M1/H-24), and anti-CD62L-PE (MEL-14), anti-DR5 (MD5.1), anti-TNFR1 (55R-286) was from Abcam. To detect surface receptors, cells were incubated with 0.015 µg of either anti-DR-5-PE or anti-TNFR1-PE for 30 min. 7-Aminoactinomycin D (7-AAD; eBioscience) was added to each tube just before collection of cells. For the annexin V/7-AAD assay, whole spleen cells or FACS-sorted CD4+CD44sh T cells (> 2 × 10^7) were stained with anti-CD4-allophycocyanin and anti-CD44-PE and then were washed in annexin buffer and the cells were then collected by centrifugation. Staining and FACS collection was done as described (26) except that 2 µl of Annexin V-FITC was added to each pellet to saturate the cells. Lymphocytes with low forward-scatter, which were annexin V+ and 7-AAD-, were included in the lymphocyte gate, and then a gate was set on CD4+CD44sh T cells. The annexin V+CD4+CD44sh T cells included cells in both early and late stages of apoptosis.

Cell culture

CD4+ T cells were enriched from the spleens of mice using MACS sorting (Miltenyi Biotec). Where indicated, the CD4+ T cell population was FACS sorted to isolate CD4+CD44sh or CD4+CD44th T cells.

For anti-CD3 stimulation, naive CD4+ T cells were plated at a density of 10^6 cells/ml in T cell medium (TCM) (26), which normally contains 2.5 ng/ml IL-2. However, for anti-CD3 activation, cells were cultured in TCM without IL-2 (± 20 ng/ml IFN-γ), with soluble anti-CD3 (1 µg/ml) and anti-CD28 (10 µg/ml) for 3 days. Viable cells were then isolated on a Ficoll gradient and plated on anti-CD3-coated plates for 20 h (± 20 ng/ml IFN-γ). An aliquot of anti-CD3-stimulated cells was used for apoptosis measurements and the remaining cells were used for RNA preparation and RT-PCR. All cytokines used in the cell cultures were purchased from R&D Systems.

To inhibit ROS-mediated apoptosis in activated CD4+ T cells, a superoxide dismutase mimetic, manganese (III) tetrakis 4-benzoic acid (MnTBAP; Calbiochem) was used. CD4+ T cells were MACS sorted from the spleens of day 21 WT and IFN-γ KO BCG-infected mice. MnTBAP stock, prepared in 0.1 M NaOH, was added to the CD4+ T cells. Apoptosis of CD4+CD44th T cells was measured after 24 h with the annexin V and 7-AAD assay.

Hydrogen peroxide (H2O2) was used to induce apoptosis in activated CD4+ T cells from day 21 BCG-infected IFN-γ KO mice. Sorted CD4+ T cells (>95% CD44sh) were cultured in TCM with H2O2 at a concentration of 15 µM, and IFN-γ was added to indicated wells at a concentration of 20 ng/ml. Apoptotic death was measured after 4 h using the annexin V and 7-AAD assay.

For sensitization of CD4+ T cells to TRAIL, TNF-α, and NO-donor S-nitroso-N-acetyl-penicillamine (SNAP)-mediated apoptosis, MACS-sorted CD4+ T cells were prepared from day 21 BCG-infected IFN-γ KO mice. Sorted CD4+ T cells (>95% CD44sh) were cultured in TCM with H2O2 at a concentration of 15 µM, and IFN-γ was added to indicated wells at a concentration of 20 ng/ml. Apoptotic death was measured after 4 h using the annexin V and 7-AAD assay.

For sensitization of CD4+ T cells to TRAIL, TNF-α, and NO-donor S-nitroso-N-acetyl-penicillamine (SNAP)-mediated apoptosis, MACS-sorted CD4+ T cells were prepared from day 21 BCG-infected IFN-γ KO mice and cultured in TCM for 16 h with IL-2 (2.5 ng/ml) + 10 ng/ml IFN-γ. Cells were washed three times with TCM and TCM ± SNAP (Calbiochem), ± rTRAIL, or ± TNF-α was added. After 32 additional hours of culture, cells were harvested, stained with anti-CD4, anti-CD44, annexin 7-AAD, or JC-1 and analyzed by flow cytometry. For experiments on Fig. 5, D and F, cells were prepared as above, cultured in medium ± IFN-γ, washed three times, then cultured with 50 ng/ml TRAIL or TNF-α.

Cell and RNA preparation for RT-PCR

CD4+ T cells from naive or BCG-infected WT and IFN-γ KO mice on days 0, 14, and 21 were MACS sorted from pooled spleen cells. Naive
CD4<sup>+</sup>CD4<sup>44<sup>high</sup></sup> and BCG-infected CD4<sup>+</sup>CD4<sup>44<sub>low</sub></sup> T cells were further isolated by FACS sorting. RNA was isolated from ≥5 × 10<sup>5</sup> sorted CD4<sup>+</sup>CD4<sup>44<sub>high</sub></sup> T cells (or CD4<sup>+</sup>CD4<sup>44<sub>low</sub></sup> T cells) using an RNasey mini kit (Qiagen). Genomic DNA was eliminated with DNase. The RNA concentration and purity was determined by measuring OD at 260 and 280 nm.

