Brief Definitive Report

Lineage-specific Requirement for Signal Transducer and Activator of Transcription (Stat)4 in Interferon γ Production from CD4+ Versus CD8+ T Cells

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Summary

$CD4^+$ and $CD8^+$ T cells exhibit important differences in their major effector functions. $CD8^+$ T cells provide protection against pathogens through cytolytic activity, whereas $CD4^+$ T cells exert important regulatory activity through production of cytokines. However, both lineages can produce interferon (IFN)-γ, which can contribute to protective immunity. Here we show that $CD4^+$ and $CD8^+$ T cells differ in their regulation of IFN-γ production. Both lineages require signal transducer and activator of transcription (Stat)4 activation for IFN-γ induced by interleukin (IL)-12/IL-18 signaling, but only $CD4^+$ T cells require Stat4 for IFN-γ induction via the TCR pathway. In response to antigen, $CD8^+$ T cells can produce IFN-γ independently of IL-12, whereas $CD4^+$ T cells require IL-12 and Stat4 activation. Thus, there is a lineage-specific requirement for Stat4 activation in antigen-induced IFN-γ production based on differences in TCR signaling between $CD4^+$ and $CD8^+$ T cells.

Key words: Stat4 • interferon γ • T lymphocytes • interleukin 12 • interleukin 18

Interferon (IFN)-γ enhances defense against bacterial and viral pathogens (1) and is produced by both innate and adaptive immune cells, including NK cells, $CD8^+$ T cells, and $CD4^+$ T cells. NK cells are an important source of IFN-γ in early infection, and can secrete IFN-γ upon initial activation (2). In contrast, $CD4^+$ cells produce little IFN-γ on primary activation and require cytokine-dependent differentiation to acquire this capacity (3, 4). Further, $CD4^+$ T cells exhibit a developmental dichotomy diverging to either an IFN-γ–producing (Th1) or IL-4–producing (Th2) phenotype (5, 6). A similar paradigm was recently extended to $CD8^+$ T cells, where Tc1 and Tc2 subsets develop in response to conditions that induce Th1 and Th2 subsets (7–10). However, unlike Th2 cells, Tc2 cells retain the capacity for IFN-γ production, although reduced compared with Tc1 cells, and produce quantitatively less IL-4 relative to their CD4 counterpart (7).

For $CD4^+$ T cells, Th1/Th2 polarization involves IL-12 and IL-4 signaling via the JAK-STAT Janus kinases/signal transducer and activator of transcription) pathway (11–15). The role of IL-12 in Th1 development was established using both IL-12– and Stat4-deficient mice (12, 13, 16). IL-12 p40-deficient mice had impaired NK responses and lower IFN-γ production from $CD4^+$ T cells (16), but IFN-γ secretion by LAK cells and generation of allo-specific CTL were unimpeded. These studies suggested IL-12–independent pathways in NK cells and CTLs. Stat4-deficient mice exhibit reduced but not absent IFN-γ production (12, 13), but the source was not apparent, since these studies used unseparated splenocytes and polyclonal activation. In sum, these studies suggest not all cell types are entirely IL-12 and Stat4 dependent for IFN-γ production.

The TCR pathway has been considered the only physiologic stimuli for IFN-γ induction in $CD4^+$ T cells. However, IL-12 and IL-18 were shown to induce IFN-γ production in Th1 cells by a TCR-independent mechanism (17). The pathways activated by TCR and IL-12/IL-18 treatment are differentially sensitive to Cyclosporin A inhibition and appear to induce IFN-γ transcription through activation of distinct sets of factors (18). Thus the requirements for Stat4 and IL-12 in IFN-γ production may depend on the activating stimulus as well as the cell type.

