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The Aryl Hydrocarbon Receptor Promotes IL-10 Production by NK Cells

Sagie Wagage,* Beena John,* Bryan L. Krock, † Aisling O’Hara Hall,* Louise M. Randall,*† Christopher L. Karp,‡ M. Celeste Simon, † and Christopher A. Hunter*

The cytokine IL-10 has an important role in limiting inflammation in many settings, including toxoplasmosis. In the present studies, an IL-10 reporter mouse was used to identify the sources of this cytokine following challenge with *Toxoplasma gondii*. During infection, multiple cell types expressed the IL-10 reporter but NK cells were a major early source of this cytokine. These IL-10 reporter+ NK cells expressed high levels of the IL-12 target genes T-bet, KLRG1, and IFN-γ, and IL-12 depletion abrogated reporter expression. However, IL-12 signaling alone was not sufficient to promote NK cell IL-10, and activation of the aryl hydrocarbon receptor (AHR) was also required for maximal IL-10 production. NK cells basally expressed the AHR, relevant chaperone proteins, and the AHR nuclear translocator, which heterodimerizes with the AHR to form a competent transcription factor. In vitro studies revealed that IL-12 stimulation increased NK cell IL-10 levels, and the AHR and AHR nuclear translocator were required for optimal production of IL-10. Additionally, NK cells isolated from *T. gondii*-infected Ahr-/- mice had impaired expression of IL-10, which was associated with increased resistance to this infection. Taken together, these data identify the AHR as a critical cofactor involved in NK cell production of IL-10.

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Abbreviations used in this article: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; FicZ, 6-formylindolo[2,3-b]carbazole; KLRG1, killer cell lectin-like receptor subfamily G member 1; LAK, lymphokine-activated killer cell; MFI, mean fluorescence intensity; PEC, peritoneal exudate cell; STag, soluble Toxoplasma Ag; WT, wild-type.

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Toxoplasma gondii* is an apicomplexan parasite that induces highly Th1-polarized immune responses characterized by the production of IL-12 and IFN-γ, which are required to control parasite growth (1, 2). Appropriate regulation of this Th1 response is critical for surviving infection, which has been illustrated by reports that IL-10−/− mice infected with *T. gondii* control parasite burdens but succumb to immune-mediated pathology (3, 4). Although CD4+ T cells contribute to this pathology these cells are also a critical source of IL-10 during toxoplasmosis. Consequently, mice in which T cells are unable to express IL-10 also develop immune-mediated tissue pathology when challenged with *T. gondii* (5). Additionally, IL-10−/− Rag2−/− mice reconstituted with CD4+ T cells that are capable of producing IL-10 survive *T. gondii* infection whereas their counterparts given IL-10−/− CD4+ T cells do not (6). Although these results indicate that CD4+ T cells are an important source of IL-10 that protects against fatal immune-mediated pathology during toxoplasmosis, a number of other cell types produce IL-10 during this infection. The biological relevance of innate sources of IL-10 was suggested by the finding that IL-10−/− SCID mice, which lack B and T cells, exhibit improved survival following *T. gondii* infection compared with SCID mice (7). Recent studies have shown that NK cells can produce IL-10 and are a biologically relevant source of this cytokine during toxoplasmosis (8). NK cells are also a source of IL-10 in other murine models of infection, as NK cell IL-10 promotes increased parasite burdens during visceral leishmaniasis and limits the magnitude of the CD8+ T cell response during murine CMV infection (8–10). Taken together, these reports indicate major biological functions for NK cell–derived IL-10 in a variety of viral, bacterial, and parasitic infections.

Recent studies have identified effects of aryl hydrocarbon receptor (AHR) signaling on multiple aspects of the immune response, including IL-10 production (11). The AHR is a ligand-activated transcription factor that interacts with a structurally diverse array of ligands, which comprise synthetic compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin and endogenous molecules, which include certain tryptophan and arachidonic acid metabolites (12). AHR activity was initially studied for its role in mediating tetrachlorodibenzo-p-dioxin–induced toxicity. However, a number of recent studies have identified multiple effects of AHR signaling on the immune system, most notably in Th17 cells and innate lymphoid cells (13–18). In contrast to its effects in promoting the expression of the effector cytokines IL-22 or IL-17 in these cells, the AHR has also been shown to promote the production of IL-10. Thus, in type 1 regulatory T cells, the AHR interacts with the transcription factor c-Maf to promote IL-10 expression (11). Ahr−/− dendritic cells and macrophages also exhibit defects in IL-10 production (19, 20). Importantly, AHR activity appears to play a role in the response to *T. gondii*, as Ahr−/− mice show increased susceptibility to this challenge, possibly due to immune-mediated pathology (21).

