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Strong TCR Signaling, TLR Ligands, and Cytokine Redundancies Ensure Robust Development of Type 1 Effector T Cells

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T cell effector function is a central mechanism of adaptive immunity, and accordingly, protection of the host against pathogens. One of the primary effector molecules produced by T cells in response to such pathogens is the cytokine, IFN-γ. Although the signaling pathways associated with the production of IFN-γ are well established, disparate in vivo and in vitro results indicate that distinct pathways may become more prominent dependent upon the nature of the infection, inflammatory milieu and tissue localization. We have examined the roles and requirements of the major IFN-γ-inducing pathways in vivo and in vitro, specifically: strength of TCR signal; paracrine release of IL-12, IL-23, and IL-18; and autocrine production of IFN-γ. Our data show a dynamic interaction between these activation pathways, which allows the host a degree of flexibility and redundancy in the induction of IFN-γ. Upon strong signaling through the TCR, IL-12, IL-18, and IL-23 play negligible roles in the induction of IFN-γ, whereas autocrine IFN-γ is an important component in sustaining its own secretion. However, the absence of any one of these factors during a weaker TCR signal, results in strikingly impaired T cell IFN-γ production. Of note, TLR-activated dendritic cells (DCs) were capable of overcoming the absence of a strong TCR signal, IL-12, IL-23, or IL-18 revealing an important additional mechanism for ensuring a robust IFN-γ response. Our findings clarify the hierarchical requirements of the major IFN-γ inducing pathways and highlight the important role TLR ligand-activated DCs have to preserve them. The Journal of Immunology, 2006, 176: 7180–7188.

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3 Abbreviations used in this paper: DC, dendritic cell; WT, wild type; VLP, virus-like particle; LCMV, lymphocytic choriomeningitis virus.
T cells produce IFN-γ, even in the absence of polarizing cytokines and a strong TCR signal. Taken together, this study highlights the individual roles of TCR signal strength, IL-12, IL-23, IFN-γ, and IL-18 in the induction of effective IFN-γ responses by T cells, providing a platform for the interpretation of both in vivo and in vitro IFN-γ responses.

Materials and Methods

Mice

C57BL/6 wild-type (WT) mice were obtained from Charles River Breeding Laboratories. IL-12p35- and IL-12p40-deficient mice were backcrossed over eight times onto a C57BL/6 background and bred in BioSupport animal facility. IL-12-deficient mice were obtained from The Max Planck Institute for Immunobiology. SMARTA-2, lymphocytic choriomeningitis virus (LCMV) GP13 TCR transgenic mice (GLNPGIDYKVGYFQKS VEFD) (18) and TCR7, LCMV GP33 TCR transgenic mice (KAVYN FATM) (19), were backcrossed onto C57BL/6 background over six generations. Mice were maintained under specific pathogen-free at BioSupport in isolated ventilated cages. Animal used in experiments were between ages 8 and 10 wk. All experiments were performed with permission from the Zürich Animal Ethics Committee.

Peptides, virus-like particles (VLPs), and oligonucleotides

LCMV glycoprotein peptides GP13, GP33, and V4Y have been described previously (20, 21). The production and purification of recombinant p13-VLP is described previously (20). Phosphorothioate-modified CpG-containing oligonucleotide was synthesized by Microsynth. The following oligonucleotide sequence was used 1668pt (5′-TCC ATG ACC TTC GTG AAT AAT-3′).

