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A Novel Immunoregulatory Function for IL-23: Inhibition of IL-12 Dependent IFN- γ Production

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Summary

Most studies investigating the function of IL-23 have concluded that it promotes IL-17 secreting T cells. While some reports have also characterized IL-23 as having redundant pro-inflammatory effects with IL-12, we have instead found that IL-23 antagonizes IL-12 induced secretion of IFN- γ . When splenocytes or purified populations of T cells are cultured with IL-23, IFN- γ secretion in response to IL-12 is dramatically reduced. The impact of IL-23 is most prominent in CD8 T cells, but is also observed in NK and CD4 T cells. Mechanistically, the IL-23 receptor is not required for this phenomenon, and IL-23 inhibits signaling through the IL-12 receptor by reducing IL-12 induced signal transducer and activator of transcription 4 (STAT4) phosphorylation. IL-23 is also able to reduce IFN- γ secretion by antagonizing endogenously produced IL-12 from *Listeria monocytogenes* (LM) infected macrophages. In vivo, LM infection induces higher serum IFN- γ levels and a greater percentage of IFN- γ +CD8⁺ T cells in IL-23p19 deficient mice as compared to wild-type mice. This increase in IFN- γ production coincides with increased LM clearance at days 2–3 post-infection. Our data suggest that IL-23 may be a key factor in determining the responsiveness of lymphocytes to IL-12 and their subsequent secretion of IFN- γ .

Keywords

cytokines; innate immunity; *Listeria monocytogenes*; CD8 T cells

Introduction

IL-23 is an IL-12 family cytokine which is composed of the IL-12p40 subunit and a novel cytokine subunit, p19. The heterodimeric structure of IL-23 binds to a receptor complex containing the IL-12R β 1 and a novel receptor termed IL-23R [1]. Like IL-12, IL-23 is secreted by activated macrophages and dendritic cells [2] in response to gram positive and negative bacterial, viral, and fungal infections, as well as multiple other stimuli [3–11]. The majority of the research investigating the actions of IL-23 has focused on its role in maintaining populations of IL-17 secreting cells as well as the possibility of directly inducing IL-17 and IL-22 secretion from a variety of cells types (for reviews see [2,12]). Initially, however, IL-23 was found to have some overlapping functions with IL-12. In a previous report, stimulation of human CD4 T cells with anti-CD3 and anti-CD28 resulted in IFN- γ secretion which could be significantly enhanced by IL-12 and slightly enhanced by

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

IL-23 [1]. Contrary to this, using the murine system several studies have suggested that IL-23 does not induce production of IFN- γ in CD4 T cells [13,14]. In the current paper, we also provide evidence that murine CD4 and CD8 T cells do not respond to IL-23 by secreting IFN- γ .

IL-23 has been shown to play a role in infectious diseases, autoimmunity and cancer. Mice deficient in IL-23 show increased susceptibility to *Mycobacterium tuberculosis*, *Klebsiella pneumoniae*, *Citrobacter rodentium*, *Toxoplasma gondii*, *Salmonella enterica* and *Cryptococcus neoformans* [15–23]. Increased susceptibility to these infections was linked to the ability of IL-23 to regulate the production of IL-17 and IL-22, although during certain infections, IL-23 does not regulate both cytokines. Contradictory roles for IL-23 in tumor progression have been reported. One paper showed that tumors engineered to secrete IL-23 did not grow as well as control tumors and were less metastatic [14]. In contrast, data from another group indicated that IL-23 could promote tumor incidence by reducing the infiltration of CD8 T cells into the tumor site [24]. This article also demonstrated that IL-23 can negatively influence the activity of cytotoxic T cells, which suggests that IL-23 may be involved in the negative regulation of CD8 T cell functions.

IL-12, on the other hand, has been shown to coordinate innate and adaptive immune responses in a distinctly pro-inflammatory fashion by increasing the proliferation, cytolytic activity, and secretion of IFN- γ from CD4 and CD8 T cells (for review see [25]). The production of IFN- γ by multiple cell types results in enhanced Th1 immune responses by activating macrophages to be bactericidal, increasing MHC class I and class II expression, and inhibiting proliferation of Th2 cells [26,27]. This secretion of IFN- γ has been shown to be essential for survival during infection with several pathogens, including the gram positive intracellular bacterium, *Listeria monocytogenes* (LM) [28]. Our recent data documents the rapid, innate production of IFN- γ from memory CD8 T cells, as well as NK cells, stimulated with IL-12 and IL-18 [29–31]. Importantly, memory CD8 T cells specific for ovalbumin (OT-I T cells) were shown to secrete IFN- γ independent of T cell receptor ligation, in response to IL-12 and IL-18 that was produced during infection with wild-type LM. These ovalbumin-specific CD8 T cells were able to provide substantial innate immune protection from LM infection when transferred into IFN- γ deficient mice [30]. Collectively, these studies demonstrate the crucial role of IFN- γ , induced by IL-12 and IL-18, in controlling infections with intracellular pathogens.

While IL-23 was initially reported to have overlapping effector functions with IL-12, we present data which show that IL-23 can negatively regulate IL-12 induced IFN- γ production, particularly in CD8 T cells, both in vitro and in vivo. The ability of IL-23 to regulate IL-12 induced IFN- γ production did not require IL-17A, IL-17F, IL-22, or the IL-23 receptor. Interestingly, IL-23 was able to inhibit signaling through the IL-12 receptor by reducing the phosphorylation of signal transducer and activator of transcription 4 (STAT4).