In this study, we used an IL-10 reporter mouse to characterize the cell types that express IL-10 during toxoplasmosis and identified...
NK cells as a major source of this regulatory factor. During acute infection, NK cells that expressed the IL-10 reporter had higher levels of the IL-12 target genes IFN-γ, T-bet, and killer cell lectin-like receptor subfamily G member 1 (KLRG1) than did reporter-negative NK cells, and IL-12 depletion abrogated NK cell IL-10 reporter expression. However, in vitro studies using NK cells suggested that IL-12 was not sufficient to induce IL-10 and that AHR activation contributed to optimal IL-10 production. NK cells basally expressed AHR transcripts and these were increased following stimulation with IL-12. IL-10 production by in vitro–expanded NK cells (lymphokine-activated killer cells, or LAKs) was enhanced by augmenting AHR activity and decreased in the presence of AHR inhibitors. LAKs genetically deficient for the AHR or the AHR nuclear translocator (ARNT), which dimerizes with the AHR to form a competent transcription factor, were impaired in their ability to produce IL-10. Finally, NK cells isolated from Ahr–/– mice that had been infected with T. gondii exhibited defects in IL-10 expression. These data identify the AHR as a critical cofactor involved in the ability of IL-12 to promote NK cell production of IL-10, suggesting that AHR ligands can serve as signals that allow NK cells to sense and respond to their environment.

Materials and Methods

Mice and infections

Vert-X mice were provided by Dr. Christopher L. Karp (previously at the Cincinnati Children’s Hospital Medical Center, Cincinnati, OH). Ahr–/– mice that had been backcrossed onto a C57BL/6 background for 21 generations were obtained from Dr. Christopher A. Bradfield (University of Wisconsin School of Medicine and Public Health, Madison, WI). Tissues from Vav-Cre Ahr–/– mice and control mice were provided by M. Celeste Simon (University of Pennsylvania, Philadelphia, PA). RAG1–/– mice and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DX5+ NK1.1+CD3– splenocytes from the National Cancer Institute Animal Production Program (Frederick, MD). All mice were bred and housed in specific pathogen-free facilities at the University of Pennsylvania in accordance with institutional guidelines. For infections, Me99 cysts were harvested from the brains of chronically infected CBA/J mice and experimental animals were injected i.p. with 20 cysts. For IL-12 depletion, mice were injected i.p. with 1 mg anti-IL-12p40 (clone C17.8) or control rat IgG (Sigma-Aldrich, St. Louis, MO) 1 d before infection and 3 d after infection.

Cell isolation

Lymphocytes were isolated from the liver as described previously (22). Spleens were dissociated through a 40-μm filter and RBcs were lysed with 0.86% ammonium chloride (Sigma-Aldrich) in sterile water. Peritoneal exudate cells (PECs) were collected by lavaging the peritoneal cavity with 7 ml PBS. Bone marrow was isolated by flushing femurs and tibias with PBS, followed by RBC lysis.

LAK generation

For the production of LAKs from RAG1–/– mice, bone marrow cells were plated at 1 x 10⁶ cells/ml in 10 ml complete RPMI 1640 (10% heat-inactivated FCS, 2 mM glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 1 mM sodium pyruvate, 1% nonessential amino acids, 5 x 10⁻⁵ M 2-ME) and 4 x 10⁵ U/ml recombinant human IL-2 (Proleukin; Novartis, Basel, Switzerland). Cells were supplemented with IL-2 on days 3 and 5 or days 4 and 6 after plating. LAKs were collected and stimulated on day 7. For the generation of LAKs from Vert-X mice, Ahr–/– mice, Vav-Cre Ahr–/– mice, or controls, NK cells were enriched from the bone marrow or spleen using the EasySep mouse NK cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada). CD3+ NK1.1+ cells were sorted on a FACSaria (BD Biosciences, San Jose, CA) and cultured for 1 wk in complete RPMI 1640 with recombinant human IL-2.