TCR transgenic T cell-DC coculture

Naive SMARTA-2 and TCR7 mice were sacrificed and spleens were removed. CD4+ and CD8+ T cells, respectively, were isolated by MACS bead separation following manufacturers instructions (Miltenyi Biotec) and were used to be 90% CD4+ CD62L+ by or CD8+ CD62L+ by subsequent FACs analysis. DCs were isolated from spleens of naive WT, IL-12p35-, IL-12p40-, or IL-18-deficient mice as described previously (22). Isolated T cells (6.5 × 10^6 cells/well) and DCs (1.4 × 10^6 cells/well) were cultured in 96-well plates in the presence of GP13, GP33, or V4Y peptide at the indicated concentrations. In the indicated cultures, anti-IFN-γ Ab (clone AN-18) was added to reach the final concentration of 10 μg/mL. For activation, PGE (1 μM) was added to the culture for 2 h, followed by washing in medium and Ag-pulsing. Alternatively, bone marrow-derived DCs were generated as previously described (23), activated overnight with 1 μM CpG and supernatant was added to the culture. The same experimental set up was applied on splenic DCs. On day 3 of culture, cells were activated in the presence of PMA and ionomycin for 4 h. IFN-γ and IL-4 production was determined by flow cytometry or ELISA as described previously. Alternatively, for day 5 cultures, cells were restimulated at day 3 with freshly isolated DCs and GP13 or GP33 peptide at the same concentration used in the primary stimulation. In the indicated cultures, cyclosporin A (Sigma-Aldrich) was added on day 3 at a final concentration of 100 ng/mL. On day 5 of culture, cells were activated with PMA and ionomycin for 4 h and cytokine production was determined by flow cytometry or ELISA. For long cultures (10 days) cells were harvested at day 4 after primary stimulation, rested for 3 days in medium containing IL-2 and restimulated for additional 3 days with freshly isolated DCs and GP13 or GP33 peptide at the same concentration used at the onset of the culture. Finally, cells were activated in the presence of PMA and ionomycin as described previously.

Intracellular cytokine staining and FACS analysis

Cells from in vitro culture were stimulated with 10^{-7} M PMA and 1 μg/ml ionomycin for 4 h at 37°C in IMDM. For the final 2 h, 10 μg/ml brefeldin A was added to the cultures to retain cytokines in the cytoplasm. Therefore, cells were washed with PBS/0.1% BSA and CD8+ T cells were surface stained with PE-labeled anti-CD8 mAb and FITC-labeled anti-Vα2 mAb (eBioscience). Next, cells were washed with PBS/0.1% BSA, and then again in PBS and fixed with 2% paraformaldehyde for 20 min at room temperature. Fixed cells were incubated in permeabilization buffer (0.5% saponin, 0.2% PBS, 0.05% anti-IL-4 mAb and allophycocyanin-labeled anti-IFN-γ mAb for CD4+ T cells or only allophycocyanin-labeled anti-IFN-γ mAb for CD8+ T cells (eBioscience) for 30 min at room temperature. Cells were washed twice in permeabilization buffer, and then resuspended in PBS/0.1% BSA and analyzed by flow cytometry (FACSCalibur; BD Biosciences) and FlowJo software (Tree Star). Lymph node cells from immunized mice were first surface-stained with PE-labeled anti-Ly5.1 mAb (BD Biosciences), fixed and subsequently incubated with permeabilization buffer containing allophycocyanin-labeled anti-IFN-γ mAb for 30 min at room temperature. After extensive washing, cells were resuspended in PBS/0.1% BSA and analyzed by FACs.

ELISA measurement of IFN-γ

Supernatant was collected on days 3, 5, or 10 of T cell-DC coculture and analyzed for IFN-γ. The 96-well plates (Maxisorp; Nunc) were coated with anti-IFN-γ (BD Biosciences) at 5 μg/ml in 50 μL PBS overnight at 4°C. Between each of the following steps, plates were washed five times with PBS. Coated plates were blocked with PBS/1% BSA for 2 h at room temperature. Samples from individual cultures were serially diluted in PBS/0.1% BSA, followed by incubation at room temperature for 2 h. Thereafter, alkaline phosphatase-labeled goat anti-IFN-γ (eBioscience) was added at room temperature for 2 h, followed by the addition of the substrate p-nitrophenyl phosphate (Sigma-Aldrich). OD was determined at 405 nm using a SpectraMax spectrophotometer (Bucher Biotech).