Results

IL-23 Does Not Induce IFN- γ from Murine Lymphocytes

A previous report found that IL-23 was able to enhance IFN- γ secretion from human CD4 T cells that were differentiated with anti-CD3 and anti-CD28 for 3–6 days in vitro [1]. In order to determine if murine CD8 T cells would respond to IL-23 in a similar fashion by enhancing the production of IFN- γ , purified CD8⁺ T cells from naïve wild-type C57Bl/6 mice (WT B6) were differentiated for 6 days in vitro with plate bound anti-CD3 and anti-CD28 in the presence of IL-12, IL-23, or no additional cytokines. Consistent with previously published data [1], including IL-12 during the differentiation of CD8 T cells led to an increase in the concentration of IFN- γ in culture supernatants of CD8⁺ T cells that were

either unstimulated (Figure 1A) or stimulated for 5 hours with PMA and ionomycin (Figure 1B). Likewise, IL-12 increased the percentage of CD8⁺ T cells that were determined to be IFN- γ ⁺ by flow cytometry in both unstimulated (Figure 1D) and PMA and ionomycin stimulated (Figure 1E) cultures. In contrast, IL-23 impacted neither the production of IFN- γ by CD8⁺ T cells nor the percentage of IFN- γ ⁺ CD8⁺ T cells in unstimulated or PMA and ionomycin stimulated cultures (Figure 1A,B,D,&E). Furthermore, the same pattern of results was observed in purified CD4⁺ T cells (Figure 1C&F): while IL-12 was able to increase IFN- γ production when present during differentiation, IL-23 was not.

Our previously published data show that that IL-12 and IL-18 work synergistically with each other to induce IFN- γ secretion [29,30]. To determine if the IL-12 family member, IL-23, could likewise work synergistically with IL-18 to induce IFN- γ production, splenocytes from WT B6 mice were cultured overnight with IL-12, IL-23, or neither cytokine, with and without IL-18. The data show that while IL-12 and IL-18 increase the percentage of CD8⁺ T cells (Figure 2A), CD4⁺ T cells (Figure 2B), NK cells (Figure 2C), and splenocytes (Figure 2D) that are IFN- γ ⁺, IL-23 does not impact the percentage of IFN- γ ⁺ cells in the cultures with or without IL-18. Interestingly, these data also show that IL-23 neither induced the differentiation of CD8⁺ or CD4⁺ T cells to produce IFN- γ , nor did it directly induce IFN- γ , even in the presence of IL-18.

IL-23 Inhibits IL-12 Induced IFN- γ Production

IL-23 was not found to induce IFN- γ in cultures of splenocytes stimulated overnight by cytokines alone or in cultures of purified T cells differentiated for six days with anti-CD3, anti-CD28 and cytokines (Figures 1 and 2). To test the impact of IL-23 in combination with IL-12 on IFN- γ production, splenocytes were cultured overnight with IL-12, or IL-12 and IL-18, with or without IL-23. As seen in Figure 3 A–C, IL-23 did not facilitate IFN- γ production when combined with IL-12. Instead, IL-23 inhibited IL-12 induced IFN- γ production from CD4⁺ T, CD8⁺ T, and NK cells stimulated with IL-12 and IL-18 or IL-12 alone. CD8⁺ T cells exhibited the most dramatic inhibition of IFN- γ ⁺ cells, showing an approximately 50% reduction in the percentage of CD8⁺ T cells that were IFN- γ ⁺. IL-23, however, did not impact the IFN- γ response to IL-18 alone. Furthermore, IL-23 reduced the amount of IFN- γ protein levels in the supernatants of splenocyte cultures stimulated with IL-12 or IL-12 and IL-18 (Figure 3D). These unique findings suggest that IL-23 is negatively regulating IL-12 induced effector functions.

Titration of this IL-23 effect determined that both 10 and 100 ng/ml IL-23 were able to negatively regulate CD8⁺ T cell IFN- γ production in response to IL-12 and IL-18 (Figure 4A). When the concentration of IL-12 in the culture was titrated, both 10 and 100 ng/ml IL-23 were able to reduce the percentage of CD8 T cells (Figure 4B) and total splenocytes (Figure 4C) that were producing IFN- γ in response to 0.5, 5, and 50 ng/ml IL-12. Thus, IL-23 was able to negatively regulate IFN- γ production in response to even a five-fold higher concentration of IL-12. Previous research has shown that IL-2 or IL-15 can enhance IL-12 and IL-18 induced IFN- γ production from CD8⁺ T cells [29]. To more thoroughly investigate the negative regulatory capacity of IL-23 on IFN- γ production, splenocytes were cultured overnight with IL-12, IL-12 and IL-18, or neither cytokine, with or without IL-2 or IL-15 (Figure 4D–E and Table 1). In accordance with previously published data, IL-2 and IL-18 facilitated IL-12 induced IFN- γ production from CD8⁺ T cells (Figure 4D–E) [29]. The addition of both 10 and 100 ng/ml IL-23 to the cultures resulted in the inhibition of IFN- γ production in cultures that included IL-12. The percent reduction of IFN- γ ⁺ CD8⁺ T cells was calculated for 10 and 100 ng/ml IL-23 (Table 1). While the addition of IL-2, IL-15, and IL-18 increases the IFN- γ response to IL-12, the regulatory capacity of IL-23 was generally greatest when IFN- γ production was predominantly due to IL-12 stimulation (Table 1).