Stimulation of LAKs or NK cells isolated from infected mice

LAKs were harvested and plated at a concentration of 1–2 x 10⁶ cells/ml on 96-well plates (BD Biosciences). The cells were cultured in complete RPMI 1640 (or complete IMDM where indicated). LAKs were stimulated with a concentration of 5 ng/ml IL-12 (eBioscience, San Diego, CA) unless otherwise stated, 2000 U/ml Proleukin, 30 μM CH-223191 (Calbiochem, Darmstadt, Germany), 0.625 μM flavone (Sigma-Aldrich), 0.31 μM a-naphthoflavone (Sigma-Aldrich), 300 nM 6-formylindolo[2,3-b]carbazole (Enzo Life Sciences, Farmingdale, NY), or DMSO (Sigma-Aldrich) as a vehicle control. After 48 h, supernatants were collected and levels of IL-10 or IFN-γ were assayed by ELISA. For analysis of IL-10/GFP expression, cells were surface stained and run on a FACSCount II (BD Biosciences). For analysis of IL-10 production from NK cells in infected mice, DX5+ NK1.1+ CD3– cells were sorted from spleens. The purified NK cells were plated at a concentration of 2 x 10⁶ cells/ml on 96-well plates, stimulated for 48 h with 50 ng/ml PMA and 1 μM ionomycin, and cytokine levels were measured by ELISA.

ELISAs

For IL-10 ELISAs, Inmulon 4HBX plates (Thermo Fisher Scientific, Waltham, MA) were coated with anti–IL-10 (clone JES-2A5; BD Pharmingen San Diego, CA), blocked in 5% FBS in PBS, and loaded with samples. Biotinylated anti–IL-10 (clone JES-16E3) was used for detection followed by peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA), TrueBlue (KPL, Gaithersburg, MD), and tetramethylbenzidine stop solution (KPL). For IFN-γ ELISAs, plates were coated with anti–IFN-γ (clone AN-18; eBioscience) and loaded with samples. Biotinylated anti–IFN-γ (clone R4–6A2; eBioscience) was used for detection, followed by peroxidase-conjugated streptavidin and ABTS (KPL). For IL-12p40 ELISAs, plates were coated with anti–IL-12p40 (clone C17.8), followed by detection using biotinylated anti–IL-12p40 (clone C15.6).

RNA preparation and PCR

To evaluate the expression of the AHR and its chaperone proteins, LAKs were stimulated for 6 h or NK1.1+ DX5– cells were sorted from the spleens of infected Vert-X mice. To analyze cytokine expression by NK cells from infected wild-type (WT) or Ahr–/– mice, DX5+ NK1.1+ CD3– cells were sorted from liver lymphocyte preparations. All sorting was performed on a FACSaria or FACS Vantage SE (BD Biosciences). RNA was isolated using the RNAeasy Mini kit (Qiagen, Valencia, CA). Samples were treated with DNase (Promega, Madison, WI), and cDNA was generated using reagents from Invitrogen (Grand Island, NY). For RT-PCR analysis, cDNA samples were amplified with SYBR Green (Applied Biosystems, Carlsbad, CA) on the ABI 7500 Fast real-time PCR system (Applied Biosystems). Primers for AHR and β-actin transcripts were purchased from Invitrogen. Primers for AHR chaperone protein and ARNT transcripts were generated using Primer 3 and obtained from Integrated DNA Technologies (Coralville, IA). The following sequences were used for p23 (5′-TGATCCCTAACAGCAGCTAA-3′ and 5′-AATTGTATGTTCCGGCTTCTT-3′), Hsp90 (5′-AGGAGGATCGAGAAGAAAAG-3′ and 5′-ACAGCGGACCTGTGTT-3′), ARNT (5′-TGCCACTCTGCTACTGCTG-3′ and 5′-GAACATGCTGCTACCTGGAA-3′), and ARAP (5′-CTCGAGATGTCGGCAAGAAC-3′ and 5′-GAAGTGGAGAAGGCTTCTTA-3′). The following sequences were used for IL-10 (5′-ACCTGTCCTACCTTGCT-3′ and 5′-GGTGGCCAAGCTTACTCCGGA-3′), IFN-γ (5′-CTTCCCTGACACAGGACCCG-3′ and 5′-TGACCTGTGTTCTGGAACG-3′), and HPRT (5′-AACTTTTATGTCCTGAGG-3′). Amplification was calculated using the 2–ΔΔCt method (23).