**Real-time quantitative RT-PCR**

cDNA was prepared using Superscript II reverse transcriptase using 500 ng of RNA. Quantitative PCR was performed using ABI 7700 and reagents from Applied Biosystems. The primer sequences for SYBR Green quantitative PCR are listed in Table I, as are the expected lengths and melting temperatures. The fold induction of each gene in activated CD4<sup>+</sup>/H9004 T cells was not influenced by IFN-γ KO. 10<sup>6</sup> spleen cells were resuspended in 150 μl of TCM, incubated 15 min with 2.5 μg/ml JC-1 at 37°C. After FACS col-
Thy 1.2+ donor-derived CD4+CD44high cells and the annexin and 7-AAD assay was done to detect the percentage of gated cells that were apoptotic and dead. \( \Delta \phi \) of the cells was measured using the fluorescent lipophilic cationic JC-1 dye as described above. A control group of BCG-infected IFN-γ receptor KO mice was not used for adoptive transfer studies. The control mice were used to measure annexin V and JC-1 fluorescence of CD4+CD44high T cells within BCG-infected IFN-γ receptor KO mice.

### Results

**IFN-γ regulates the transcription of many diverse apoptosis-pathway genes in activated CD4+ T cells**

During BCG infection, mice generate a large population of activated CD4+ T cells. These cells are enlarged and express surface markers characteristic of effector T cells: CD4+CD44high CD62Llow, as we have previously shown (26). A large population of CD4+ T cells with an “effector phenotype” (32, 33) was not evident in 6- to 10-wk-old naive WT or IFN-γ KO mice, suggesting that the majority of splenic effector CD4+ T cells were induced by infection of the mice with BCG. We have previously characterized the kinetics of expansion and contraction of the responding CD4+ effector T cell population during BCG infection (26). In WT mice, the CD4+ effector T cell population in the spleens expanded 10-fold (from 2.6 \( \times \) 10^6 cells/spleen to 25.5 \( \times \) 10^6 cells/spleen) between days 0 and 23. After reaching a peak, the WT CD4+ effector T cells contracted to 6.46 \( \times \) 10^6 cells/spleen by day 35 of infection. This expansion and contraction of CD4+ effector T cells occurred during persisting BCG infection in the spleens. The BCG CFU per spleen ranged between 2 \( \times \) 10^7 CFU on day 1, 3 \( \times \) 10^6 CFU on day 21, to 3 \( \times \) 10^5 CFU on day 35. Therefore, in WT mice, the expansion and contraction of the splenic CD4+ T effector cells occurred despite the persisting BCG organisms in the spleen. We used the polyclonal CD4+ T cell effector population for analysis because our experiments required large numbers of unfixed cells that cannot be isolated from BCG-infected mice using standard methods for detecting Ag-specific T cells, e.g., intracellular cytokine secretion assays.

In WT mice, the activated splenic CD4+CD44high effector T cell population (hereafter called “CD4+ T cells,” unless otherwise stated) exhibited an increasing percentage of apoptotic (annexin V+) cells between days 14 and 21 of BCG infection, as expected (26) (Fig. 1A). Although there was a high percentage of annexin V+CD4+ T cells at the peak of the expansion phase, the WT splenic CD4+ effector T cell population was not entirely killed during the contraction phase (26). In IFN-γ KO mice, the percentage of apoptotic CD4+ T cells decreased significantly between days 14 and 21 of BCG infection (Fig. 1A), indicating an essential role for IFN-γ in promoting apoptosis of the effector CD4+ T cell population.

To gain further insight into the role of IFN-γ during CD4+ T cell apoptosis, we first did a microarray analysis for expression of apoptosis genes in activated WT and IFN-γ KO CD4+ T cells taken from BCG-infected spleens (data not shown). We then measured the expression of a subset of apoptosis-pathway genes in WT and IFN-γ KO CD4+ T cells. The CD4+CD44high T cell population was sorted ex vivo from BCG-infected WT and IFN-γ KO spleens on days 14 and 21. Quantitative RT-PCR was used to measure transcripts of apoptosis-pathway genes. IFN-γ and many diverse proapoptotic transcripts were coordinately up-regulated during CD4+ T cell activation in WT CD4+ T cells (Fig. 1B). In contrast to WT, IFN-γ KO-activated CD4+ T cells exhibited little induction of most of the transcripts above the levels expressed in naive WT and IFN-γ KO CD4+ T cells. There was no difference in either apoptosis or expression of apoptosis-related genes in naive CD4+CD44high T cells sorted from naive WT and IFN-γ KO mice (Fig. 1, A and B). Also addition of IFN-γ to WT or IFN-γ KO naive CD4+ T cells had no effect on their apoptosis or gene expression (X. Li and D. K. Dalton, unpublished data).