The induction of cytokine secretion from T cells can also be accomplished via TCR ligation. To further investigate if the negative regulatory capacity of IL-23 on IFN- γ production was exclusive to IL-12 stimulation, splenocytes were stimulated through their TCR and IFN- γ production was measured in the presence or absence of IL-23. Splenocytes from naive WT B6 mice were stimulated with anti-CD3, and T cells from OT-I TCR transgenic mice on a RAG-1 deficient background (OT-I RAG-1KO) infected six months prior with vaccinia virus expressing full-length ovalbumin protein (VV/OVA) were stimulated with SIINFEKL (the amino acid sequence of the cognate antigen for OT-I T cells). Table 1 summarizes the percent reduction in IFN- γ +CD8+ T cells for the above mentioned culture conditions. In both circumstances, stimulation through the TCR with either a stimulatory antibody or cognate antigen/MHC complex induced IFN- γ production that was not regulated by the presence of IL-23. Thus, IL-23 appeared to be specifically negatively regulating signals through the IL-12 receptor.

IL-23 Regulates IFN- γ Production and Bacterial Clearance during LM Infection

We have previously shown that LM infected macrophages induce the production of IFN- γ from lymphocytes through the secretion of IL-12 and IL-18 [29,30]. To determine if IL-23 was able to inhibit IFN- γ production in response to endogenously produced IL-12 and IL-18, splenocytes were co-cultured with LM infected macrophages with and without IL-23. Again, IL-23 dose-dependently inhibited both the percentage of IFN- γ +CD8+ T cells (Figure 5A), and the level of IFN- γ protein in the culture supernatants (Figure 5B).

To investigate the ability of IL-23 to negatively regulate IFN- γ production during LM infection in vivo, IL-23p19 deficient mice (IL-23p19KO) and WT B6 mice were infected with LM and sacrificed at days 1, 2, or 3 post-infection (p.i.). IL-23p19KO mice had higher serum IFN- γ levels (Figure 6A), increased percentages of IFN- γ + CD8+ T cells (Figure 6B), and increased percentages of IFN- γ + splenocytes (Figure 6C) at days 1, 2, and 3 p.i. with LM. Thus, removing IL-23 during infection enhanced the IFN- γ response to LM. A slight decrease in bacterial CFUs in the spleen (Figure 6D) and liver (Figure 6E) of IL-23p19KO mice corresponded with the increased production of IFN- γ during infection. IFN- γ is an essential cytokine in the immune response against LM, and facilitated IFN- γ production during infection may have aided in the clearance of the pathogen [28,30]. In conclusion, the ability to secrete IL-23 during early LM infection is correlated with lower IFN- γ production and subsequent hindered clearance of this pathogen.

IL-23 Negatively Regulates IFN- γ Production by Acting Directly on T Cells

IL-23 is not only able to act on lymphocytes, but also macrophages and dendritic cells [32,33]. The IL-23R has been reported to be expressed on several cell types including T cells, macrophages, and dendritic cells [34]. Thus, it is possible that an intermediate cell type may be responding to IL-23 in vivo or in the bulk splenocyte cultures and subsequently impacting the IFN- γ secretion from CD8+ T cells. In order to determine if IL-23 was acting directly on CD8+ T cells, bulk cells from spleen or lymph nodes, or purified CD8+ T cells from these tissues, were cultured with IL-12 and IL-18 with or without IL-23. The percentages of IFN- γ + CD8+ T cells from all cultures were negatively regulated by IL-23 (Figure 7 A–D). Therefore, an intermediate cell type does not appear to be required for IL-23 to inhibit IL-12 induced IFN- γ production from CD8+ T cells. In experiments designed to more accurately define the phenotype of the CD8+ T cells which were susceptible to negative regulation by IL-23, we found that cells with an activated/memory phenotype (CD44 high expressing cells) were responsive to IL-23 mediated decreases in IFN- γ secretion (data not shown). This result was predictable due to the fact that only activated and memory CD8+ T cells respond to IL-12/IL-18 by secreting IFN- γ [29,30]. Thus, IL-23 is able to directly bind to activated and/or memory CD8+ T cells to regulate

their cytokine production. Subsequent experiments determined that IL-23 does not induce apoptosis or proliferation of T cells or splenocytes after an overnight culture (data not shown). Thus, while IL-23 is negatively regulating IL-12 induced IFN- γ from activated/memory CD8 T cells by acting directly on CD8 T cells themselves, it is not doing so by changing the numbers of live CD8 T cells in the cultures. Instead, the activity of the CD8 T cells appears to be altered by IL-23.

The IL-23 Receptor is not Required for IL-23 to Inhibit IL-12 Induced IFN- γ Production