Adaptive transfer and in vivo activation of transgenic T cells

Ly5.1+ TCR transgenic CD4+ and CD8+ T cells were purified by magnetic separation (MACS, Miltenyi Biotec) and incubated with a final concentration of 2.5 mM CFSE (Molecular Probes) for 7 min, followed by extensive washing in medium before transfer. Labeled T cells (5 × 10^6) were resuspended in 20 μl PBS and injected into the tail vein of C57BL/6, IL-12p35-, IL-12p40-, or IL-18-deficient mice. After 24 h, recipients were immunized s.c. in the hind-leg flank with p13-VLPs and CpG oligonucleotides or p33 peptide with CpG oligonucleotides as described previously (20). On day 3 after immunization, total cell suspensions from inguinal lymph nodes were prepared and cells (3 × 10^7 per well) were restimulated with 1 μM GP13 or GP33 peptide for 6 h at 37°C in IMDM. For the final 3 h, 10 μg/ml brefeldin A was added to the cultures to retain cytokines in the cytoplasm. Thereafter, cells were stained and analyzed by flow cytometry as described previously.

Results

IL-12 and IL-18 are not required for IFN-γ production by CD4+ and CD8+ T cells in vitro

IL-12 can clearly induce Th1 cell differentiation and IFN-γ production, yet whether it is strictly required remains controversial. In addition, the closely related cytokine, IL-23, has been shown to induce IFN-γ production by human T cells, although recent publications question these findings in mice, instead postulating a role for IL-23 in inducing IL-17 production (24, 25). The cytokine IL-18 can also drive IFN-γ production, although its role appears to be limited to stimulation of previously differentiated cells. We sought to clarify the respective roles of these cytokines in CD4+ and CD8+ T cell differentiation using an in vitro TCR transgenic T cell-DC coculture system. CD11c+ DCs were isolated from spleens of naive WT or IL-12p35-deficient mice, and cultured in the presence of either naive CD4+ TCR transgenic SMARTA2 (Fig. 1A) or CD8+ TCR transgenic TCR7 T cells (Fig. 1B) and their corresponding specific peptides GP13 or GP33 of the LCMV glycoprotein, respectively (18, 19). After 3 days, cells were restimulated with PMA/ionomycin and intracellular cytokine staining for IFN-γ was performed. Alternatively, cells were harvested after 4 days of primary antigenic stimulation, rested in medium with IL-2 for 3 days before secondary stimulation with freshly isolated DCs. Restimulation with PMA/ionomycin was performed after an additional 3 days. Our results show that IFN-γ production, both at day 3 and 10 of culture, was comparable irrespective of whether the CD4+ or CD8+ T cells were activated by WT or IL-12p35-deficient DCs, indicating that in this system, IL-12 was superfluous for development and maintenance of type 1 effector responses (Fig. 1, A and B). Because IL-12p35-deficient DCs were still capable of producing IL-23, we isolated splenic CD11c+DCs...
from mice deficient in IL-12p40 (which lack both IL-12 and IL-23), and cultured them with the transgenic T cells and peptide. Similar to the response driven by IL-12p35-deficient DCs, the development of IFN-γ/H9253 producing CD4/H11001 and CD8/H11001 cells was normal in the combined absence of both IL-12 and IL-23 (Fig. 1, A and B), indicating that IL-23 is not involved in triggering Th1 responses, nor does it compensate for the absence of IL-12 in inducing IFN-γ production in CD4/H11001 and CD8/H11001 T cells. Of note, we also found that supplementing the cultures with rIL-23 had no influence upon T cell IFN-γ production (data not shown).

We next assessed the role of IL-18 in this coculture system, and in line with the results obtained using IL-12p35- and IL-12p40-deficient DCs, IL-18-deficient DCs were similarly capable of inducing IFN-γ production by CD4/H11001 and CD8/H11001 T cells (Fig. 1, A and B). These results were confirmed by measuring IFN-γ secretion in culture supernatant. IFN-γ was produced in similar amounts at days 3 or 10 of culture independently of the presence of IL-12, IL-23 or IL-18 (Fig. 1C). Lower amounts of IFN-γ in the 10 day CD8/H11001 T cell cultures was likely due to activation induced cell death, and was comparable irrespective of the type of DC used for activation.