We next tested gene expression in anti-CD3-activated CD4+ T cells to ask whether IFN-γ-dependent expression of apoptosis-related genes was peculiar to only CD4+ T cells in BCG-infected mice. Naïve WT and IFN-γ KO CD4+ T cells were enriched and then activated in vitro with anti-CD3 and anti-CD28. After 3 days, the viable activated cells from these cultures were enriched and then restimulated with immobilized anti-CD3 to promote activation-induced cell death (34). After 20 h of anti-CD3 restimulation, activated WT CD4+ T cells had nearly twice the percentage of apoptotic and dead cells compared with activated IFN-γ KO CD4+ T cells (Fig. 1C). Addition of exogenous IFN-γ to IFN-γ KO CD4+ T cells during the 20 h restimulation with anti-CD3 increased the percentage of annexin V+ cells of the IFN-γ KO CD4+ T cells (Fig. 1C). RT-PCR analysis of apoptosis-pathway genes showed that exogenous IFN-γ added to IFN-γ KO CD4+ T cells during restimulation also induced the transcription of proapoptotic Bcl-2 genes, TNF-superfamily genes, several caspases, and the adaptor molecule Apaf-1 (Fig. 1C). Our data confirm and extend a previous report showing that IFN-γ induced caspase-3 and -8 expression in activated CD3-stimulated T cells in vitro (28). Our new data show that IFN-γ-dependent expression of caspases-3 and -8 in vitro is part of a more extensive role for IFN-γ in sensitizing CD4+ T cells to apoptosis. An unexpected and novel result was that IFN-γ up-regulated expression of apoptosis-related molecules in the Bcl-2 family of genes in both anti-CD3- and BCG-stimulated CD4+ effector T cells. These data indicate that IFN-γ-dependent up-regulation of apoptosis pathway genes in CD4+ T cells is not peculiar to BCG infection, but also occurs during activation of CD4+ T cells by anti-CD3.

**IFN-γ regulates protein expression of apoptosis-related molecules**

Various genes of interest were further analyzed for protein expression/enzyme activity in WT and IFN-γ KO CD4+CD44high T cells taken ex vivo from BCG-infected mice. The enzyme activity of caspases-9, -8, and -3 was significantly greater in WT CD4+ T cells compared with IFN-γ KO cells (Fig. 2A). Caspase-9 is activated in the apotosome after cytochrome c is released from damaged mitochondria (19). This suggested that WT CD4+ T cells may undergo mitochondria-pathway apoptosis during BCG infection. Additionally, the proapoptotic proteins Bax, Bim, and Bid, which promote mitochondrial pathway apoptosis, were expressed 5- to 9-fold higher in FACS-sorted WT CD4+CD44high T cells compared with IFN-γ KO cells (Fig. 2B). The antiapoptotic Bcl-2 protein was expressed 2-fold higher in WT CD4+ T cells compared with IFN-γ KO cells. Two other intracellular proteins involved in mitochondria-mediated apoptosis, Apaf-1, a major component of the apotosome, and caspase-9, were also expressed in an IFN-γ-dependent manner in CD4+ T cells (Fig. 2B). Some proteins could be induced by culturing IFN-γ KO CD4+ T cells with exogenous IFN-γ (Fig. 2B). The data indicate that, during BCG infection, IFN-γ up-regulated both transcripts and proteins of components of the intracellular apoptotic machinery in CD4+ effector T cells.

WT, but not IFN-γ KO, CD4+ T cells underwent apoptosis that was associated with loss of \( \Delta \psi _m \)

We next investigated the role of IFN-γ in the mitochondrial pathway of apoptosis in WT and IFN-γ KO CD4+ T cells. A characteristic feature of Bim- and Bax-associated apoptosis is the loss of \( \Delta \psi _m \), indicating damage to the mitochondria. Such damage can be detected with the cationic dye, JC-1, which emits fluorescence at
590 nm when it is aggregated within undamaged mitochondria. Thus, a loss of fluorescence at 590 nm indicates that the mitochondrial membranes are damaged and permeable (35). More than 50% of WT activated CD4$^{+}$/H11001 T cells from BCG-infected mice exhibited low JC-1 fluorescence (FL-2 low), indicating that these cells had damaged mitochondrial membranes (Fig. 3, A and C). In contrast, activated CD4$^{+}$/H11001 T cells from BCG-infected IFN-$\gamma$/H9253 KO mice had JC-1 fluorescence profiles similar to those of healthy naive CD4$^{+}$ CD44$^{low}$ T cells, in which the majority of cells had intact mitochondrial membranes (Fig. 3, B and C).