In this study, we directly compared the $CD4^+$ and $CD8^+$ T cells for IL-12 and Stat4 requirements in IFN-γ induction through the TCR or IL-12/IL-18 pathways. We find that $CD4^+$ and $CD8^+$ T cells exhibit lineage-specific differences in requiring Stat4-activation for IFN-γ production. Specifically, both $CD4^+$ and $CD8^+$ T cells require Stat4 in IL-12/IL-18 induction of IFN-γ, but only $CD4^+$ T cells require Stat4 for TCR induced IFN-γ production.

Materials and Methods

Animals. Stat4-deficient DO11.10 TCR-transgenic mice have been described previously (12, 19). 2C TCR transgenic mice (20) were obtained from Dr. T. Hansen (Washington University, St. Louis, MO).

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Cytokines, Antibodies, and Other Reagents. Recombinant human IL-2, IL-4, IL-12, and KJ1-26 (21) were used as previously described (19). Recombinant murine IL-18 (Research Diagnostics Inc.) was used at 50 ng/ml. Anti–IL-12 (TOSH) (22) and anti–IFN-γ (H22) (from Dr. R.D. Schreiber, Washington University, St. Louis, MO) were used at 10 μg/ml. Anti-CD3 (2C11) (from Dr. A. Shaw, Washington University, St. Louis, MO) was coated at 10 μg/ml for primary stimulations and 1 μg/ml for secondary stimulation, and anti-CD28 (PV1) (from Dr. Carl June, Naval Medical Research Institute, Bethesda, MD) was used at 1 μg/ml. All other staining reagents were purchased from PharMingen.

T Cell Purification and Cultures. Sorted $CD4^+$ DO11.10 T cells (2×10^5/ml) were activated with 0.3 μM OVA peptide (OVA), IL-2, IL-12, and irradiated BALB/c splenocytes as previously described (3). In other experiments, DO11.10 splenocytes (3×10^6/ml) were activated with OVA, IL-2, IL-12 (Th1), or IL-4 (Th2) as indicated in the figure legends. $CD8^+$ T cells were sorted from spleen and lymph node cells of 2C mice and activated (2×10^5/ml) using irradiated BALB/c splenocytes (1.5×10^6/ml). $CD4^+$ and $CD8^+$ T cells were sorted from spleen and lymph node cells of Stat4-deficient or wild-type mice, and stimulated (4×10^5/ml) with irradiated C57BL/6 splenocytes (4×10^6/ml), IL-2, and the indicated cytokines and antibodies.

ELISA and Intracellular Cytokine Staining. IFN-γ was measured by ELISA as previously described (3). Intracellular cytokine staining was performed as described elsewhere (18, 23). T cells were stimulated overnight with OVA and either irradiated APCs or plate-bound anti-CD3, and Brefeldin A (10 μg/ml; Epicenter Technologies) was added for the final 4 h. Cells were harvested, washed, and stained for CD4, CD8, and KJ1-26 as indicated in the figure legends. After washing, cells were fixed, washed, permeabilized, and stained for IFN-γ.

