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IL-23 Is Required for the Development of Severe Egg-Induced Immunopathology in Schistosomiasis and for Lesional Expression of IL-17[1]

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In infection with the trematode helminth *Schistosoma mansoni*, the severity of CD4 T cell-mediated hepatic granulomatous and fibrosing inflammation against parasite eggs varies considerably in humans and among mouse strains. In mice, either the natural high pathology, or high pathology induced by concomitant immunization with schistosome egg Ags (SEA) in CFA (SEA/CFA), results from a failure to contain a net proinflammatory cytokine environment. We previously demonstrated that the induction of severe immunopathology was dependent on the IL-12/IL-23 common p40 subunit, and correlated with an increase in IL-17, thus implying IL-23 in the pathogenesis. We now show that mice lacking the IL-23-specific subunit p19 are impaired in developing severe immunopathology following immunization with SEA/CFA, which is associated with a marked drop of IL-17 in the granulomas, but not in the draining mesenteric lymph nodes, and with a markedly suppressed SEA-specific IFN-γ response regulated by a striking increase in IL-10. The granulomas are characterized by a significant reduction in Gr-1$^+$ cell recruitment and by alternative macrophage activation. Taken together, these results demonstrate that IL-23 per se is not necessary for the generation of IL-17-producing T cells, but is essential for the development of severe schistosome egg-induced immunopathology, and its absence cannot be overcome with other possible compensatory mechanisms. ***The Journal of Immunology,*** **2008, 180: 2486–2495.**

Granulomatous and fibrosing inflammation against parasite eggs is the main pathology in infection with schistosome helminths (1, 2). The lesions are mediated and orchestrated by CD4 T cells specific for egg Ags, and in the case of the species *Schistosoma mansoni*, localize to the liver and intestines. The extent of immunopathology varies widely both in humans as well as among mouse strains in an experimental model (3–6). In genetically predisposed low pathology mouse strains, such as the C57BL/6 (BL/6), an early Th1-type response readily gives way to a Th2-type response, whereas in high pathology strains, such as the C3H or CBA, the Th1 response lingers along with the Th2 response into the chronic phase of the disease (7). Significantly, a high pathology phenotype with remarkable similarity to its natural counterpart can also be induced in BL/6 mice by concomitant immunization with soluble schistosome egg Ags (SEA)[3] in CFA (SEA/CFA) (8–10).

IL-12, together with the transcription factor T-bet, is a most critical element in the induction of Th1 responses (11). IL-12 is a heterodimeric cytokine composed of two subunits, p40 and p35; however, the finding that the p40 subunit can also associate with a distinct p19 subunit to form IL-23 has added diversity and complexity to the initiation of inflammatory processes (12). Both IL-12 and IL-23 are produced by the same set of accessory cells—mainly activated dendritic cells and macrophages—but each of them drives different and independent subsets of proinflammatory CD4 T cells with distinct signature cytokines and functions: IL-12 induces Th1 cells that produce IFN-γ, thereby activating macrophages to kill intracellular pathogens, whereas IL-23 promotes the so-called Th17 cells, characterized by producing IL-17, IL-6, TNF-α (13, 14) and by mediating chronic inflammation in autoimmune diseases, including experimental allergic encephalomyelitis (15, 16), collagen-induced arthritis (17, 18), inflammatory bowel disease (19), and systemic sclerosis (20), as well as in transplant rejection (21).

Using schistosome-infected IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ BL/6 mice, we previously demonstrated that the exacerbation of egg-induced immunopathology afforded by immunization with SEA/CFA is not dependent on IL-12p35, but fails to materialize in the absence of IL-12p40 (10). The SEA/CFA-induced pathology correlated with increased levels of IL-17 and a decline in IL-5 and IL-10, but was independent of IFN-γ. Importantly, naturally high pathology CBA mice similarly exhibited elevated IL-17, and in both models the severe hepatic lesions could be reduced by in vivo treatment with mAb against IL-17 (10). These results, in agreement with the observations made in the autoimmune diseases, suggested that the IL-23/IL-17 pathway, and not the IL-12/IFN-γ pathway, was critical for the development of high pathology in schistosomiasis, indirectly implying a need for IL-23 in the Th17 cell-mediated immunopathology. More recent studies, however, cast a doubt on the importance of IL-23 by demonstrating that Th17 cells can be generated by a combination of IL-6 and TGF-β with IL-23 acting as a survival or expansion factor for the cells of this lineage (22–24). IL-21 has also been shown to facilitate Th17 cell development (25, 26).