Mitochondria-mediated apoptosis also releases reactive oxygen species from the permeable mitochondria into the cytosol (36). Cells undergoing mitochondria-mediated apoptosis can be rescued from death if intracellular ROS are neutralized in vitro (37). To neutralize ROS, we cultured highly enriched CD4$^{+}$/H11001 T cells with a synthetic antioxidant, MnTBAP. Neutralizing intracellular ROS with MnTBAP rescued WT CD4$^{+}$ T cells from apoptosis (Fig. 3D). In contrast, neutralizing intracellular ROS had less effect on IFN-$\gamma$/KO CD4$^{+}$ T cells, which remained viable in culture (Fig. 3D). These data indicate that IFN-$\gamma$ is required for intracellular...
ROS-associated mitochondria-mediated apoptosis of CD4+ T cells during BCG infection.

We next investigated the responses of IFN-γ KO CD4+ T cells to exogenous ROS. IFN-γ KO CD4+ T cells were cultured in several conditions, then apoptosis was measured (Fig. 3E). TCM ± IFN-γ had little effect on isolated IFN-γ KO CD4+ T cells. Medium with hydrogen peroxide increased the apoptosis of IFN-γ KO CD4+ T cells. However, IFN-γ enhanced hydrogen peroxide-mediated apoptosis of IFN-γ KO CD4+ T cells (Fig. 3E).

These data indicate that IFN-γ sensitizes CD4+ T cells to apoptosis by exogenous ROS in vitro.

We next tested whether ROS has an in vivo role in apoptosis of CD4+ T cells. Mice with a genetic lesion in the NADPH subunit gp91phox (gp91phox KO mice) cannot produce myeloid cell-derived ROS (38). This strain of mice was used to test the in vivo role of exogenous ROS in apoptosis. Unexpectedly, CD4+ T cells from day 21 BCG-infected gp91phox KO mice exhibited significantly lower caspase-9 activity than those from WT mice (p < 0.006, representative of two to three experiments).

We next tested whether ROS has an in vivo role in apoptosis of CD4+ T cells. Mice with a genetic lesion in the NADPH subunit gp91phox (gp91phox KO mice) cannot produce myeloid cell-derived ROS (38). This strain of mice was used to test the in vivo role of exogenous ROS in apoptosis. Unexpectedly, CD4+ T cells from day 21 BCG-infected gp91phox KO mice exhibited significantly lower caspase-9 activity than those from WT mice (p < 0.006, representative of two to three experiments).
increased apoptosis (data not shown) and caspase-9 activity (Fig. 3F) compared with WT cells. Thus, although CD4+ T cells are capable of being killed by ROS in vitro, NADPH oxidase-derived ROS are not required in vivo for caspase-9 activity in CD4+ T cells during BCG infection.

The gp91phox KO mice have been reported to produce increased amounts of NO (39). We next determined the role of NO in promoting caspase-9 activity in CD4+ T cells in vivo. CD4+ T cells from BCG-infected iNOS KO mice had significantly lower caspase-9 activity compared with cells from BCG-infected WT mice. However, caspase-9 activity in iNOS KO CD4+ T cells was intermediate between that of WT and IFN-γ KO CD4+ T cells (Fig. 3F). This suggested there are other IFN-γ-dependent molecules that can also trigger the mitochondria-mediated pathway of T cell apoptosis in vivo.

Several proapoptotic molecules trigger the intrinsic mitochondria-mediated pathway of apoptosis in CD4+ T cells from BCG-infected mice

We next tested the effect of several cell-extrinsic molecules on causing mitochondrial damage of CD4+ T cells. MACS-sorted IFN-γ KO CD4+ T cells were cultured with IFN-γ, and then with a NO donor molecule, SNAP. Treatment of CD4+ T cells with SNAP resulted in significantly increased damage to the mitochondrial membranes (ΔΨm) compared with cells cultured without SNAP (Fig. 4A), and resulted in an increased percentage of dead cells at a concentration of 250 μM SNAP (Fig. 4B). This formally demonstrates that exogenous NO can cause mitochondrial membrane damage and death of CD4+ T cells. Additionally, the death ligands TNF-α and TRAIL also caused significant mitochondrial damage to activated CD4+ T cells (Fig. 4, C and D). These results indicate that several cell-extrinsic molecules can cause damage to the mitochondrial membranes in CD4+ T cells.