T cell polarization

CD4+ T cells were sorted from the spleens and lymph nodes of C57BL/6 mice. Cells were cultured in complete IMDM for 4 d on plates coated with 1 μg/ml anti-CD3 (eBioscience), with soluble 1 μg/ml anti-CD28 (eBioscience). Th17 cells were cultured under previously described conditions (24). Th1 cells were stimulated with 20 ng/ml IL-12 and 10 μg/ml anti-IL-4 (clone 11B11).

IL-12 expression in infected mice

Serum was collected 5 d after infection and IL-12p40 levels were assayed by ELISA. Splenocytes were cultured at 1 x 10⁶ cells/ml in complete RPMI 1640 with or without 62 μg/ml soluble Toxoplasma Ag (STAg). Supernatants were collected after 48 h and IL-12p40 was detected by ELISA. PECs were incubated with brefeldin A (Sigma-Aldrich) and GolgiStop (BD Biosciences) for 6 h, surface stained, and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Cells were permeabilized with 0.5% saponin (Sigma-Aldrich) and stained for IL-12.

Abs and flow cytometry

For surface staining, samples were washed in flow cytometry buffer containing 1% BSA (Sigma-Aldrich) and 2 mM EDTA (Invitrogen) in PBS, fixed with 2.4% and normal rat IgG (Invitrogen), and stained with mAbs. For detection of IFN-γ expression by NK cells, splenocytes were...
surface stained and stimulated with PMA and ionomycin with brefeldin A (Sigma-Aldrich) for 4 h. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, and stained for IFN-γ. For the detection of T-bet and Ki67, cells were surface stained and then stained intracellularly for T-bet and Ki67 using the Foxp3/transcription factor staining buffer set (eBioscience). For the detection of GFP in fixed cells from Vert-X mice, cells were stained intracellularly for GFP using an anti-GFP Ab from eBioscience. For the detection of GFP in fixed cells from Vert-X mice that are KLRG1+. T-bet and Ki67 using the Foxp3/transcription factor staining buffer set (25).

Preparation kit (Roche, Indianapolis, IN). Parasite DNA levels were determined by RT-PCR as previously described (25). DNA was isolated from the PECs using the High Pure PCR Template Preparation kit (Roche, Indianapolis, IN). Parasite DNA levels were determined by RT-PCR as previously described (25).

Parasite burdens

DNA was isolated from the PECs using the High Pure PCR Template Preparation kit (Roche, Indianapolis, IN). Parasite DNA levels were determined by RT-PCR as previously described (25).

Statistical analysis

Statistical significance was determined using paired or unpaired Student t tests, which were performed using Prism software (GraphPad Software La Jolla, CA). For Figs. 3B, 5C, 5H, 5I, and 6f paired t tests were used to determine significance using pooled data from multiple experiments. In this analysis, the results from each individual experiment were paired.

Results

Characterization of cellular sources of IL-10 during toxoplasmosis

Whereas CD4+ T cells are an essential source of IL-10 during infection, studies in IL-10−/− SCID mice have suggested that the production of IL-10 by innate cell populations promotes susceptibility to T. gondii (6, 7). To identify innate and adaptive sources of IL-10, Vert-X IL-10/GFP reporter mice, which have been used previously to assess IL-10 expression (8, 26, 27), were challenged with the Me49 strain of T. gondii. Five days following infection, NK cells constituted the largest fraction of total IL-10–expressing cells in the spleen and liver, which are two of the main sites of mature NK cell localization (28) (Fig. 1A). The numbers of splenic IL-10/GFP+ cells are shown in Supplemental Fig. 1. These results indicated that NK cells are a major source of IL-10 during early infection, in agreement with previous findings (8).

Further characterization of NK cells in the Vert-X mice revealed that infection led to a global increase in CD11c and CD69 expression and that these markers of NK cell activation were ex-