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[3] Abbreviations used in this paper: SEA, schistosome egg Ag; MLN, mesenteric lymph node; Ct, cycle threshold; GC, granuloma cell; MLNC, MLN cell; INOS, inducible NO synthase.

Table I. *Primers used in this study*

Primer Name	Forward	Reverse	Accession Number
p19	CCAGCAGCTCTCTCGGAATC	GATTCATATGTCCCGCTGGTG	
p40	ACAGCACCAGCTTCTTCATCAG	TCTTCAAAGGCTTCATCTGCAA	
p35	CACCCTTGCCCTCCTAAACC	CACCTGGCAGGTCCAGAGA	
IL-17	ACCGCAATGAAGACCCTGAT	CAGGATCTCTTGCTGGATGAGA	
IFN-γ	GGATATCTGGAGGAACTGGCAA	TGATGGCCTGATTGTCTTTCAA	
IL-6	ACACATGTTCTCTGGGAAATCGT	AAGTGCATCATCGTTGTTCATACA	
IL-4	CTCATGGAGCTGCAGAGACTCTT	CATTCATGGTGCAGCTTATCGA	
IL-13	TGACCAACATCTCCAATTGCA	TTGTTATAAAGTGGGCTACTTCGATTT	
IL-10	TTTGAATTCCCTGGGTGAGAA	GCTCCACTGCCTTGCTCTTATT	
IL-21			NM..021782.2
CXCL1			NM..008176.1
CXCL2			NM..009140.2
CCL11			NM..011330.1
YM1			NM..009892.1
Arginase			NM..007482.2
INOS			NM..00440483.m1

To more precisely address the role of IL-23 in high pathology schistosomiasis, we now examined mice lacking the IL-23p19 subunit, which are unable to produce IL-23, while retaining the ability to make IL-12. Unlike the IL-12p40$^{-/-}$ mice, the schistosome-infected IL-23p19$^{-/-}$ mice displayed a more modest but still significant increase in pathology in response to immunization with SEA/CFA, which correlated with a rise in IL-17 production in the mesenteric lymph nodes (MLN), but not in the hepatic lesions themselves. Thus, our findings indicate that IL-23 critically contributes to the immunopathology, but by itself is not essential for the development of Th17 cells in schistosomiasis.

Materials and Methods

Mice, infection, and immunization

IL-23p19-deficient (IL-23p19$^{-/-}$) mice were produced as previously described (15) and bred in-house. C57BL/6 (BL/6) mice, 5–6 wk old, were purchased from The Jackson Laboratory. All mice were maintained in the Animal Facility at Tufts University School of Medicine in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. Mice were infected by i.p. injection with 80 cercariae of *S. mansoni* (Puerto Rico strain), which were obtained from infected *Biomphalaria glabrata* snails, provided to us by Dr. F. Lewis (Biomedical Research Institute, Rockville, MD, through National Institutes of Health/National Institute of Allergy and Infectious Diseases Contract N01-AI-55270). Some mice were immunized by s.c. injection with 50 μg of SEA emulsified in CFA (SEA/CFA), as previously described (8). Treatment of BL/6 mice with SEA/CFA causes marked exacerbation of their egg-induced immunopathology; either SEA or CFA by themselves are ineffective (8). SEA was prepared as previously described (27).

Real-time quantitative RT-PCR

Total RNA from individual livers and MLN of schistosome-infected mice were isolated using RNA STAT-60 (Tel-Test) according to manufacturer's instructions. After isopropanol precipitation, total RNA was re-extracted with phenol:chloroform:isoamyl alcohol (25:24:1; Sigma-Aldrich) using phase-lock light tubes (Eppendorf). Total RNA (5 μg) was subjected to treatment with DNase (Roche Molecular Biochemicals) according to the manufacturer's instructions to eliminate possible genomic DNA contamination. DNase-treated total RNA was reverse-transcribed using Superscript II (Invitrogen Life Technologies) according to manufacturer's instructions. Primers shown in Table I were designed using Primer Express (Applied Biosystems). Other primers, for which sequences are unavailable, were obtained commercially from Applied Biosystems. Real-time RT-PCR on 10 ng of cDNA from each sample was performed using either of two methods. In the first method, two gene-specific unlabeled primers were used at 400 nM in a PerkinElmer SYBR Green real-time quantitative PCR assay using an ABI 5700 instrument (Applied Biosystems). In the second method, two unlabeled primers at 900 nM each were used with 250 nM FAM-labeled probe (Applied Biosystems) in a TaqMan real-time quantitative PCR on an ABI 7700 sequence detection system. The absence of genomic DNA contamination was confirmed using primers that recognize the genomic region of the CD4 promoter. Ubiquitin levels were measured in a separate reaction and used to normalize the data. Using the mean cycle threshold (Ct) value for ubiquitin and the gene of interest for each sample, the equation 1.8 e (Ct ubiquitin − Ct gene of interest) × 10^4 was used to obtain the normalized values (16).