**IFN-γ up-regulated death receptors on CD4+ T cells and sensitized the cells to apoptosis by TRAIL and TNF-α**

Consistent with the difference in gene transcription, a higher percentage of WT CD4+ T cells expressed cell-surface DR5 (Fig. 5A) and TNFR1 (Fig. 5B) compared with IFN-γ KO cells ex vivo. After 16 h of culture with IFN-γ, the surface expression of DR5 on IFN-γ KO CD4+ T cells was up-regulated by IFN-γ in a dose-dependent manner (Fig. 5C). IFN-γ also sensitized CD4+ T cells to apoptosis by rTRAIL (Fig. 5D). That is, cells cultured in IFN-γ and then in TRAIL were significantly more apoptotic than cells cultured first in medium and then with TRAIL. Additionally, TRAIL protein was expressed at higher levels in WT CD4+ CD44+B cells compared with IFN-γ KO cells as shown by an immunoblot (Fig. 5G). Furthermore, IFN-γ induced the expression of TNFR1 on IFN-γ KO CD4+ T cells in a dose-dependent manner (Fig. 5E). IFN-γ also sensitized CD4+ T cells to TNF-α-mediated apoptosis (Fig. 5F). These results indicate that IFN-γ sensitized activated CD4+ T cells to undergo apoptosis, at least in part, by up-regulating protein expression of DR5, TNFR1, and TRAIL in CD4+ T cells.

**Mice with gene deletions of DR5, TNFR1, and iNOS had significantly lower apoptosis and caspase activity of CD4+ T cells during BCG infection**

To test the in vivo roles of molecules that were characterized in vitro, we used gene KO mice. CD4+ T cells from WT and IFN-γ KO BCG-infected mice were analyzed in parallel with CD4+ T cells from BCG-infected DR5 KO, TNFR1 KO, and iNOS KO mice in multiple experiments (Fig. 6). The percentage of CD4+ CD44+B cells that were annexin V+ and had active caspases 3 and 8 was significantly lower in DR5 KO, TNFR1 KO, and iNOS KO mice compared with WT mice. The previous in vitro data suggested that signals from NO, TRAIL, and TNF-α trigger the mitochondria-mediated pathway of apoptosis in activated CD4+ T cells. Mitochondrial membrane damage activates caspase-9 in the apoptosome after cytochrome c is released (19, 40). Caspase-9 activity was also significantly lower in CD4+ CD44+B cells that were annexin V+ and had active caspases 3 and 8 in WT BCG-infected iNOS, DR5, and TNFR1 KO mice compared with WT cells. Decreased caspase-9 activity in the iNOS, TNFR1, and DR5 gene KO mice supports the idea that cell extrinsic signals from NO, TNF-α, and TRAIL are mediators of mitochondrial damage to activated CD4+ T cells in vivo during BCG infection. It has been suggested that caspase-9 serves to amplify signals when caspase-8 has been activated via the death receptors (41). These data indicate that at least three IFN-γ-induced proapoptotic signals individually contribute to promoting apoptosis and caspase enzyme activity in CD4+ T cells.

**Expression of the IFN-γ receptor on activated CD4+ T cells was required for apoptosis in WT BCG-infected recipients**

The in vitro experiments above showed that IFN-γ acted directly on purified IFN-γ KO CD4+ T cells to sensitize them to apoptosis...
Groups of three gene knockout mice were infected with BCG in parallel with groups of three WT and IFN-γ KO mice. On day 21 of BCG infection, the spleen cells were isolated and prepared for FACS. A gate was set on CD4+CD44high T cells that had active caspases-3, -8, and -9 and was measured using fluorescent molecules that bind to the active site of each enzyme. Each point is the mean and SD of six to nine mice per genotype done in two to three independent experiments. The values for CD4+CD44high T cells in each group of gene knockout mice were compared with the values in WT CD4+CD44high T cells. A. Percent of cells that were annexin V+ (***, p < 0.001 for IFN-γ KO, DR5 KO, and TNFR1 KO; p ≤ 0.012 for iNOS KO). B. Percent of cells with active caspase-8 (***, p < 0.0002). C. Percent of cells with active caspase-3 (**, p < 0.0001), D. Percent of cells with active caspase-9 in KO mice (***, p < 0.009; *, p < 0.04). Percentage for each group was compared with WT group using the Mann-Whitney U test.