In addition to binding to its own receptor (IL-12R β 1/IL-23R heterodimer), IL-23 may also bind to the IL-12R β 1 subunit of the IL-12 receptor, and act as a receptor antagonist. The IL-12p40 homodimer is able to bind to the IL-12R, and in doing so act as an antagonist [35]. Thus, there is reason to believe that IL-23 may behave in this fashion. If IL-23 is able to bind to the IL-12 receptor, and functionally antagonize it, the IL-23 receptor would not be needed for the ability of IL-23 to negatively regulate IL-12 induced IFN- γ production. In order to investigate this possibility, splenocytes were cultured with IL-12 (Figure 8A), or IL-12 and IL-18 (Figure 8B), with or without IL-23, in the presence or absence of an IL-23 receptor neutralizing antibody. These data suggest that IL-23 is able to inhibit IL-12 induced IFN- γ production even when it is unable to bind the IL-23 receptor: the percentage of IFN- γ + CD8+ T cells was reduced by IL-23 in all culture conditions. Thus, signaling through the IL-23 receptor is not necessary for IL-23 to antagonize IL-12 induced IFN- γ secretion. This finding argues against the possibility of another IL-23 induced molecule, such as IL-17A, IL-17F, or IL-22, mediating the IL-23 induced suppression of IFN- γ secretion. Indeed, IL-17A levels in these overnight cultures were below the level of detection (data not shown). Furthermore, while IL-23 was able to induce IL-22 and IL-17F secretion from splenocytes in an overnight culture, the IL-23 receptor neutralizing antibody prevented the secretion of both of these cytokines (Figure 8C&D). Yet, it had no impact on the ability of IL-23 to negatively regulate IL-12 induced IFN- γ production. IL-23 must signal through the IL-23 receptor in order to induce IL-17 or IL-22 secretion, however the IL-23 receptor was not needed for IL-23 to antagonize IL-12 induced IFN- γ secretion.

Signaling through the IL-12 receptor, and the resulting production of IFN- γ , has been shown to be dependent on the phosphorylation of STAT4 [36,37]. If IL-23 could inhibit the activation of STAT4, this would provide further support of the notion that IL-23 is able to bind to and antagonize the IL-12R β 1 subunit of the IL-12 receptor. In order to test this hypothesis, naive splenocytes were cultured for 30 min with or without IL-12 and either 0, 10, or 100 ng/ml IL-23. As shown in Figure 9, IL-12 increased the percentage of phosphoSTAT4+ splenocytes and CD8+ T cells, and IL-23 was able to reduce the percentage of IL-12 induced phosphoSTAT4+ cells in a dose dependent manner. These data strengthen the argument that IL-23 may be able to influence cell responsiveness to IL-12 by acting on a receptor other than the IL-23 receptor, most likely through binding to the IL-12 receptor, thus inhibiting the phosphorylation of STAT4.

Discussion

The focus on IL-23 has predominantly been its role in the maintenance, differentiation, or stimulation of IL-17 secreting cells [2,12,13,20,38–41]. A previous report also stated that IL-23 had overlapping effector functions with IL-12, and found that IL-23 could increase IFN- γ production during the differentiation of human CD4+ T cells [1]. However, we determined that IFN- γ production was not increased when IL-23 was present during the differentiation of murine CD4+ or CD8+ T cells. Furthermore, overnight stimulation with IL-23 did not induce IFN- γ production from murine lymphocytes. Instead of confirming overlapping effector functions with IL-12, the current study provides evidence that IL-23 plays yet another novel role in coordinating immune responses by negatively regulating

IL-12 induced IFN- γ production from lymphocytes, particularly CD8⁺ T cells. While IL-2, IL-15, and IL-18 all enhance IL-12 induced IFN- γ production, the inhibition by IL-23 was strongest when IFN- γ production was predominantly due to IL-12. Furthermore, TCR-induced IFN- γ was not regulated by IL-23. Additional *in vivo* experiments with LM found that mice deficient in IL-23 had greater IFN- γ production which coincided with decreased LM spleen and liver CFU counts at early time points *pi*. *In vitro* studies determined that an intermediary population of cells was not necessary for IL-23 to reduce IL-12 induced IFN- γ production from lymphocytes. While IL-23 was able to act directly on T cells, it did not induce apoptosis or proliferation of cells after an overnight incubation, but rather the activity of the cells: IFN- γ production in response to IL-12. Interestingly, the IL-23 receptor did not appear to be required for the ability of IL-23 to inhibit this IL-12 effector function. IL-23 was, however, able to inhibit signaling through the IL-12 receptor by reducing IL-12 induced STAT4 phosphorylation. Taken together, data from the current study suggest that IL-23 plays a novel role in immune responses by negatively regulating IL-12 induced effector functions such as IFN- γ production.

The mechanism by which IL-23 is able to inhibit IL-12 induced secretion of IFN- γ is likely to be mediated through the antagonistic binding of IL-23 to the IL-12 receptor. The receptor for IL-23 is comprised of a novel IL-23R subunit and the IL-12R β 1 subunit [34], suggesting that IL-23 may have the capacity to bind the IL-12 receptor. Several pieces of experimental data support this theory. As seen in Table 1, IL-23 did not inhibit IFN- γ that was induced through the TCR or through IL-2, IL-15, and IL-18. Only when the IL-12 receptor was utilized to induce IFN- γ , did IL-23 attenuate IFN- γ production. IL-12 induced IFN- γ production requires STAT4 phosphorylation [36,37]. Importantly, our data show that IL-23 is able to inhibit IL-12 induced STAT4 phosphorylation. Further investigation into IL-12 and IL-23 receptor expression and signaling is required to fully understand how IL-23 reduces IL-12 induced IFN- γ production.

Additional explanations for the ability of IL-23 to suppress IL-12 induced IFN- γ could also exist. One possible alternative theory is that other cytokines (including: IL-17A, IL-17F, IL-22, IL-10, or IL-4) were induced by IL-23 and subsequently suppressed IFN- γ production. However, overnight stimulation of splenocytes from naive mice with IL-23 did not induce IL-17A, IL-10 or IL-4. In addition, when the IL-23 receptor was neutralized, the production of IL-22 and IL-17F was significantly decreased in response to IL-23, while the ability of IL-23 to inhibit IL-12 induced IFN- γ was not impacted.