Cell preparations

Livers and MLN were removed aseptically from 7-wk infected mice. Granuloma cells (GC) were obtained by homogenization of the livers in a Waring blender, isolation of granulomas by 1 g of sedimentation, extensive washing and enzymatic digestion with 1 mg/ml collagenase type H, from *Clostridium histolyticum* (Sigma-Aldrich). Single-cell suspensions from MLN were prepared by teasing the tissues in complete RPMI 1640 medium (cRPMI) supplemented with 10% FCS (Atlanta Biologicals), 4 mM L-glutamine, 80 U/ml penicillin, 80 μg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES, 1× non-essential amino acids (all obtained from BioWhittaker), and 0.1% 2-ME. Erythrocytes were lysed with Tris ammonium chloride buffer (pH 7.2; Sigma-Aldrich) for 15 min on ice. Cells were washed and live cells that excluded trypan blue were counted and resuspended at the desired concentrations in cRPMI. For purification of CD4$^+$ cells, MLN cells (MLNC) were negative selected on CD4 MACS columns (Miltenyi Biotec) following the manufacturer's instructions. The resulting cell preparations were >94% CD4$^+$ cells as determined by flow cytometry.

Cell cultures and cytokine determinations

Bulk cell suspensions (5 × 10^6 cells/ml) from hepatic granulomas and MLN, or purified CD4$^+$ cells from MLN (1 × 10^6 cells/ml) plus normal irradiated syngeneic splenic APC (4 × 10^6 cells/ml), were incubated in the presence or absence of 15 μg/ml SEA. Anti-IL-10 mAb (clone JS5-2A5; BD Biosciences) were added into some cultures of MLNC at a concentration of 1 or 5 μg/ml. After 48 h, the culture supernatants were removed, filtered, and stored at −36°C until analysis by ELISA. For the detection of IL-6, IL-17, IL-21, and IFN-γ, mAb, standard cytokines and protocols were obtained from R&D Systems, and for the detection of IL-5, IL-10, and TGF-β, from BD Biosciences.

Flow cytometry

GC were stained ex vivo for flow cytometry analysis using FITC-conjugated anti-Gr-1 (clone RB6-8C5; BD Biosciences) and allophycocyanin-conjugated anti-CCR3 mAb (R&D Systems) following a protocol described previously (28). Labeled cells were acquired on a FACSCalibur flow cytometer using the CellQuest software version 3.2.1 (BD Biosciences). Data were analyzed using the WinList 5.0 software (Verity Software House). Unstained cells and cells stained with irrelevant isotype-matched Abs were included as controls to assess the amount of nonspecific staining.

Histopathology and morphometric analysis

Liver samples were fixed in 10% buffered formalin and processed for routine histopathologic analysis. Five-micrometer sections were stained with H&E and the extent of granulomatous inflammation around schistosome

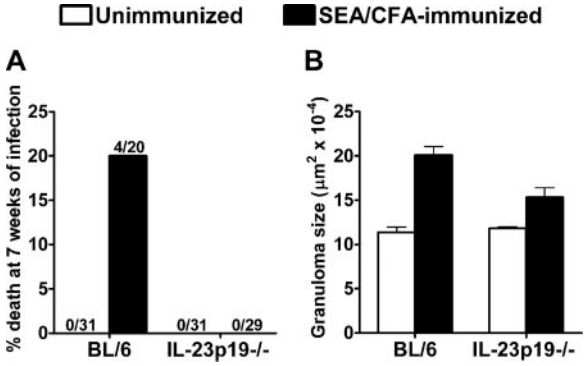

FIGURE 1. Mortality and hepatic immunopathology in 7-wk schistosome-infected BL/6 and IL-23p19$^{-/-}$ mice. *A*, Mortality by 7 wk postinfection occurs only in the SEA/CFA-immunized BL/6 mice ($p = 0.0003$). Indicated are the numbers of dead mice over total in group. *B*, Hepatic granulomatous inflammation is significantly lower in the infected SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice but significantly higher in SEA/CFA-immunized IL-23p19$^{-/-}$ than in their unimmunized counterparts (both $p < 0.05$). Mortality data are pooled from seven separate experiments and the immunopathology from three separate experiments with three to six mice per group.

eggs was measured by computer-assisted morphometric analysis using Image-Pro Plus software (Media Cybernetics). The lesions were assessed on coded slides by an observer unaware of the experimental setting. To reflect more accurately the true shape and dimension of the granulomas, only those with a visible central egg were counted. A minimum of 10 granulomas were scored per liver section. Granuloma sizes are expressed as means of areas measured in micrometers squared ± SEM.