We considered it plausible that any role for IL-12, IL-23, or IL-18 in the induction of IFN-γ production may be overcome through autocrine activation by IFN-γ. We therefore neutralized IFN-γ activity during the 3 days of culture and then determined the proportion of IFN-γ-producing cells by flow cytometry. Neutralization of IFN-γ during the culture period reduced the proportion of IFN-γ-producing CD4/H11001 (Fig. 2A) and CD8/H11001 T cells (Fig. 2B), regardless of whether the activating DCs produced IL-12, IL-23, or IL-18 (Fig. 2 and data not shown). Taken together, these data show that activation of T cells via the TCR, in addition to autocrine production of IFN-γ, is sufficient for differentiation into IFN-γ-producing cells. However, these data did not clarify why IL-12, IL-23, and IL-18 production is important in other model systems (26–31).
CD4<sup>+</sup> and CD8<sup>+</sup> transgenic T cells display normal proliferation and IFN-γ production in the absence of IL-12 or IL-18 in vivo

To establish whether these cytokines were essential for in vivo T cell differentiation, we next performed adoptive transfer studies using CD4<sup>+</sup> and CD8<sup>+</sup> transgenic T cells injected into IL-12p35<sup>-/-</sup>, IL-12p40<sup>-/-</sup>, or IL-18-deficient recipients. CD4<sup>+</sup> or CD8<sup>+</sup> T cells specific for the GP13 or GP33 peptide, respectively, were labeled with CFSE and injected i.v. into Ly5.2<sup>+</sup> WT or cytokine-deficient recipients. On day 1 after transfer, mice were immunized s.c. with GP13 coupled to VLP or GP33 peptide (20). Three days after immunization, total cell suspensions were prepared from draining lymph nodes, and after peptide-specific restimulation, IFN-γ production in the absence of IL-12 or IL-18 was detectable in every cycle at comparable frequencies between WT and deficient mice (Fig. 3, A). The proliferation at each cell division was assessed by flow cytometry. The transferred CD4<sup>+</sup> transgenic T cells, identified through Ly5.1<sup>+</sup> staining, underwent several rounds of proliferation as shown by CFSE dilution, and no difference was seen between WT, IL-12p35<sup>-/-</sup>, IL-12p40<sup>-/-</sup>, or IL-18-deficient recipient mice (Fig. 3A). IFN-γ production was detectable in CD4<sup>+</sup> T cells that had proliferated for at least four rounds, and was comparable in both WT and deficient mice. Comparatively, Ly5.1<sup>+</sup> CD8<sup>+</sup> T cells underwent six rounds of divisions within this timeframe and IFN-γ production was detectable in every cycle at comparable frequencies between WT and deficient mice (Fig. 3, B and C). Taken together, these data demonstrated that CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation and differentiation into IFN-γ producing effector cells in this in vivo system was independent of IL-12, IL-23, and IL-18.

IL-12 and/or IL-18 can drive IFN-γ production in the absence of TCR signaling

Considering two different pathways for IFN-γ promoter activation have been described: one mediated by signaling through the TCR; the other mediated by the synergistic effect of IL-12 and IL-18 in an Ag-independent manner (1), we hypothesized that one pathway might act to compensate for the other. To address this question we used the immunosuppressor cyclosporin A, which inhibits signaling downstream of the TCR (32). CD8<sup>+</sup> TCR transgenic T cells were incubated for 3 days with specific-peptide pulsed-DCs as described in the prior section. On day 3, T cells were restimulated with specific peptide and freshly isolated WT or deficient DCs, in the presence or absence of cyclosporin A. After a further 2 day culture, we assessed IFN-γ secretion in culture supernatant by ELISA and intracellular cytokine staining for IFN-γ was performed. IFN-γ production by CD8<sup>+</sup> T cells activated by WT DCs in the presence of cyclosporin A was reduced, but still present, as compared with nontreated effector T cells (Fig. 4). Comparatively, IFN-γ production was no longer detectable from CD8<sup>+</sup> T cells stimulated with IL-12p35<sup>-/-</sup> or IL-18-deficient DCs in the presence of cyclosporin A (Fig. 4). Under the same conditions, CD4<sup>+</sup> T cell IFN-γ production was abrogated after stimulation with DCs from each mouse strain, indicating that CD4<sup>+</sup> T cells have a greater requirement for TCR signaling (data not shown). These results show that TCR engagement plays the major role in driving effector T cell differentiation and IFN-γ secretion, and additionally provide evidence that IL-12 and IL-18 are important factors in promoting and sustaining IFN-γ production by CD8<sup>+</sup> effector cells when TCR signaling is impaired.