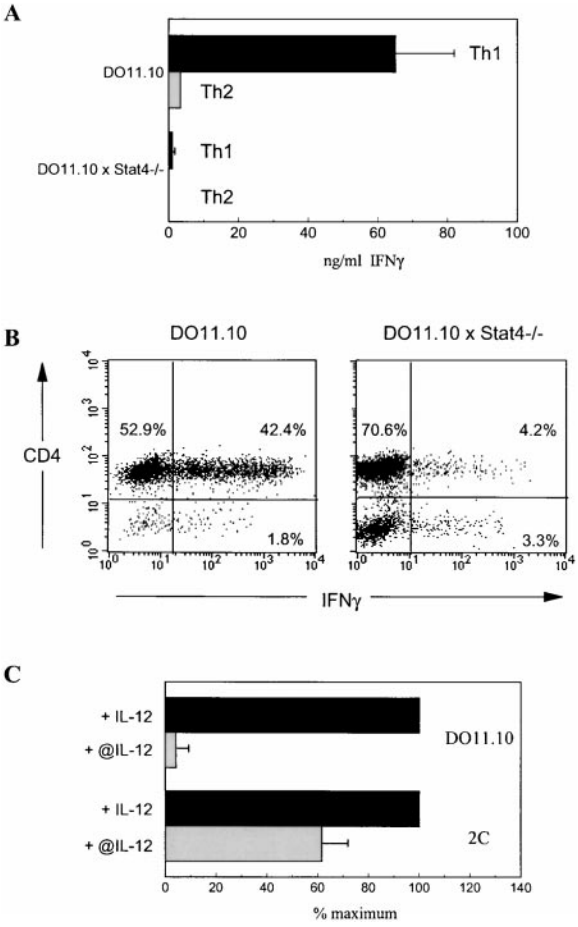

Figure 1. $CD4^+$ T cells exhibit strict requirement for IL-12 and Stat4 in antigen-induced IFN-γ production. (A) Splenocytes from DO11.10 or DO11.10 × Stat4-deficient (Stat4−/−) were cultured with IL-2 and OVA, and either IL-12 (Th1) or IL-4 (Th2) as indicated for 7 d. Cells were restimulated with irradiated BALB/c splenocytes and OVA for 40 h and supernatants were assayed for IFN-γ. Data shown are the mean ± SD of four replicate determinations and is representative of three similar experiments. (B) Cells were treated as above except Brefeldin A was added for the final 4 h of a 19-h restimulation. Samples were stained with FITC-conjugated anti-CD4 and biotin-conjugated KJ1-26 followed by cychrome-streptavidin and PE-conjugated anti–IFN-γ as described in Materials and Methods. Analysis gates were set on live $KJ1\text{-}26^+$ cells. Quadrants are based on isotype control staining. (C) Sorted DO11.10 $CD4^+$ T cells were stimulated with OVA/APCs, IL-2, and either IL-12 (black bars) or anti–IL-12 (gray bars). Sorted $CD8^+$ T cells from 2C mice were stimulated with APCs, and either IL-12 (black bars) or anti–IL-12 (gray bars). After 6 d, equal numbers of DO11.10 and 2C T cells were reactivated in the absence of cytokines for 40 h and IFN-γ was measured. Data are the mean ± SD of IFN-γ production represented as a percentage of the IL-12–treated condition (% maximum) from four independent experiments.

Results

$CD4^+$ and $CD8^+$ T Cells Have Distinct Requirements for IL-12 and Stat4 in the Production of IFN-γ. Previous analyses of Stat4-deficient mice reported five- to sixfold reduced IFN-γ production based in part on polyclonal cellular activation of unseparated splenocytes (12, 13). To examine the requirement for Stat4 in antigen-specific $CD4^+$ T cells, we used DO11.10 TCR-transgenic mice crossed to either wild-type or Stat4-deficient backgrounds. Splenocytes from unimmunized mice were primed in vitro and induced toward Th1 and Th2 phenotypes (4) (Fig. 1 A). As expected, wild-type DO11.10 T cells primed in the presence of IL-12 generated high levels of IFN-γ upon secondary stimulation. In contrast, Stat4-deficient DO11.10 T cells primed with IL-12 generated nearly 100-fold less IFN-γ, confirming that Stat4 has a significant role in $CD4^+$ T cells for IFN-γ production.

After in vitro priming, clonotype-positive ($KJ1\text{-}26^+$) T cells from wild type DO11.10 transgenic mice are predominantly $CD4^+$. However, in Stat4-deficient mice, as much as 25% of the $KJ1\text{-}26^+$ T cells are $CD4^-$ (Fig. 1 B) and $CD8^-$ (not shown) after in vitro priming. Double-negative T cells have been reported to exhibit differences in Th1/Th2 regulation, with impaired Th2 development (24, 25). Thus, we wished to assess production of IFN-γ in $CD4^+$ and $CD4^-$ T cells using intracellular cytokine staining (Fig. 1 B). Wild-type DO11.10 T cells produced abundant intracellular IFN-γ production, whereas Stat4-deficient DO11.10 T cells showed a significantly lower percentage of IFN-γ–producing cells with lower mean fluorescence intensities relative to wild-type T cells (Fig. 1 B). Of the Stat4-deficient DO11.10 T cells, 6% of $CD4^+$ cells produced IFN-γ, whereas 13% of $CD4^-$ negative cells produced IFN-γ, implying that in $KJ1\text{-}26^+$ T cells, $CD4^+$ cells are more Stat4 dependent for IFN-γ production than are $CD4^-$ cells.