Statistical analysis

ANOVA followed by the Tukey test for multiple comparisons or the χ^2 test were used to determine the statistical significance of the differences among groups. Results differing with a $p < 0.05$ were considered significant. Each individual experiment was conducted with groups of three to eight mice.

Results

Decreased mortality and reduced hepatic egg immunopathology in schistosome-infected, SEA/CFA-immunized IL-23p19$^{-/-}$ mice

The role of IL-23 in the schistosome infection was investigated in IL-23p19$^{-/-}$ mice concomitantly immunized with SEA/CFA, a regimen that causes marked exacerbation of egg-induced immunopathology with sharp increase in IL-17 and death typically ensuing at 7 wk postinfection (8, 10). This model, which bears substantial resemblance to the natural high pathology, was used because the IL-23p19$^{-/-}$ mice are on a low pathology BL/6 background. Unlike the BL/6 wild-type mice, all SEA/CFA-immunized IL-23p19$^{-/-}$ mice survived the 7 wk schistosome infection (Fig. 1*A*), as was the case in IL-12p40$^{-/-}$ mice examined in another study (10). However, unlike the IL-12p40$^{-/-}$ mice, in which there was no pathology exacerbation following immunization with SEA/CFA, the IL-23p19$^{-/-}$ mice consistently responded to this challenge with a small but significant increase in the hepatic immunopathology (Fig. 1*B*). No deaths or differences in the low pathology were observed in the schistosome-infected, unimmunized IL-23p19$^{-/-}$, or BL/6 mice.

In infected, SEA/CFA-immunized IL-23p19$^{-/-}$ mice, IL-17 *gene expression is profoundly inhibited in the livers, but not in the MLN*

The use of mice lacking specific and shared subunits of IL-12 and IL-23 made it possible to begin understanding the pathogenic roles of Th1 vs Th17 subsets in a number of disease models including schistosomiasis (10). Analysis of mRNA transcripts by real-time RT-PCR confirmed that IL-23p19 (Fig. 2*A*), but not IL-12p40 (Fig. 2*B*) and IL-12p35 (Fig. 2*C*) were indeed absent in both the liver as well as MLN from the schistosome-infected, SEA/CFA-immunized, or unimmunized IL-23p19$^{-/-}$ mice.

We previously demonstrated that pathology exacerbation following immunization with SEA/CFA correlates with increased levels of IL-17 in GC and MLNC irrespective of the presence or absence of IFN-γ (10). In agreement with these observations, there were no mRNA IL-17 transcripts in the livers with smaller granulomas of SEA/CFA-immunized IL-23p19$^{-/-}$ mice in comparison with similarly treated BL/6 mice, and there were no IL-17 transcripts in the livers of either unimmunized mouse groups (Fig. 3*A*). In contrast, IFN-γ mRNA levels were not significantly different in these mouse groups (Fig. 3*B*). IL-6 was increased (Fig. 3*C*), and IL-21 was not different (Fig. 3*D*) in the SEA/CFA-immunized mice. IL-4 and IL-13 were significantly reduced in BL/6 mice after immunization, but this difference was considerably less pronounced in the IL-23p19$^{-/-}$ mice (Fig. 3, *E* and *F*). IL-10 was also decreased in the SEA/CFA-immunized mouse groups (Fig. 3*G*).

Examination of mRNA levels from the MLN of the same mouse groups unexpectedly revealed that, in contrast to the livers, there