FIGURE 5. IFN-γ up-regulated the surface expression of TNFR1 and DR5 in isolated IFN-γ KO CD4+ T cells and sensitized the cells to apoptosis by exogenous TRAIL and TNF-α. Spleen cells were prepared from day 21 BCG-infected WT and IFN-γ KO mice. The cells were stained directly ex vivo with Abs to detect surface expression of DR5 and TNFR1. Each bar or point is the mean and SD of cells from mice taken ex vivo or cultured as indicated in figure. WT CD4+CD44high T cells exhibited an increased percentage of (A) DR5+ and (B) TNFR1+ cells compared with IFN-γ KO CD4+CD44high T cells (**, p < 0.0001, t test). C. CD4+CD44high T cells were prepared from the spleens of BCG-infected IFN-γ KO mice and the cells were cultured in vitro with indicated concentrations of IFN-γ. After 16 h of culture, the expression of DR5 on CD4+CD44high T cells was measured by FACS. Shown is the percent of cells that are DR5+. D. CD4+CD44high T cells were prepared from IFN-γ KO spleens and were cultured in either medium or IFN-γ as in Materials and Methods. Shown is the percentage of CD4+CD44high T cells that were annexin V− (**, p ≤ 0.005; t test). E. Pure IFN-γ KO CD4+CD44high T cells from day 21 BCG-infected spleens were cultured for 16 h with IFN-γ and FACS was used to measure TNFR1 expression. F. Cells were cultured as described in Materials and Methods with IFN-γ or not. Shown is the percentage of cells that were annexin V− in each condition. (**, p ≤ 0.003: Student’s t test). G. An immunoblot of cell lysates was done to detect the expression of TRAIL in CD4+CD44high T cells. The same procedures and samples were used as were described in the Fig. 2B legend and Materials and Methods. Numbers above the blots are the relative intensities of full-length TRAIL + soluble TRAIL in each sample compared with the amount in ex vivo IFN-γ KO CD4+CD44high T cells arbitrarily set to a value of 1.

FIGURE 6. Mice with genetic lesions in DR5, TNFR1, and iNOS exhibited significantly lower percentages of activated CD4+ T cells that were apoptotic and that had active caspases during BCG infection. Groups of three gene knockout mice were infected with BCG in parallel with groups of three WT and IFN-γ KO mice. On day 21 of BCG infection, the spleen cells were isolated and prepared for FACS. A gate was set on CD4+CD44high T cells and the percentage of cells that were apoptotic was measured by annexin V staining. The percentage of CD4+CD44high T cells that had active caspas-3, -8, and -9 was measured using fluorescent molecules that bind to the active site of each enzyme. Each point is the mean and SD of six to nine mice per genotype done in two to three independent experiments. The values for CD4+CD44high T cells in each group of gene knockout mice were compared with the values in WT CD4+CD44high T cells. A. Percent of cells that were annexin V+ (***, p < 0.001 for IFN-γ KO, DR5 KO, and TNFR1 KO; p ≤ 0.012 for iNOS KO). B. Percent of cells with active caspase-8 (***, p < 0.0002). C. Percent of cells with active caspase-3 (**, p < 0.0001). D. Percent of cells with active caspase-9 in KO mice (***, p < 0.009; *, p < 0.04). Percentage for each group was compared with WT group using the Mann-Whitney U test.

by exogenous ROS, NO, TRAIL, and TNF-α. We next investigated the in vivo role of IFN-γ signaling on CD4+ T cells in the following replicate experiments. Cohorts of WT and IFN-γ receptor KO mice were infected with BCG. The CD4+ T cells in BCG-infected IFN-γ receptor KO mice received no signals from IFN-γ during T cell activation within BCG-infected IFN-γ receptor KO mice. On day 17, the CD4+ T cells (70% CD44 high) from three BCG-infected IFN-γ receptor KO mice were MACS sorted, pooled, and transferred into three different BCG-infected WT Thy 1.1 recipients. On day 21, CD4+ T cells in the recipient spleens were analyzed by flow cytometry. A gate was set on Thy 1.1+ CD4+CD44high (WT recipient) and Thy 1.2+ CD4+CD44high (IFN-γ receptor KO donor) cells prepared from WT recipient BCG-infected spleens. As controls for CD4+ T cell apoptosis without IFN-γ signaling, cohorts of IFN-γ receptor KO mice were BCG infected, but the CD4+ T cells were not transferred to WT recipients. IFN-γ receptor KO donor CD4+ T cells taken from BCG-infected WT spleens had significantly lower mitochondrial membrane damage and apoptosis compared with WT CD4+ T cells within the same spleens (Fig. 7). In fact, the IFN-γ receptor KO donor CD4+ T cells were as viable and their mitochondria as undamaged in WT BCG-infected spleens as they were in control IFN-γ receptor KO BCG-infected spleens (Fig. 7). These data support the idea that IFN-γ acts directly on activated CD4+ T cells to
FIGURE 7. IFN-γ receptor (IFN-γ R) KO-activated CD4+ T cells did not undergo apoptosis in BCG-infected WT spleens. Groups of WT and IFN-γ R KO mice were infected with BCG. On day 17, the IFN-γ R KO spleens were prepared and pooled. CD4+ T cells were enriched by MACS sorting, and 10^7 CD4+Thy 1.2+ IFN-γ R KO donor cells were injected by i.v. route into three individual WT B6-congenic Thy 1.1+ recipient mice. On day 21, the WT spleens were prepared and annexin V and 7-AAD and JC-1 staining was done. Shown is the percentage of the donor and host spleen cells, gated on CD4+CD44+ Thy 1.2+, or Thy 1.1+ cells that were (A) JC-1+high and (B) annexin V+. Also shown (IFN-γ R KO) are values from CD4+CD44+ Thy 1.2+ T cells in BCG-infected IFN-γ R KO mice where CD4+ T cells were not transferred into WT recipients. Values were obtained in two independent experiments and significance was measured using the Mann-Whiney test (**, p = 0.0022).

sensitize them to mitochondrial damage and apoptosis during BCG infection.