These and other results suggest that IFN-γ production may be regulated differently in various T cell lineages (16, 26, and Carter, L.L., unpublished observations). Therefore, we wished to compare $CD4^+$ and $CD8^+$ T cells from TCR-transgenic mice for their dependence on IL-12 for driving IFN-γ production (Fig. 1 C). $CD8^+$ or $CD4^+$ T cells were sorted from 2C TCR-transgenic mice or DO11.10 mice, respectively, and primed with antigen in the presence of IL-12 or anti–IL-12 antibody for 6 d, restimulated, and assessed for IFN-γ production. $CD4^+$ DO11.10 T cells produced high IFN-γ when primed with IL-12, but virtually undetectable IFN-γ when primed with anti–IL-12 antibody (Fig. 1 C). In contrast, 2C $CD8^+$ T cells produced high levels of IFN-γ even when primed in the presence of anti–IL-12 antibody, with IFN-γ production being reduced only twofold relative to cells primed with IL-12. Thus, $CD8^+$ T cells show significant IL-12–independent IFN-γ production, whereas $CD4^+$ T cells do not.

In the mouse, Stat4 is uniquely activated by IL-12 (11, 27, 28). Therefore, IL-12–independent IFN-γ production by $CD8^+$ T cells suggests either that Stat4 activation is IL-12 independent or that IFN-γ production is Stat4 independent. To distinguish these possibilities, we analyzed purified $CD4^+$ and $CD8^+$ T cells from Stat4-deficient and wild-type BALB/c mice. T cells were primed in the presence of IL-12 with either allogeneic stimulators or plate-bound anti-CD3 and anti-CD28 (Fig. 2). When primed and reactivated with allogeneic stimulators (Fig. 2, left), Stat4-deficient $CD4^+$ T cells produced very little IFN-γ. In comparison, Stat4-deficient $CD8^+$ T cells produced significantly more IFN-γ, although the level observed was reduced three- to fourfold relative to the wild-type $CD8^+$ control. When T cells were reactivated with anti-CD3 (Fig. 2, middle), Stat4-deficient $CD4^+$ T cells remained poor IFN-γ producers, whereas Stat4-deficient $CD8^+$ T cells produced IFN-γ at levels similar to wild-type $CD8^+$ controls (Fig. 2, middle). When T cells were primed with anti-CD3/anti-CD28 and IL-12, and reactivated with anti-CD3, Stat4-deficient $CD4^+$ T cells again produced very low levels of IFN-γ, whereas Stat4-deficient $CD8^+$ T cells produced high levels of IFN-γ (Fig. 2, right).

We extended these results with intracellular cytokine staining (Fig. 3). Purified $CD4^+$ and $CD8^+$ T cells from Stat4-deficient and wild-type mice were primed in the presence of IL-12 using either APCs or anti-CD3/anti-CD28, and reactivated with APCs (Fig. 3 A) or anti-CD3 (Fig. 3 B). $CD4^+$ T cells again showed a strict requirement for Stat4 in IFN-γ production with both forms of activation. In contrast, Stat4-deficient $CD8^+$ T cells produced abundant IFN-γ with either form of activation. With anti-CD3 treatment, equivalent percentages of Stat4-deficient and wild-type $CD8^+$ T cells produced IFN-γ, whereas with activation by APCs, IFN-γ^+ Stat4-deficient $CD8^+$ T