IFN-γ production in the absence of IL-12 or IL-18 is not regulated by Ag concentration

Because TCR signaling appears to be the primary mediator of IFN-γ responses, we next assessed the influence of Ag concentration. The 3 day coculture experiments shown in Fig. 1 were performed at the Ag concentration of 1 μM. In the CD4<sup>+</sup> transgenic T cell-DC coculture system, this concentration of Ag leads to the development of Th1, IFN-γ-producing cells, whereas a lower Ag concentration of 1 nM results in the differentiation of IL-4-producing Th2 cells (22, 33). In the CD8<sup>+</sup> TCR transgenic T cell-DC coculture system, lower concentrations of Ag lead to reduced IFN-γ production, but no secretion of IL-4. We sought to determine whether the concentration of Ag itself regulated the requirement for IL-12 and IL-18 in the induction of IFN-γ-producing cells. Thus, we reduced the amount of antigenic stimulation by titrating down the concentration of peptide in our coculture system. There was no significant difference in IFN-γ production between CD4<sup>+</sup> T cells stimulated by WT, IL-12p35<sup>-/-</sup> or IL-18-deficient DCs (Fig. 5 A) regardless of the Ag concentration used, indicating that these cytokines are not important for Th1 effector cell differentiation even at low Ag concentration. We also investigated the role of IL-12 and IL-18 in inducing IFN-γ production by CD8<sup>+</sup> T cells at low Ag concentrations. Similar to the results found for CD4<sup>+</sup> T cells, the frequency and total number of CD8<sup>+</sup> IFN-γ-producing cells were decreased, as compared with higher concentrations of Ag.
concentrations of Ag; however, there was no difference in IFN-γ production in the absence of either IL-12 or IL-18 (Fig. 5B). A similar result was obtained by measuring IFN-γ secretion in the culture supernatant (Fig. 5C).

**IL-12 and IL-18 are required for IFN-γ production by CD8⁺ T cells when signaling through the TCR is mediated by a weak agonist peptide**

It was surprising that although TCR signaling was crucial for IFN-γ production in the absence of polarizing cytokines, titrating the concentration of peptide did not influence T cell differentiation. We hypothesized that the quality, rather than the quantity of TCR signaling might be crucial. We thus used altered peptide ligands so that a high- or low-affinity signal could be delivered in the same TCR transgenic T cell-DC coculture system (34). Altered peptide ligands for the CD4⁺ GP13-transgenic T cells have yet to be identified, so we performed a CD8⁺ transgenic T cell-DC coculture with WT, IL-12p35⁻⁻ or IL-18-deficient DCs and the weak agonist peptide, V4Y. This modified peptide interacts with the TCR with a much lower affinity than the full agonist peptide GP33 (21). After 3 days of culture with GP33 or V4Y, supernatants were collected and an IFN-γ ELISA was conducted. As expected, comparable amounts of IFN-γ were produced in the WT, IL-12p35⁻⁻, or IL-18-deficient DC-T cell cultures upon stimulation with GP33 peptide (Fig. 6A). However, IFN-γ production by CD8⁺ transgenic T cells was dramatically reduced, as compared with the WT control, when these cells were stimulated by IL-12p35⁻⁻ or IL-18-deficient DCs presenting the weak agonist V4Y (Fig. 6A). This result was confirmed by intracellular cytokine staining (Fig. 6B). Notably, addition of exogenous IL-12 was sufficient to fully restore IFN-γ production by T cells under these conditions (data not shown). Our data support the hypothesis that signaling via the TCR alone drives IFN-γ production by T cells when the strength of TCR signaling is weak. Taken together with the results shown in Fig. 5, these data show that the strength of TCR signaling that compensates for the absence of IL-12 and IL-18 depends more on the “quality” of the TCR signal rather than the “quantity.”