FIGURE 1. Phenotype of IL-10/GFP+ and IL-10/GFP− NK cells following infection. Vert-X IL-10 reporter mice were infected with T. gondii for 5 d. (A) Percentages of different cell types within the total IL-10/GFP+ population in the spleen, PECs, and liver of infected Vert-X mice, represented as pie graphs. Average percentages were calculated from two to three independent experiments, with a total of 8–10 mice. Various cell types were distinguished based on the expression of surface receptors: NK cells (DX5+ NK1.1+CD3+CD19+), CD4+ T cells (CD3+CD4+), CD8 T cells (CD3+CD8+), CD4+CD8+ T cells (CD3+CD4+CD8+), and B cells (CD19+CD5+). (B) Expression of CD69 and CD11c on splenic NK cells from naive and infected Vert-X mice. Results are representative of three independent experiments, with a total of 5 naive and 11 infected mice. (C) The expression of Ki67, IFN-γ, T-bet, and KLRG1 by total splenic NK cells from naive Vert-X mice and the IL-10/GFP+ or IL-10/GFP− NK cells from infected mice. Cells were restimulated with PMA/ionomycin to evaluate IFN-γ production. Data are representative of two to three independent experiments with a total of eight to nine infected mice and four to five naive mice. (D) Frequency of splenic IL-10/GFP+ and IL-10/GFP− NK cells from infected Vert-X mice that produce IFN-γ following PMA/ionomycin restimulation. Data are pooled from two experiments with a total of eight mice. (E) The MFI of T-bet in splenic IL-10/GFP+ and IL-10/GFP− NK cells from infected Vert-X mice. Data are pooled from three experiments with a total of nine mice. (F) Frequencies of IL-10/GFP− NK cells or IL-10/GFP+ NK cells from infected Vert-X mice that are KLRG1+. Data are pooled from three independent experiments with a total of nine mice.
pressed similarly by IL-10/GFP+ and IL-10/GFP− NK cells (Fig. 1B) (29, 30). The levels of Ki67, a marker of proliferating cells, also increased in IL-10/GFP+ and IL-10/GFP− NK cells from infected mice (Fig. 1C). However, several other markers were differentially expressed by IL-10/GFP+ and IL-10/GFP− NK cells. Following PMA/ionomycin stimulation, a higher percentage of IL-10/GFP− NK cells produced IFN-γ than did IL-10/GFP+ NK cells (Fig. 1C, 1D). Most NK cells in naive mice stained positive for the transcription factor T-bet, but T-bet expression increased following infection (Fig. 1C) and IL-10/GFP+ NK cells expressed the highest levels of T-bet (Fig. 1E). Both IL-10/GFP+ and IL-10/GFP− NK cells from infected mice expressed high levels of KLRG1, but a significantly higher percentage of IL-10/GFP+ cells were KLRG1+ (Fig. 1C, F). The marked increases in NK cell expression of CD69 and CD11c following infection indicated that both IL-10/GFP+ and IL-10/GFP− NK cells had become activated, but IL-10/GFP+ NK cells expressed higher levels of IFN-γ, T-bet, and KLRG1, which are targets of IL-12 signaling (31, 32).

IL-12 induces NK cell IL-10 expression

The finding that NK cells are a major source of IL-10 in early infection raised the question of which signals induce IL-10 expression by this population. Previous work has shown that IL-12 promotes NK cell IL-10 production during toxoplasmosis (8), consistent with the finding that IL-10/GFP+ NK cells expressed high levels of IL-12 target genes. To examine the effects of IL-12 signaling on NK cells during infection, Vert-X mice were treated with IL-12–depleting Abs and challenged with T. gondii. Depletion of IL-12 in infected Vert-X mice abrogated NK cell IL-10 reporter expression (Fig. 2A), in agreement with previous studies (8). NK cells in infected control and anti–IL-12p40-treated mice upregulated expression of CD69 and CD11c (Fig. 2B). However, IL-12 depletion antagonized the infection-induced upregulation of KLRG1 and Ki67 (Fig. 2C–E). T-bet mean fluorescence intensities (MFIs) were also reduced following IL-12 depletion (Fig. 2C, 2F). These results suggested that IL-12 signaling during infection promoted NK cell IL-10 expression and proliferation. In agreement with this, infected mice that had been treated with IL-12–depleting Abs had fewer total splenic NK cells than did infected controls (data not shown).

To study the factors that regulate NK cell expression of IL-10 in vitro, IL-2–activated LAKs were used, which were generated as previously described (33). To evaluate IL-10 production by these cells, LAKs were stimulated with different combinations of cytokines for 48 h. In the absence of stimulation, these cells did not produce detectable levels of IL-10, and stimulation with IL-2 or IL-12 alone induced little IL-10 secretion (Fig. 3A). However, when treated with the combination of IL-2 and IL-12, LAKs produced high levels of IL-10 and IFN-γ as previously reported (34). Similar results were observed with NK cells freshly isolated from the spleens of RAG1−/− mice (data not shown). A number of other cytokines, including the IL-12 family members IL-23 and IL-27, did not induce IL-10 production from LAKs when used in various combinations with IL-2 or IL-12 (data not shown).