Discussion
In this study, we investigated the molecular mechanisms for IFN-γ-dependent apoptosis of CD4+ effector T cells during BCG infection of mice. We propose the following model for apoptosis of activated CD4+ T cells during BCG infection. As the CD4+ T cells become activated by BCG infection, they produce IFN-γ. As the activated CD4+ T cells produce IFN-γ, it acts directly on the CD4+ T cells to prepare them to respond to proapoptotic signals. The activated CD4+ T cells are conditioned by IFN-γ to respond to proapoptotic signals in several ways (Fig. 8). IFN-γ up-regulates important components of the intracellular apoptosis machinery in activated CD4+ T cells. These include Bax, Bid, Bim, Apaf-1, Bcl-2, and several caspase enzymes. IFN-γ also directly acts on activated CD4+ T cells to prepare them to respond to proapoptotic signals by inducing the expression of the TRAIL receptor DR5, and TNFR-1. Finally, IFN-γ promotes the expression of cell-extrinsic proapoptotic signals TRAIL and TNF-α from CD4+ T cells, as well as, NO and TNF-α from activated macrophages. These molecules are capable of damaging the mitochondria and causing apoptosis in vitro. Mice with genetic deficiencies in these apoptosis pathways also had impaired caspase-9 activity. Caspase-9 is usually activated in the apoptosis after the mitochondria have been damaged (19, 40). This supports the in vivo roles of these pathways in promoting mitochondria-mediated apoptosis. It has been reported that the IFN-γ receptor 2, required for signaling by IFN-γ, is down-regulated in vitro by IFN-γ in activated CD4+ T cells (42). Our data suggest IFN-γ has its effects on CD4+ T cells before their down-regulation of IFN-γ receptor 2.

IFN-γ is also required for controlling the growth of the BCG organisms (26). A dual role of IFN-γ for controlling the growth of BCG and for promoting apoptosis of the critical CD4+ effector T cells probably reflects a regulatory network involving negative feedback inhibition. Once IFN-γ has been secreted, the activated CD4+ T cells have performed their effector functions of recruiting macrophages to the sites of infection and activating the macrophages. In a classical negative feedback loop, the end product shuts down its own production. Secretion of IFN-γ by CD4+ T cells sensitizes them to die, thereby shutting down IFN-γ production. Secretion of IFN-γ also stimulates immune cells to produce proapoptotic and cytotoxic molecules such as NO, TNF-α, and TRAIL, which contribute to the death of CD4+ effector T cells. Because production of IFN-γ and subsequent activation of macrophages is the desired outcome of the Th1 immune response, negative feedback inhibition can explain, at least in part, the paradoxical pro and anti-inflammatory activities of IFN-γ. The production of IFN-γ by CD4+ T cells induces a robust proinflammatory response while at the same time promoting the shutdown of the CD4+ T cells that are orchestrating the response.

The relationship between T cell contraction and persisting bacteria has been previously addressed by several studies. Our previous study demonstrated that in WT mice with BCG infection, the splenic effector CD4+ T cell population expands and contracts despite the continuing presence of large numbers of BCG in the spleens of infected mice (26). We have now shown that activated IFN-γ receptor KO CD4+ T cells did not undergo increased apoptosis even when transferred into WT mice, where the BCG growth is under immunological control. In a different study, the duration of infection and Ag display during Listeria infection had a minimal effect on the expansion and contraction of CD4+ T cells (43). Similarly, during infection with attenuated Listeria, the Ag-specific CD4+ T cell population in IFN-γ KO mice showed a prolonged contraction phase despite complete clearance of the bacteria (27). Our study contributes to the increasing evidence suggesting that during prolonged infections IFN-γ and IFN-γ receptor signaling are more important for T cell apoptosis and contraction than is clearance of the infection.

Systemic BCG infection was used as model to study apoptosis of effector CD4+ T cells during a prolonged infection. BCG infection results in the expansion of a large population of effector CD4 T cells undergoing IFN-γ-dependent apoptosis in the spleens.
of WT, but not IFN-γ KO, mice. This model allowed us to study the molecular mechanisms of IFN-γ-dependent apoptosis of the effector CD4+ T cell population. We have previously shown that a substantial population of effector CD4+ T cells persists in the WT spleens after expansion and contraction of the effector CD4+ T cell population, despite the persisting mycobacteria (26). Similarly, during chronic infection with Mycobacterium tuberculosis a stable population of effector CD4+ T cells persists in the lungs despite large numbers of persisting mycobacteria (44).