In vivo data during LM infection further suggest that the presence of IL-23 suppresses IFN- γ production. Previously published data has shown that innately produced IL-12 and IL-18 lead to the IFN- γ production observed during early time points *p.i.* with LM [42]. We present data here that mice specifically lacking the p19 subunit of the IL-23 protein (IL-23p19KO mice) have increased IFN- γ production at days 1, 2, and 3 *p.i.* with LM. Data from other laboratories has determined that mice lacking IL-22 or the IL-17 receptor A do not show alterations in IFN- γ production during LM infection [22,43]. Thus, while a deficiency in IL-23 enhances innate IFN- γ production during LM infection, the absence of IL-22 or IL-17 receptor signaling does not.

IL-23 may impact a broad range of IL-12 induced effector functions. The immune responses driven by IL-12 are numerous and include: inducing IFN- γ secretion; inducing the proliferation, clonal expansion, and cytolytic activity of lymphocytes; and promoting the differentiation and proliferation of naive CD4 T cells into the Th1 phenotype [25,44,45]. In the current study, we provide data that IL-23 can regulate IL-12 induced IFN- γ secretion in lymphocytes, including CD8 T, CD4 T, and NK cells. Furthermore, in the presence of IL-12, IL-23 has been shown to suppress the development of a Th1 response [46].

Previously published reports also show that IL-23 can influence additional CD8 T cell effector functions. In a tumor model, IL-23 negatively reduced both the number and cytolytic activity of CD8 T cells within the cancerous tissue [24]. Furthermore, IL-17 secreting CD8 T cells, which are maintained by IL-23, have been found to not be cytolytic (our unpublished data) [47,48]. Taken together, the data clearly indicate that IL-23 can impact a broad range of lymphocyte effector functions, frequently in an opposing direction to that of IL-12.

The balance between IL-12 and IL-23 production during infection or disease will likely tailor the ensuing adaptive immune response. Greater production of IL-12 would lead to a Th1 response, dominated by IFN- γ and macrophage activation, likely reducing the development of IL-17 secreting T cells. Greater production of IL-23 would lead to the development of IL-17 secreting T cells, neutrophil recruitment, and potential suppression of IL-12 induced effector functions, such as IFN- γ secretion and cytolytic activity. Although these pathways are not mutually exclusive, it is evident that there is cross-regulation between the pathways [40,49–52]. In the current study, we provide evidence that IL-23 is also capable of inhibiting the IL-12 induced effector function of IFN- γ production from murine CD8 and CD4 T cells. Through this action, IL-23 would be able to inhibit a Th1 response, and thus the subsequent production of IFN- γ . A reduction in IFN- γ would then allow for a more robust Th17 response. Utilizing strategies to effectively modulate the production of IL-12 versus IL-23 could be useful for vaccine development or for therapies to blunt pathologic or autoimmune T cell responses.

Materials and Methods

Mice

WT B6 mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) or Taconic (Germantown, NY). IL-23p19KO mice backcrossed on a WT B6 genetic background were generously provided by Dr. Nico Ghilardi at Genentech, Inc. and have previously been described [53]. C57BL/6-Ly5.1/Cr (Ly5.1) mice and OT-I RAG-1KO mice were housed by Dr. James Forman at the University of Texas Southwestern Medical Center. Mice between 6 to 12 weeks of age were housed with food and water ad libitum in sterile microisolator cages with sterile bedding at the University of North Texas Health Science Center AAALAC accredited animal facility. All animal experiments were conducted under the approval of the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center.

Cell Lines, Cell Culture, and Reagents

The J774 macrophage line was grown in DMEM (Invitrogen-Gibco, Carlsbad, CA) supplemented with 10% FCS (Atlanta Biologicals, Norcross, GA), L-glutamine, vitamins, and penicillin/streptomycin. Spleens and lymph nodes were processed as previously described [54]. For studies where purified populations of CD8+ and CD4+ T cells were cultured, the BD IMag™ anti-mouse CD8 α (53-6.7) and anti-mouse CD4 (GK1.5) magnetic particles were used to positively select the cells, following the manufacturer's instructions (BD PharMingen, San Jose, CA). A purity of 98% CD8+ or CD4+ was achieved. The purified CD8+ or CD4+ T cell cultures, the bulk lymph node cell or splenocyte cultures, and the J774-splenocyte co-cultures, were all cultured as previously described [54]. Where indicated, cells were cultured with 10 ng/ml IL-2 (Peprotech, Rocky Hill, NJ), varying concentrations of IL-12 (Peprotech), 10 ng/ml IL-18 (MBL International, Woburn, MA), varying concentrations of IL-23 (eBiosciences, San Diego, CA), 10 μ g/ml plate-bound anti-CD3 ϵ or the indicated concentration of soluble anti-CD3 ϵ (BD PharMingen; clone 145-2C11), 1 μ g/ml soluble anti-CD28 (BD PharMingen; clone 37.51), varying

concentrations of the ovalbumin derived SIINFEL peptide (provided by Dr. James Forman), and 10 µg/ml anti-IL-23R neutralizing antibody (R&D Systems, Minneapolis, MN; clone 258010). Where indicated, cells were stimulated with 50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 500 ng/ml Ionomycin (EMD, Gibbstown, NJ) for the last 5 hrs of the culture.