**FIGURE 2.** Phenotype of NK cells following IL-12 depletion during infection. Vert-X IL-10 reporter mice were infected with T. gondii for 5 d and given control rat IgG or anti–IL-12p40. NK cells were gated as DX5+ NK1.1+CD3− CD19− cells. (A) IL-10/GFP expression by NK cells in the spleens of Vert-X mice that were given rat IgG or anti–IL-12p40. Numbers represent the means ± SD from four independent experiments, with a total of 13–16 mice per group. (B and C) Expression of CD69, CD11c, KLRG1, Ki67, and T-bet by NK cells from naive Vert-X mice or infected Vert-X mice that were given rat IgG or anti–IL-12p40. Numbers represent the means ± SD from two to four independent experiments, with a total of 13–16 mice per group. (D) KLRG1 MFIs of KLRG1+ NK cells from infected Vert-X mice given rat IgG or anti–IL-12p40. Data are pooled from four independent experiments. (E) Frequency of Ki67+ NK cells from infected Vert-X mice given rat IgG or anti–IL-12p40. Data are pooled from four independent experiments. (F) T-bet MFIs of NK cells from infected Vert-X mice given rat IgG or anti–IL-12p40. Data are pooled from four independent experiments.
AHR and NK cells express the AHR

Although these studies highlighted the critical role of IL-12 in inducing NK cell IL-10 expression, optimal cytokine production by NK cells typically requires multiple signals. Therefore, additional pathways may have contributed to IL-10 induction in these cells. One candidate was signaling by the AHR, which promotes IL-10 production by macrophages, dendritic cells, and type 1 regulatory T cells (11, 19, 20). Indeed, LAKs that had been stimulated with IL-2 and IL-12 in the culture medium IMDM, which contains high levels of AHR ligands (35), secreted significantly more IL-10 than did LAKs that were cultured in RPMI 1640 (Fig. 3B). The production of IL-10 was not affected by the type of media used (Fig. 3B). Because this result suggested that AHR signaling promoted IL-10, NK cell expression of transcripts for the AHR as well as the p23, Hsp90, and ARA9 chaperone proteins that associate with the AHR were then evaluated (Fig. 4A). LAKs basally expressed transcripts for the AHR, p23, Hsp90, ARA9, and ARNT (Fig. 4A), which heterodimerizes with the AHR in the nucleus to form a competent transcription factor. Although the expression of most AHR chaperone proteins and ARNT was not affected by IL-12, this treatment did result in a ~3-fold increase in AHR mRNA within 6 h (Fig. 4A). Transcripts for the AHR, p23, Hsp90, ARA9, and ARNT were also detected in NK cells that had been freshly isolated from the spleens of RAG1−/− mice (Fig. 4B). Because Th17 cells express high levels of AHR mRNA compared with other T cell subsets, polarized Th17 and Th1 cells were included in these experiments as controls (14, 15, 36). These results suggest that NK cells are able to respond to AHR signaling and that their ability to do so is enhanced by IL-12 stimulation. Interestingly, NK cells isolated from T. gondii-infected mice 5 d after infection expressed reduced levels of AHR transcripts compared with cells from naive mice (data not shown). These data are consistent with the idea that stimulation with AHR ligands can lead to a down-regulation of AHR expression (37).

Effect of AHR signaling on IL-10 and IFN-γ production by LAKs

To determine the effects of AHR signaling on NK cell cytokine production, LAKs were stimulated with AHR inhibitors in the presence of IL-2 and IL-12 and supernatants were assayed for IL-10 and IFN-γ. The effects of these inhibitors on LAK IFN-γ expression were variable: two of the inhibitors, flavone and α-naphthoflavone, had no significant effect on IFN-γ production, whereas treatment with CH-223191 led to decreased IFN-γ secretion (Fig. 5A). No difference in survival was seen between LAKs treated with DMSO as a vehicle control or cells stimulated with CH-223191 (data not shown). IFN-γ transcript expression also decreased following stimulation with CH-223191 (Fig. 5B). To further investigate the effects of AHR signaling on NK cells, LAKs were stimulated in vitro with the high-affinity AHR ligand FICZ, which can form intracellularly from tryptophan following exposure to UV light (38, 39). LAKs stimulated with FICZ in the presence of IL-2 and IL-12 produced similar levels of IFN-γ as did LAKs treated with the vehicle control (Fig. 5C). Because possible off-target effects with the use of pharmacological agents as AHR agonists or antagonists were a concern, LAKs were generated from mice genetically deficient for the AHR and WT controls (Fig. 5D). Following stimulation with IL-2 and IL-12, Ahr−/− LAKs secreted less IFN-γ than did LAKs from WT mice. This reduction was statistically significant (p < 0.02) when comparing the pooled percentages of Ahr−/− LAK IL-10 production relative to WT LAKs from three separate experiments. No survival differences were observed between WT and Ahr−/− LAKs (data not shown). Taken together, the results from these studies suggest a role for AHR signaling in the ability of LAKs to produce IFN-γ.