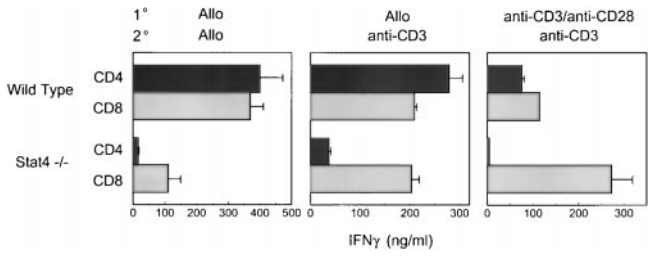

Figure 2. $CD8^+$ T cells exhibit Stat4-independent IFN-γ production. $CD4^+$ (black bars) and $CD8^+$ (gray bars) T cells were sort-purified from pooled lymph nodes and spleens of wild-type or Stat4-deficient (Stat4 −/−) mice and stimulated with IL-2, IL-12, and irradiated allogeneic splenocytes (H-2^b) (left and middle) or plate-bound anti-CD3 and anti-CD28 (right). After 6 d, cells were harvested and reactivated at 4×10^5/ml with irradiated allogeneic APCs (left) or plate-bound anti-CD3 (middle and right), and IFN-γ was measured after 40 h. Data in left and middle panels are the mean ± SD pooled from six independent experiments. Data in right panel are the mean ± SD from one of two experiments.

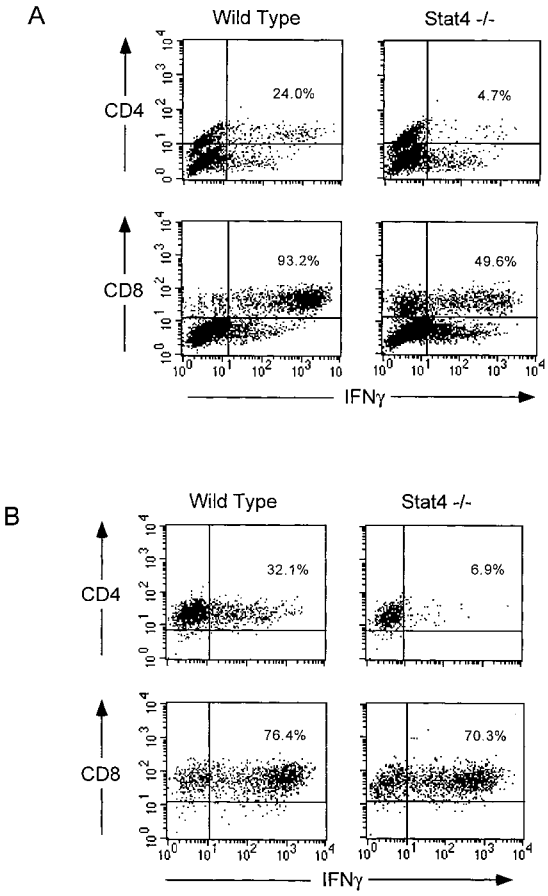

Figure 3. $CD4^+$ and $CD8^+$ T cells have distinct requirements for Stat4 in TCR-induced IFN-γ production. $CD4^+$ and $CD8^+$ T cells were sorted from lymph nodes and spleens of wild-type or Stat4-deficient mice and stimulated in the presence of IL-2 and IL-12 with either irradiated allogeneic splenocytes (A) or plate-bound anti-CD3 and anti-CD28 (B). After 5 d, cells were restimulated with irradiated allogeneic APCs (A) or plate-bound anti-CD3 (B) for 17 h with Brefeldin A added for the final 5 h. Samples were stained with FITC-conjugated anti-CD4 or anti-CD8, fixed, permeabilized, and stained with PE-conjugated anti–IFN-γ. Syngeneic splenocytes were used as stimulators for a specificity control and no IFN-γ^+ cells were detected (data not shown). Gates for analysis excluded dead cells and quadrants were set based on isotype control staining. The percentages displayed indicate the frequency of cells positive for IFN-γ in the $CD4^+$ (top) or $CD8^+$ (bottom) population, rather than the percentage of total cells. Data shown are representative of three independent experiments.

cells were reduced twofold. Thus, in contrast to $CD4^+$ T cells, $CD8^+$ T cells show significant Stat4-independent IFN-γ production, which is most apparent with direct TCR-mediated cellular activation.