**TLR ligand licensed DCs provide innate signals which ensure IFN-γ production by T cells**

An important characteristic of pathogens, known to modulate the quality of adaptive immune responses, is the binding of pathogen-associated molecular patterns to TLRs (35). We thus examined whether TLR ligand mediated DC activation influenced the requirement for the IFN-γ-inducing pathways described. We performed a coculture with WT and IL-12p35⁻⁻ or IL-18-deficient DCs...
that were activated with the TLR ligand CpG for 2 h before incubation with CD8\(^+\) T cells and V4Y. Upon stimulation with TLR-matured DCs, IFN-\(\gamma\) production was comparable between T cells irrespective of the weak TCR signal and the absence of IL-12 or IL-18 (Fig. 6C). We next assessed whether the TLR ligand-activated DCs influenced T cell IFN-\(\gamma\) production through the production of a soluble factor. Accordingly, the CD8\(^+\) T cell coculture was performed as described above, in the presence of supernatant from CpG-activated DCs. IFN-\(\gamma\) production by CD8\(^+\) T cells was restored by supernatant from CpG-activated DCs, but not “resting” DCs that had not received a TLR stimulus (Fig. 6D). Notably, conditioned supernatant from IL-12p35-, or IL-18-deficient DCs similarly restored IFN-\(\gamma\) production (data not shown), indicating an additional soluble factor was capable of supporting T cell IFN-\(\gamma\) production. Addition of neutralizing Abs against IL-12 or IL-18 in the cultures with CpG activated DCs confirmed these cytokines were not responsible for TLR-induced IFN-\(\gamma\) production, although supplementing the cultures with IL-18 and IL-12 did restore the response (data not shown). Taken together, these data show that activation of DCs by TLR ligands provides an important additional safeguard for the production of IFN-\(\gamma\) by T cells.

**Discussion**

IFN-\(\gamma\) plays a central role in adaptive immune responses against intracellular pathogens and tumors. Both Ag-dependent and Ag-independent pathways to induce IFN-\(\gamma\) production have been identified, however it remains unclear exactly how these pathways interact and their respective importance. In an attempt to clarify their roles, we have assessed the differential requirements for strength of TCR signaling together with autocrine IFN-\(\gamma\) stimulation, IL-12, IL-23, and IL-18 in promoting IFN-\(\gamma\) production. We show that provided TCR-Ag binding occurs with high affinity (strong TCR signal), IL-12, IL-23, and IL-18 are dispensable for the development of IFN-\(\gamma\)-producing effector cells in vitro and in vivo. These cytokines are only required for IFN-\(\gamma\) production in primary or secondary responses in which TCR-Ag affinity is weak (weak TCR signal) (Fig. 6).
Considering supplementation of cultures with IL-23 did not restore IFN-γ production (data not shown), the defect in IFN-γ production upon weak TCR stimulation in the IL-12p40-deficient mice likely results from the absence of IL-12, not IL-23. Of note, autocrine IFN-γ production played a central role in promoting IFN-γ production by both CD4+ and CD8+ T cells, implicating it high in the hierarchy of IFN-γ-inducing factors.