The effects of AHR signaling on IL-10 production by LAKs were also evaluated. All three of the AHR inhibitors significantly reduced LAK IL-10 secretion in response to IL-2 and IL-12 (Fig. 5E). Flavone and α-naphthoflavone have been shown to act as AHR agonists at high concentrations (40), and their effects on IL-10 expression were concentration-dependent, with maximal inhibition of IL-10 seen when LAKs were treated with intermediate concentrations of these inhibitors (data not shown). Similarly, stimulation with CH-223191, the most effective AHR inhibitor used in these studies (40), also led to a decrease in the level of IL-10 mRNA expressed by LAKs (Fig. 5F). This was corroborated by using LAKs generated from Vert-X mice, which expressed low levels of AHR ligands (35), and secreted significantly less IL-10 than did LAKs that were cultured in RPMI 1640 (Fig. 3B). The production of IFN-γ was not affected by the type of media used (Fig. 3B). Because this result suggested that AHR signaling promoted IL-10, NK cell expression of transcripts for the AHR as well as the p23, Hsp90, and ARA9 chaperone proteins that associate with the AHR were then evaluated (Fig. 4A). LAKs basally expressed transcripts for the AHR, p23, Hsp90, ARA9, and ARNT (Fig. 4A), which heterodimerizes with the AHR in the nucleus to form a competent transcription factor. Although the expression of most AHR chaperone proteins and ARNT was not affected by IL-12, this treatment did result in a ~3-fold increase in AHR mRNA within 6 h (Fig. 4A). Transcripts for the AHR, p23, Hsp90, ARA9, and ARNT were also detected in NK cells that had been freshly isolated from the spleens of RAG1−/− mice (Fig. 4B). Because Th17 cells express high levels of AHR mRNA compared with other T cell subsets, polarized Th17 and Th1 cells were included in these experiments as controls (14, 15, 36). These results suggest that NK cells are able to respond to AHR signaling and that their ability to do so is enhanced by IL-12 stimulation. Interestingly, NK cells isolated from T. gondii-infected mice 5 d after infection expressed reduced levels of AHR transcripts compared with cells from naive mice (data not shown). These data are consistent with the idea that stimulation with AHR ligands can lead to a down-regulation of AHR expression (37).
on its interaction with ARNT. Collectively, these studies indicated that AHR activity is required for optimal IL-10 expression by LAKs in response to stimulation with IL-2 and IL-12.

Role of the AHR in the innate response to T. gondii

To determine the role of the AHR on NK cell responses in vivo, Ahr<sup>−/−</sup> mice were challenged with T. gondii and their innate immune responses were evaluated. Analysis of IL-12 production revealed that infection led to comparable serum levels of IL-12 in WT and Ahr<sup>−/−</sup> mice (Fig. 6A). Dendritic cells from these mice also expressed similar levels of IL-12 (Fig. 6B, 6C), and splenocytes isolated from infected WT and Ahr<sup>−/−</sup> mice produced comparable levels of IL-12 upon stimulation with STAγ (Fig. 6D), indicating that infected Ahr<sup>−/−</sup> mice had no early defect in IL-12 production. Infected Ahr<sup>−/−</sup> and WT mice also had similar numbers of NK cells (Fig. 6E). These populations expressed comparable levels of CD69, CD11c, T-bet, KLRG1, and Ki67 (Fig. 6F, 6G). However, NK cells isolated from Ahr<sup>−/−</sup> mice had marked reductions in their levels of IL-10 mRNA compared with cells from WT mice (Fig. 6H). Ahr<sup>−/−</sup> NK cells isolated from infected mice were also impaired in their ability to secrete IL-10 following stimulation with PMA/ionomycin ex vivo (Fig. 6I). Although IL-10 plays a critical role in limiting infection-induced immunopathology, IL-10 signaling can also promote increased pathogen burdens by attenuating inflammatory responses and suppressing antimicrobial activity. Accordingly, infected Ahr<sup>−/−</sup> mice had decreased parasite burdens during the acute phase of infection (Fig. 6J). Collectively, these results indicated that although T. gondii infection induced AHR-independent activation of NK cells, optimal NK cell expression of IL-10 was dependent on AHR activity.