Since APCs can produce IL-12 and IL-18 (4, 29, 30), T cell activation using APCs could engage both the TCR and the IL-12/IL-18 pathway for IFN-γ production. Therefore, we asked if these pathways were differentially Stat4 dependent in $CD4^+$ and $CD8^+$ T cells (Fig. 4). Purified $CD4^+$ and $CD8^+$ T cells from Stat4-deficient and wild-type mice were primed with IL-12 and allogeneic APCs and reactivated on day 6 with either anti-CD3 or IL-12/IL-18. In response to anti-CD3, wild-type $CD4^+$, but not Stat4-deficient $CD4^+$, T cells produced IFN-γ. As above, both wild-type and Stat4-deficient $CD8^+$ T cells produced IFN-γ. However, in response to IL-12/IL-18 treatment, both $CD4^+$ and $CD8^+$ Stat4-deficient T cells failed to produce IFN-γ. Thus, the IL-12/IL-18 pathway for IFN-γ production is strictly Stat4 dependent in both $CD4^+$ and $CD8^+$ T cells. In contrast, the TCR-induced pathway for IFN-γ production is Stat4 dependent only in $CD4^+$, and not $CD8^+$, T cells.

Discussion

Previous observations have suggested the existence of both IL-12–dependent and –independent pathways for IFN-γ production (12, 13, 16, 31–33). However, since few of these studies analyzed purified cell types, the effects of Stat4 in specific lineages were potentially obscured. Furthermore, recent studies have demonstrated that IFN-γ gene transcription can be activated by two distinct signaling pathways, one by TCR signaling and other by IL-12 and IL-18 (18), and these pathways were not individually examined in the previous studies. Therefore, the aim of this study was to analyze differences between $CD4^+$ and $CD8^+$ T cells in their regulation of these two pathways for IFN-γ production.

In this paper, we make several new observations. First, we show that the IL-12/IL-18 pathway for induction of IFN-γ operates in $CD8^+$ as well as $CD4^+$ T cells. Second, we formally demonstrate that the IL-12/IL-18 pathway is strictly Stat4 dependent in both $CD4^+$ and $CD8^+$ T cells. Third, we have identified an unexpected difference between $CD4^+$ and $CD8^+$ T cells in TCR signaling. Specifically, $CD4^+$ T cells produce IFN-γ in a completely Stat4-dependent manner, whereas $CD8^+$ T cells are Stat4 independent for TCR-induced IFN-γ production.

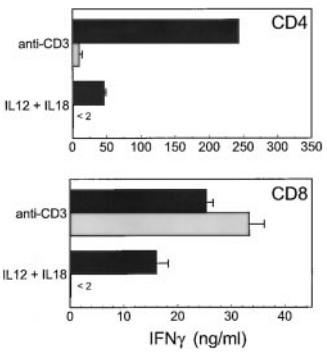

Figure 4. $CD8^+$ T cells possess both Stat4-independent (TCR) and Stat4-dependent (IL-12/IL-18) pathways for IFN-γ induction. $CD4^+$ (top) and $CD8^+$ (bottom) T cells were sorted from lymph nodes and spleens of wild-type (black bars) or Stat4-deficient (gray bars) mice and stimulated with irradiated allogeneic splenocytes, IL-2, and IL-12. After 5 d, cells were harvested and equal cell numbers were restimulated by the addition of IL-12 and IL-18 or plate-bound anti-CD3 for 40 h, and IFN-γ was measured by ELISA. Data are the mean ± SD of four independent experiments.