Our findings reveal a clear hierarchy in the importance of the two different pathways for IFN-γ promoter activation, with TCR signaling playing the dominant role. Such redundancies, or fail-safe mechanisms, are in line with the important role IFN-γ plays in protective immunity. Depending on the nature of the infection, these different pathways are likely to be important to a greater or lesser extent, potentially underlying the disparate results regarding the role of IL-12 and IL-18 in vivo and in vitro (6, 12, 13, 16, 27, 30). For example, IL-12 was shown to be dispensable for Th1 development and IFN-γ production during LCMV, vesicular stomatitis virus, and mouse hepatitis virus infection (12, 13). However, in contrast to immune responses against viruses, it is well recognized that IL-12 is required for protection against the intracellular protozoan parasites L. major (3, 26) and Toxoplasma gondii (37). IL-12-deficient mice are highly susceptible to infection with L. major, and in line with this finding, the administration of exogenous IL-12 to IL-12p40 mice has been shown to restore primary effective immune responses against L. major (4). Considering our data, the differential requirements for IL-12 in antiviral versus antiparasite responses would suggest that viral infections may lead to stronger signaling through the TCR. Indeed, both GP13 and GP33 peptides derived from LCMV drive strong Th1/Tc1 responses in cocultures (Fig. 1, A and B), whereas cocultures with LACK-specific TCR transgenic T cells drive stronger Th2 responses as compared with Th1 responses (G. Iezzi and M. Kopf, unpublished observations). Furthermore, LACK-specific cells from L. major-susceptible BALB/c mice exhibit a lower affinity for LACK and develop into Th2 cells, as compared with LACK-specific cells from resistant B10.D2 mice, which exhibit a high affinity toward LACK and become IFN-γ-producing Th1 cells (38). Although multiple mechanisms are sure to influence resistance or susceptibility to L.
-major infection, strength of TCR signal is likely to play a role in the requirement for IL-12 production. In addition to the high Ag load present during viral infections, the presence of TLR ligands also influences the requirement for IL-12 production. We found that IL-12 and IL-18 were no longer required upon weak antigenic stimulus, when Ag-presenting DCs had previously been activated with a TLR ligand. The apparent mechanism involved a soluble factor distinct from IL-12, IL-23, and IL-18. Thus, the licensing of DCs by TLR ligands (for example during viral infections) appears to compensate for weaker TCR signals, helping to ensure an effective immune response develops. IL-12 may play a more prominent role in environments lacking sufficient pathogen associated molecular patterns or other inflammatory stimuli, for example in antitumor (6, 39) or some antiparasite responses.

The IL-12-induced transcription factor, T-bet, has been identified as being critical for driving transcription of the *Ifnγ* gene in CD4⁺ T cells, whereas CD8⁺ T cells do not appear to require it to produce IFN-γ (40). Similarly, signal transducer and activator of transcription (STAT4) has been shown to be important for TCR-induced IFN-γ production by CD4⁺ but not CD8⁺ T cells (2). Considering we found TCR strength of signal was the initial defining factor leading to IFN-γ production, and that CD4⁺ and CD8⁺ T cells appear to have different thresholds for activation upon TCR stimulation (41, 42) (Fig. 3), it is plausible that the differential importance of T-bet and STAT4 described in these T cell subsets, may rather reflect their respective responsiveness to the anti-CD3 used to stimulate them. Indeed, we found that when the partial agonist, V4Υ, was used to stimulate specific CD8⁺ T cells, IL-12 was required for full IFN-γ production. Thus, careful consideration of the nature of the TCR stimulus is important for the interpretation of differences between CD4⁺ and CD8⁺ T cells.

IL-12 can act in synergy with IL-18 to induce IFN-γ production by differentiated CD4⁺ and CD8⁺ effector cells. Although IL-18-deficient mice display impaired IFN-γ production in response to LPS (27), previous publications show that IL-18 alone is not sufficient to induce Th1 differentiation, but rather supports IL-12 in this respect (16). Our data showed that CD4⁺ and CD8⁺ effector T cell differentiation and IFN-γ production occurred in the absence of IL-18 in vitro and in vivo. However, similar to the IL-12-deficient system, T cells activated by IL-18-deficient DCs exhibited impaired IFN-γ production in the absence of strong TCR signaling. Our results indicate that IL-18 plays an equally important role as IL-12, in driving primary CD8⁺ T cell IFN-γ production when the TCR signal is suboptimal. However, it remains unclear whether IL-18 and IL-12 can substitute for each other when the TCR signal is weak, or whether their synergistic action is required.

Overall, our data are supportive of a hierarchical model whereby upon strong TCR stimulation IFN-γ-producing effector T cells develop in the absence of IL-12 and IL-18, and are sustained through autocrine activation by IFN-γ. Upon weak TCR stimulation IL-12 and IL-18 become requisite for optimum IFN-γ production. Another facet of this system comes from TLR-activated DCs, which are capable of overcoming both the absence of IL-12/IL-18, and weak TCR stimulation (Fig. 7). In summary, our data indicate that TCR stimulation is the primary inducer of IFN-γ production in T cells, and that TLR-licensed DCs and the cytokines IL-12 and IL-18 play secondary supporting roles to ensure effective production of IFN-γ.

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**Disclosures**

The authors have no financial conflict of interest.

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