Discussion

Multiple studies have identified effects of AHR signaling on cells of the immune system, including Th17 cells, regulatory T cells, γδ T cells, innate lymphoid cells, dendritic cells, and macrophages (14–20, 36, 41, 42). In many of these instances, AHR activity affects cytokine production, but AHR signaling can also affect the survival or maintenance of certain immune cell populations (16–18). In the present study, we establish that AHR signaling also influences NK cell production of IL-10. NK cells basally expressed AHR transcripts, and AHR expression in LAKs increased following stimulation with IL-12. AHR signaling in turn promoted
NK cell IL-10 production in vitro and contributed to their IL-12–dependent production of IL-10 during infection with *T. gondii*. Although previous work has suggested that NK cell IL-10 limits IL-12 production during experimental toxoplasmosis (8), in the present study *Ahr*<sup>−/−</sup> mice had similar levels of IL-12p40 as did WT mice. Importantly *Ahr*<sup>−/−</sup> mice also had lower parasite burdens than did WT mice, which likely impacted their IL-12 levels.

Although this report has focused on the impact of the AHR on NK cell production of IL-10, its effects on IFN-γ expression were variable. However, a recent study has shown that the AHR promotes NK cell cytolytic activity and IFN-γ production, and that NK cells with deficient AHR activity are impaired in their ability to control tumor growth (43). Collectively, these studies indicate that the AHR has a number of important effects on NK cell function in settings of infection and cancer. Thus, AHR expression likely contributes to the ability of NK cells to sense environmental signals.

NK cells express a number of receptors that allow them to detect external cues, including cytokine receptors and activating and inhibitory receptors (44). AHR activity may provide these cells with an additional means of responding to the environment, in this case.
by promoting the production of IL-10. NK cells have been shown to produce IL-10 following infection with *Yersinia pestis*, *Listeria monocytogenes*, and murine CMV (8, 10). Additionally, during visceral leishmaniasis, NK cell IL-10 promotes increased parasite burdens (9). Indeed, in the present study, the defect in NK cell IL-10 expression in *Ahr*<sup>+/−</sup> mice correlated with decreased levels of *T. gondii* at 5 d after infection. Thus, it seems likely that AHR signaling affects NK cell function during challenge with other pathogens and thereby affects outcomes of infection.

The finding that only a subset of NK cells expressed IL-10/GFP at 5 d after infection raises the question of what accounted for the differential expression of this cytokine. These subsets may reflect possible variability in AHR expression or access to AHR ligands. Alternatively, subsets may be distinguished by differences in cytokine responsiveness. NK cells exhibit variable expression of IL-12Rb2 (45), and those cells expressing higher levels of IL-12Rb2 could be more prone to producing IL-10 in response to IL-12 stimulation. Interestingly, IL-10/GFP<sup>+</sup> NK cells also expressed high levels of T-bet and KLRG1, mirroring the phenotype of short-lived effector CD8<sup>+</sup> T cells, which express higher levels of T-bet and KLRG1 than do the CD8<sup>+</sup> T cells that give rise to long-term memory (32). Several reports have indicated that NK cells can also form memory (46–49), and the expression of T-bet and KLRG1 may distinguish short-lived and long-lived populations of NK cells.
local infections elicit immunosuppressive IL-10 production by natural killer cells. Cell Host Microbe 6: 503–512.
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... effects on CD8^+ T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27: 281–295.
Supplementary Figure 1: Numbers of IL-10/GFP$^+$ cells in infected Vert-X mice.

Vert-X mice were infected for 5 days with *T. gondii*. (A) The number of different populations of IL-10/GFP$^+$ cells in the spleens of infected Vert-X mice. (B) The total number of different populations of cells in the spleens of infected Vert-X mice. NK cells were gated as DX-5$^+$ NK 1.1$^+$CD3$^-$CD19$^-$ cells. Error bars represent standard error.