Intracellular Staining and Flow Cytometry

For cell staining, the following antibodies were used from BD Pharmingen: anti-CD3ε FITC (145-2C11), anti-CD4 PE-Cy7 (RM4-5), anti-CD44 FITC (IM7), anti-CD62L APC (MEL-14), anti-NK1.1 PE (PK136), anti-CD45.2 FITC (104), anti-CD16/CD32 (2.4G2), anti-CD8α APC (53-6.7), anti-phosphoSTAT4 PE (38/p-Stat4), and anti-IFN-γ PE or APC (XMG1.2); and from Invitrogen/Caltag: anti-CD8α PE-TR (5H10). To determine cell proliferation, cells were counted on a Z1 Coulter Particle Counter (Beckman Coulter; Fullerton, CA), and subsequently stained for cell surface markers. Apoptosis of cells was determined by staining for Annexin V and 7-AAD (BD Pharmingen).

Intracellular cytokine staining was performed as previously described [54]. To measure intracellular staining of phosphorylated STAT4, unlysed splenocytes were cultured in PBS +5%FCS with cytokines (5 ng/ml IL-12 and 10 or 100 ng/ml IL-23) for 30 min and immediately fixed and lysed using BD Phosflow Lyse/Fix buffer (BD Pharmingen). Following washing in PBS+5%FCS, cells were permeabilized in BD Phosflow Perm Buffer III. After being washed in BD Pharmingen Stain Buffer, cells were subsequently incubated with saturating amounts of the cell-surface antibodies and anti-CD16/CD32 to block Fc receptors at 4°C for 20 min, followed by a 30 min incubation at room temperature with antibodies directed against CD8α and phosphoSTAT4.

ELISA

ELISAs were conducted on filtered cell culture supernatants and serum. The following antibodies were used: plate bound purified capture antibodies, anti-IFN-γ (BD Pharmingen; clone R4-6A2; 4 µg/ml), anti IL-17A (BD Pharmingen; clone TC11-18H10; 2 µg/ml), anti-IL-22 (Peprotech; 0.5 µg/ml); biotinylated detection antibodies, anti-IFN-γ (BD Pharmingen; clone XMG1.2; 1 µg/ml), anti-IL-17A (BD Pharmingen; clone TC11-8H4.1; 2 µg/ml), anti-IL-22 (Peprotech; 0.5 µg/ml). The ELISAs were developed with streptavidin-horseradish peroxidase (BD Pharmingen); and TMB substrate reagent set (BD Biosciences, San Jose, CA). Quantification was calculated by reference to recombinant murine standards: rIFN-γ (BD Pharmingen), rIL-17A (R&D Systems), rIL-22 (Peprotech). ELISAs for IL-17F (R&D Systems DuoSet mouse IL-17F kit), IL-4 (BD Pharmingen OptEIA mouse IL-4 kit), and IL-10 (BD Pharmingen OptEIA mouse IL-10 kit) were conducted according to manufacturers instructions. Cytokine concentrations were measured at 450nm on an EL808 instrument (BioTek).

Pathogens, Infection, and Bacterial Enumeration

LM 10403 serotype 1 was maintained, and LM CFUs were measured, as previously described [54]. For in vitro infections, a MOI of 50:1 was used. J774 cells were infected for 1 hr in antibiotic free media, washed with warm PBS, and then cultured in media containing 100 µg/ml gentamycin. For in vivo infections, mice were injected i.v. with approximately 5×10^4 CFUs of LM for assays performed on days 2 and 3 p.i.. In order to have detectable cytokine responses at day 1 p.i., a higher dose of approximately 10^6 CFUs of LM was used.

In order to look at an antigen specific memory CD8 T cell response, a TCR transgenic mouse model was used. Ly5.1 mice were transferred i.v. with 3×10^6 lymph node cells from OT-I RAG-1KO mice 1 day prior to i.v. infection with 2×10^6 PFUs of VV/OVA. At six

months p.i. with VV/OVA, splenocytes from the OT-I RAG-1KO transferred Ly5.1 mice were used for experiments.

Statistical Analyses

ANOVAs or t-tests were conducted on the data. Bonferroni t-tests and Tukey-Kramer analyses were used for post-hoc analyses. LM CFU data was log transformed prior to analysis, and is represented as such in the figures. A p value of 0.05 or less was considered significant in all cases.

Abbreviations

p.i.	post-infection
LM	<i>Listeria monocytogenes</i>
WT B6	wild-type C57Bl/6 mice
BFA	Brefeldin A
VV/OVA	vaccinia virus expressing ovalbumin protein
OT-I RAG-1KO	OT-I TCR transgenic mice on a RAG-1 deficient background
BHI	brain-heart infusion
STAT4	signal transducer and activator of transcription 4

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Figure 1.

IL-12, But Not IL-23, Induces the Differentiation of IFN- γ Secreting T Cells. Purified CD8+ (A, B, D, & E) or CD4+ (C&F) T cells from naive WT B6 mice were cultured six days on plates coated with anti-CD3 and soluble anti-CD28 in IL-2 supplemented medium with 5ng/ml IL-12, 10ng/ml IL-23, or no additional cytokines. On day 6, the cells were left unstimulated (A&D) or were incubated with PMA & Ionomycin for 5 hrs (B, C, E, & F). IFN- γ concentrations in the culture supernatants were measured with an ELISA (A–C) or the cells were stained for CD8, CD4, and intracellular IFN- γ and analyzed using flow cytometry (D–F). The numbers on the flow cytometry dot plots refer to the percentage of CD8+ T cells (D&E) or CD4+ T cells (F) that were positive for IFN- γ . The data are representative of three independent experiments. For each experiment, lymph node cells from three mice were combined for purification.