Common Regulation in $CD4^+$ and $CD8^+$ T Cells for IL-12/IL-18–induced IFN-γ. Two pathways are now recognized for IFN-γ induction (17, 18), one via TCR-signaling and another through IL-12 and IL-18 that acts independently of antigen stimulation (17). The TCR- and IL-12/IL-18–induced pathways were shown to be pharmacologically distinct and to induce different transcription factors (18). In this study, we show that IL-12/IL-18 induction of IFN-γ operates in $CD8^+$ as well as $CD4^+$ T cells (Fig. 4). The existence of antigen-independent IFN-γ production by previously activated T cells from both CD4 and CD8 lineage has significant implications for immune regulation. By stimulating production of cytokines in an antigen-independent manner, this pathway allows antigen-specific T cells to operate like innate immune cells. The Stat4-dependence of the IL-12/IL-18–induced pathway in both $CD4^+$ and $CD8^+$ lineages suggest a common IFN-γ regulatory mechanism.

Distinct Regulation in $CD4^+$ and $CD8^+$ T Cells for TCR-induced IFN-γ Production. In contrast to IL-12/IL-18–induced IFN-γ, TCR-induced signaling revealed a striking difference in the requirement for Stat4 between $CD4^+$ and $CD8^+$ T cells. Unseparated Stat4-deficient splenocytes displayed a partial reduction in IFN-γ production in previous studies (12, 13), whereas pure populations of $CD4^+$ T cells show a much more stringent requirement for Stat4 (Fig. 1 A). In contrast, Stat4-deficient $CD8^+$ T cells generated abundant IFN-γ particularly when activated through the TCR. When $CD8^+$ T cells were activated using APCs, a partial loss of IFN-γ production was observed in Stat4-deficient $CD8^+$ T cells relative to wild-type controls (Figs. 2, left, and 3 A), suggesting that activation with APCs engages both TCR (Stat4-independent) and IL-12/IL-18 (Stat4-dependent) pathways. Activation of $CD8^+$ T cells using anti-CD3 restricts activation to the TCR (Stat4-independent) pathway, resulting in equivalent levels of IFN-γ production by Stat4-deficient and wild-type $CD8^+$ T cells. Distinct regulation of IFN-γ gene activation between $CD4^+$ and $CD8^+$ T cells has previously been suggested (26). A Stat4-independent mechanism for IFN-γ production development has recently been described (34), but as it operated only in the absence of Stat6 and in $CD4^+$ T cells, it is distinct from the pathway described here.

Differences in TCR signaling between $CD4^+$ and $CD8^+$ lineages could reside at several levels. First, $CD4^+$ and $CD8^+$ T cells may differ in expression of signaling components downstream of the TCR. For example, certain mitogen-activated protein (MAP) kinases implicated in IFN-γ induction (35, 36) could be differentially expressed or activated in $CD4^+$ versus $CD8^+$ T cells, being Stat4 dependent only in $CD4^+$ T cells. Second, chromatin accessibility of the IFN-γ gene may differ between primary $CD4^+$ and $CD8^+$ lineages. In this model, the IFN-γ gene would be accessible to TCR-induced factors independently of Stat4 in $CD8^+$ T cells, but not in $CD4^+$ T cells. However, IFN-γ

chromatin structure in $CD4^+$ versus $CD8^+$ T cells has not yet been compared. Finally, coreceptor signaling could account for the present observations. CD8 may provide a signal that bypasses a Stat4 requirement in IFN-γ production, or conversely CD4 may provide a signal imposing such a requirement. Indeed, differences between coreceptor association with src family kinase Lck have been reported (37–40), and lack of CD4 expression impairs Th2 responses (24, 25).

In summary, the study presented here makes the first distinction between $CD4^+$ and $CD8^+$ T cells for the role of Stat4 in regulation of IFN-γ expression. Given the importance of IFN-γ in responses to pathogens and in autoimmune processes, it will be important to determine the basis of these lineage-specific differences in the Stat4-requirement for IFN-γ gene regulation.

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