However, BCG is normally used as a localized s.c. vaccine to confer resistance to tuberculosis in humans and in experimental animal models (45). It is likely that the effector CD4+ T cells generated by BCG vaccination are primed locally and then enter the circulation. In this way, the effector CD4+ T cells can migrate away from the site of infection, thereby decreasing the probability of their re-exposure to Ag and the likelihood of extensive apoptosis.

Several studies support the idea that re-exposure of Th1 effector CD4+ T cells to Ag is likely to induce IFN-γ production and apoptosis. One study reported that adoptively transferred resting Th1 cells, upon re-encounter with cognate Ag, secreted IFN-γ and underwent increased apoptosis (46). Interestingly, the peak production of IFN-γ and the peak of increased apoptosis of CD4+ effector cells coincided. It has also been reported that after adoptive transfer, Th1 cells that secrete IFN-γ are rapidly eliminated from their host, whereas Th1-polarized cells that did not secrete IFN-γ persist longer in their host and mediated recall immunity (47). These studies suggest that memory T cells remaining after mycobacterial exposure might reside in a population of cells that do not express IFN-γ until they are challenged (45, 48).

BCG-infected DR5 KO, TNFR1 KO, and iNOS KO mice all had impaired CD4+ T cell apoptosis and caspase activity. However, unlike the IFN-γ KO mice (26), none of these strains accumulated excessive numbers of CD4+ CD44high T cells on day 21 of BCG infection (data not shown). Our interpretation of this data is that in knockout mice lacking a single upstream signal of apoptosis, other IFN-γ-dependent pathways can eliminate CD4+ effector T cells. Also, multiple redundant extracellular proapoptotic signals could provide an explanation for many contradictory results on whether TNF-superfamily death molecules are involved in T cell apoptosis. How -ever, multiple redundant extracellular proapoptotic signals could provide an explanation for many contradictory results on whether TNF-superfamily death molecules are involved in T cell apoptosis. Therefore, multiple redundant extracellular proapoptotic signals could provide an explanation for many contradictory results on whether TNF-superfamily death molecules are involved in T cell apoptosis.

Our data has addressed several gaps in the current knowledge of how apoptosis of activated T cells is accomplished. A previously unanswered question is: what factors induce the expression of proapoptotic Bcl-2 genes? One hypothesis is that proapoptotic Bcl-2 genes are induced by growth factor withdrawal after Ag is cleared (1). However, in prolonged infections such as BCG, Ag is not rapidly cleared, so additional mechanisms may be required to induce proapoptotic Bcl-2 genes. Our new data show that IFN-γ also induces the expression of proapoptotic Bcl-2-related genes in CD4+ T cells during BCG infection. Thus, both IFN-γ and growth factor withdrawal may individually contribute to up-regulating the Bcl-2 genes and promoting apoptosis of responding T cells.

A related question is: how does damage to the mitochondrial membranes occur during T cell apoptosis? One hypothesis is that the mitochondria-mediated pathway of apoptosis is not triggered by signals from other cells, but is a cell-intrinsic program that is induced by the activation of T cells (2). Although death receptors and NO can promote mitochondrial damage in tumor cells (50, 51), these molecules are not generally thought to promote mitochondrial damage in activated T cells (1, 2, 16, 49). Our new data indicate that the cell-extrinsic molecules NO, TRAIL, and TNF-α are capable of damaging the mitochondrial membranes of activated CD4+ T cells in vitro. In BCG-infected gene KO mice lacking components of the TRAIL and TNF-α apoptosis pathways, decreased caspase-9 enzyme activity in activated CD4+ T cells suggests a role for TNFR1 and DR5 in promoting mitochondria-mediated apoptosis in vivo. Finally, the TNF-related and Bcl-2-related pathways are thought to be independently regulated during T cell apoptosis (1, 2, 16, 17). Our new data show that Bcl-2-related molecules and TNF-superfamily molecules are coordinately up-regulated by IFN-γ in activated CD4+ T cells that are responding to BCG infection and to anti-CD3 stimulation.

In summary, the data presented herein greatly extends the known roles for IFN-γ in promoting apoptosis of activated CD4+ T cells. Our data also provide novel evidence for a coordinated IFN-γ-dependent pattern of expression of Bcl-2-related, TNF-re-lated, and other apoptosis-related molecules within the activated CD4+ T cell population. We also showed that IFN-γ acts directly on activated CD4+ T cells to sensitize them to apoptosis. IFN-γ also induced proapoptotic signals from immune cells that were capable of damaging the mitochondria of activated CD4+ T cells and thereby killing them. This study shows a new connection between many molecules that until now have been considered independent of each other during T cell apoptosis. Our results significantly advance the understanding of how activated CD4+ T cells undergo apoptosis during a persisting infection.

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Disclosures
The authors have no financial conflict of interest.

References