Figure 2.

IL-23 Does Not Work Synergistically With IL-18 To Induce IFN- γ . Splenocytes from naive WT B6 mice were cultured overnight in IL-2 supplemented media with or without 5ng/ml IL-12, IL-18, and 10 or 100ng/ml IL-23. Cells were stained for CD3, CD4, CD8, NK1.1, and intracellular IFN- γ and were analyzed using flow cytometry. A two-way ANOVA detected a significant effect of cytokine stimulation, such that IL-12 and IL-18 increased the percentage of IFN- γ + CD8+ T (A), CD4+ T (B), NK (C; NK1.1+CD3-), and splenocytes (D), $p \leq 0.05$. These data are representative of three independent experiments. An * denotes condition differs from all other stimulation conditions ($p \leq 0.05$). A + denotes IL-12 condition differs from no additional cytokines condition ($p \leq 0.05$). All data are expressed as the mean $\pm$ SEM ($n = 2/\text{group}$).

Figure 3.

IL-23 Inhibits IL-12 Induced IFN- γ Production from Lymphocytes. Splenocytes from naïve WT B6 mice were cultured overnight in IL-2 supplemented media with or without 5ng/ml IL-12, IL-18, and 10 or 100ng/ml IL-23. Cells were stained for CD4 (A), NK1.1 (B), CD8 (C), and intracellular IFN- γ and were analyzed using flow cytometry. IFN- γ protein levels in culture supernatants were measured with ELISA (D). Two-way ANOVAs detected significant effects of IL-23 concentration. These data are representative of two independent experiments. An * indicates a difference from the 0ng/ml IL-23 culture condition, $p \leq 0.05$. All data are expressed as the mean $\pm$ SEM ($n = 2/\text{group}$).

Figure 4.

Titration of the Negative Regulatory Capacity of IL-23 on IL-12 Induced IFN- γ Production. The impact of IL-23 on IL-12 induced IFN- γ production was titrated by culturing splenocytes from naive WT B6 mice overnight in IL-2 supplemented media with 5ng/ml IL-12, IL-18, and 0, 0.01, 0.1, 1, 10, and 100ng/ml IL-23 (A). The effect of IL-12 on IFN- γ induction was titrated by culturing splenocytes from naive WT B6 mice overnight in IL-2 supplemented media with 0, 10, or 100ng/ml IL-23 and 0, 0.05, 0.5, 5, 50, or 500ng/ml IL-12 (B–C). Splenocytes from naive WT B6 mice were cultured overnight with or without 5ng/ml IL-12, IL-18, and 10 or 100ng/ml IL-23 in combination with either no IL-2 (D), or with IL-2 (E). For all cultures, cells were stained for CD8 and intracellular IFN- γ , and were analyzed using flow cytometry. Two-way ANOVAs detected significant effects of IL-23 concentration. These data are representative of two independent experiments. In panels A, D, and E, an * indicates a difference from the 0ng/ml IL-23 culture condition, $p \leq 0.05$. In panels B–C, an * indicates that both 10 and 100ng/ml IL-23 differed from 0ng/ml IL-23, $p \leq 0.05$. All data are expressed as the mean $\pm$ SEM ($n = 2$ –4/group).

Figure 5.

IL-23 Counters Endogenously Produced IL-12 and IL-18 from LM Infected Macrophages. LM infected or uninfected J774 macrophages were cultured overnight with splenocytes from naive WT B6 mice and 0, 10, or 100 ng/ml IL-23. Cells were stained for CD8 and intracellular IFN- γ and analyzed with flow cytometry (A) or IFN- γ levels in the culture supernatants were measured using ELISA (B). Two-way ANOVAs detected significant effects of IL-23 concentration. These data are representative of two independent experiments. An * indicates a significant difference from 0 ng/ml IL-23, $p \leq 0.05$. All data are expressed as the mean $\pm$ SEM ($n = 2$ /group).

Figure 6.

IL-23p19KO Mice Have Increased IFN- γ Production and Facilitated Clearance of LM. WT B6 and IL-23p19KO mice were infected with approximately 10^6 CFUs of LM and sacrificed on day 1 p.i., or approximately 5×10^4 CFUs of LM and sacrificed on days 2 or 3 p.i.. IFN- γ protein levels in the serum were analyzed using ELISA (A). Splenocytes were cultured overnight with 50:1 heat-killed LM prior to being stained with antibodies against CD8 and intracellular IFN- γ and analyzed with flow cytometry (B–C). Bacterial CFUs were enumerated in the spleen (D) and liver (E). Two-way ANOVAs detected significant effects of mouse strain. These data are representative of two independent experiments. An * indicates a significant difference from WT B6 mice, $p \leq 0.05$. All data are expressed as the mean $\pm$ SEM ($n = 4$ /group).

Figure 7.

IL-23 Inhibits IFN- γ Production By Acting Directly on T Cells. Purified CD8+ lymph node cells (A), bulk lymph node cells (B), purified CD8+ splenocytes (C), or bulk splenocytes (D) from naïve WT B6 mice were cultured overnight in IL-2 supplemented medium with 5 ng/ml IL-12, with and without IL-18, and 0 or 100 ng/ml IL-23. The cells were stained with antibodies against CD8 and intracellular IFN- γ and analyzed with flow cytometry. *t*-tests detected significant effects of IL-23 concentration. An * indicates a difference from the 0 ng/ml IL-23 culture condition, $p \leq 0.05$. These data are representative of three independent experiments. Where appropriate (C–D), the data are expressed as the mean $\pm$ SEM ($n = 3$ /group). For each experiment, lymph node cells from three mice were combined for purification.

Figure 8.

The IL-23 Receptor is Not Required for The Ability of IL-23 to Negatively Regulate IL-12 Induced IFN- γ . Splenocytes from naive WT B6 mice were cultured overnight in IL-2 supplemented media with either 5 ng/ml IL-12 (A), both IL-12 and IL-18 (B), or neither IL-12 nor IL-18 (C&D). All cells were cultured with or without 100 ng/ml IL-23 and 10 μ g/ml anti-IL-23R neutralizing antibody (α IL-23R Ab). Cells were stained with antibodies against CD8 and intracellular IFN- γ and analyzed with flow cytometry (A&B) or culture supernatants were used for an IL-17F (C) or IL-22 (D) ELISA. Two-way ANOVAs detected a significant effect of IL-23 on the percentage of IFN- γ +** CD8 T cells ($p \leq 0.05$), but no significant effect of the anti-IL-23R neutralizing antibody, $p > 0.05$ (A&B). Two-way ANOVAs conducted on IL-17F and IL-22 concentrations detected a significant effect of IL-23 ($p \leq 0.05$) that was eliminated when the IL-23R neutralizing antibody was added to the culture, $p > 0.05$ (C&D). These data are representative of three independent experiments. An * denotes that the 100 ng/ml IL-23 condition differs from the 0 ng/ml IL-23 condition, $p \leq 0.05$. All data are expressed as the mean $\pm$ SEM (n = 2–4/group).

Figure 9.

IL-23 Inhibits IL-12 Induced STAT4 Phosphorylation. Splenocytes from naive WT B6 mice were cultured for 30 min with or without 5 ng/ml IL-12, with either 0, 10, or 100 ng/ml IL-23, and were immediately fixed, lysed, permeabilized, and incubated with antibodies directed against CD8 and phosphorylated STAT4 before being analyzed using flow cytometry. The numbers in the dot plots represent the percentage of phosphorylated STAT4+ cells in the culture (A). A two-way ANOVA conducted on the %phosphoSTAT4+ splenocytes and CD8+ T cells detected a significant effect of culture condition (B), $p \leq 0.05$. These data are representative of two independent experiments. An * denotes that the group differs from the 0 ng/ml IL-23 condition, $p \leq 0.05$. All data are expressed as the mean $\pm$ SEM ($n = 3/\text{group}$).

Table 1Percent reduction in IFN- γ + CD8+ T cells with IL-23^a

	Overnight Stimulation Condition	% Reduction with 10 ng/ml IL-23 mean ($\pm$ SEM)	% Reduction with 100 ng/ml IL-23 mean ($\pm$ SEM)
WT B6 splenocytes	IL-2, 12 & 18	17.54 ($\pm$ 0.85)	48.39 ($\pm$ 2.06)
	IL-2 & 12	46.59 ($\pm$ 0.95)	85.61 ($\pm$ 3.67)
	IL-15, 12 & 18	26.38 ($\pm$ 3.29)	76.17 ($\pm$ 0.97)
	IL-15 & 12	47.73 ($\pm$ 8.27)	79.96 ($\pm$ 5.74)
	IL-12 & 18	20.31 ($\pm$ 1.24)	70.65 ($\pm$ 2.86)
	IL-12	49.30 ($\pm$ 12.68)	73.00 ($\pm$ 5.82)
OT-I RAG-1KO splenocytes	0.1 μ M SIINFEKL	-2.05 ($\pm$ 1.04)	-2.53 ($\pm$ 0.81)
	1.0 μ M SIINFEKL	-1.44 ($\pm$ 1.75)	-0.21 ($\pm$ 0.25)
	10 μ M SIINFEKL	0.08 ($\pm$ 1.74)	1.23 (0.71)
WT B6 splenocytes	0.5 μ g/ml anti-CD3	9.79 ($\pm$ 5.17)	9.38 ($\pm$ 2.90)
	5.0 μ g/ml anti-CD3	5.74 ($\pm$ 8.15)	9.57 ($\pm$ 7.08)
	50 μ g/ml anti-CD3	5.58 ($\pm$ 5.39)	7.86 ($\pm$ 4.76)

^aSplenocytes were stimulated overnight in the presence of 0, 10, or 100 ng/ml IL-23 with or without IL-2, 5 ng/ml IL-12, IL-18 and IL-15 (WT B6), 0.5, 5.0 or 50 μ g/ml soluble anti-CD3 (WT B6), or 0.1, 1.0, or 10 μ M soluble SIINFEKL peptide (OT-I RAG-1KO). Cells were stained for CD8 (WT B6) or CD45.2 and CD8 (OT-I RAG-1KO), and intracellular IFN- γ , and were analyzed using flow cytometry. The percentage reduction was calculated as follows: [1 - (%IFN- γ + / CD8+ T cells for the 10 or 100 ng/ml IL-23 condition / %IFN- γ + / CD8+ T cells for the 0 ng/ml IL-23 condition) $\times$ 100]. These data are representative of two independent experiments. All data are expressed as the mean $\pm$ SEM (n = 2–4/group).