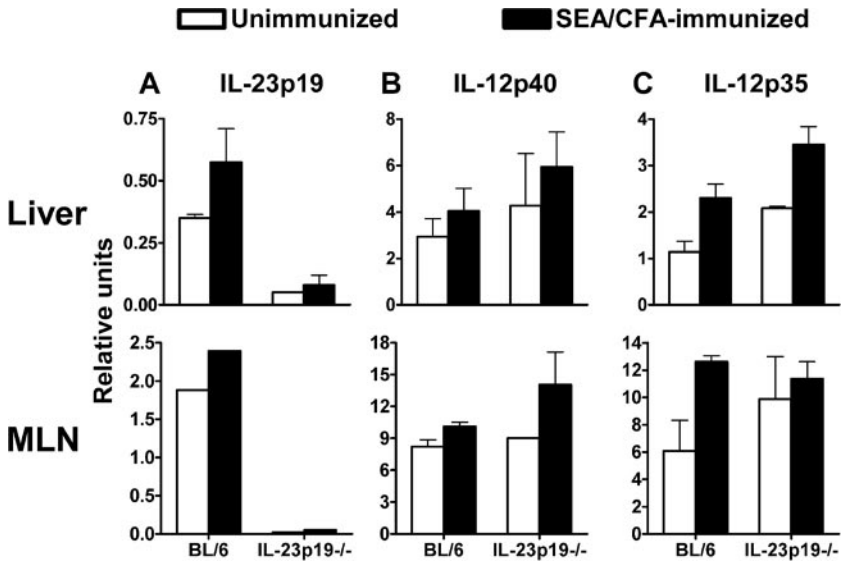

FIGURE 2. IL-23p19, IL-12p40, and IL-12p35 mRNA transcripts from livers and MLN of infected BL/6 and IL-23p19$^{-/-}$ mice. mRNA was measured by real-time RT-PCR as described in *Materials and Methods*. *A*, Liver and MLN IL-23p19 is significantly reduced in SEA/CFA-immunized and unimmunized IL-23p19$^{-/-}$ mice (both $p < 0.05$). Liver and MLN IL-12p40 (*B*) and IL-12p35 (*C*) are not significantly different in the SEA/CFA-immunized or unimmunized mouse groups. PCR data for all three transcripts were normalized to ubiquitin. Each bar represents the mean mRNA level of five to eight mice from two to three independent experiments.

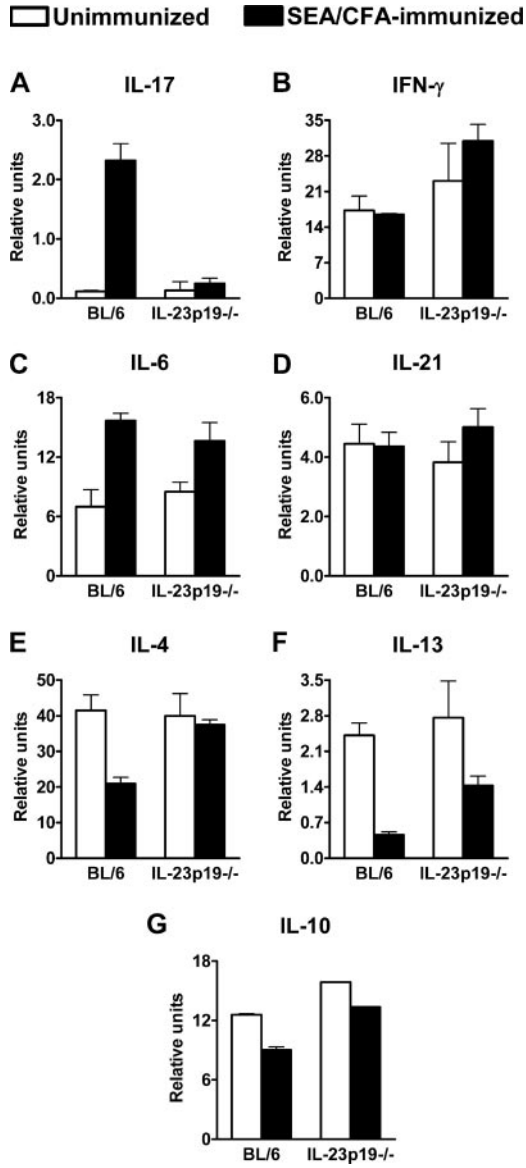

FIGURE 3. Cytokine mRNA transcripts from livers of infected BL/6 and IL-23p19$^{-/-}$ mice. mRNA was measured by real-time RT-PCR as described in *Materials and Methods*. *A*, IL-17 is significantly lower in SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice ($p < 0.001$). *B*, IFN-γ is higher in SEA/CFA-immunized IL-23p19$^{-/-}$ mice but the difference is not statistically significant. *C*, IL-6 is higher in SEA/CFA-immunized mice but not significantly different in BL/6 vs IL-23p19$^{-/-}$ groups. *D*, IL-21 is not significantly different in BL/6 and IL-23p19$^{-/-}$ mice. *E*, IL-4 is significantly higher in SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice ($p < 0.001$). *F*, IL-13 is significantly higher in SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice ($p < 0.01$). *G*, IL-10 is reduced in SEA/CFA-immunized mice but significantly higher in the IL-23p19$^{-/-}$ group ($p < 0.001$). PCR data for all seven transcripts were normalized to ubiquitin. Each bar represents the mean mRNA level ± SD of 5–12 mice from two to three independent experiments.

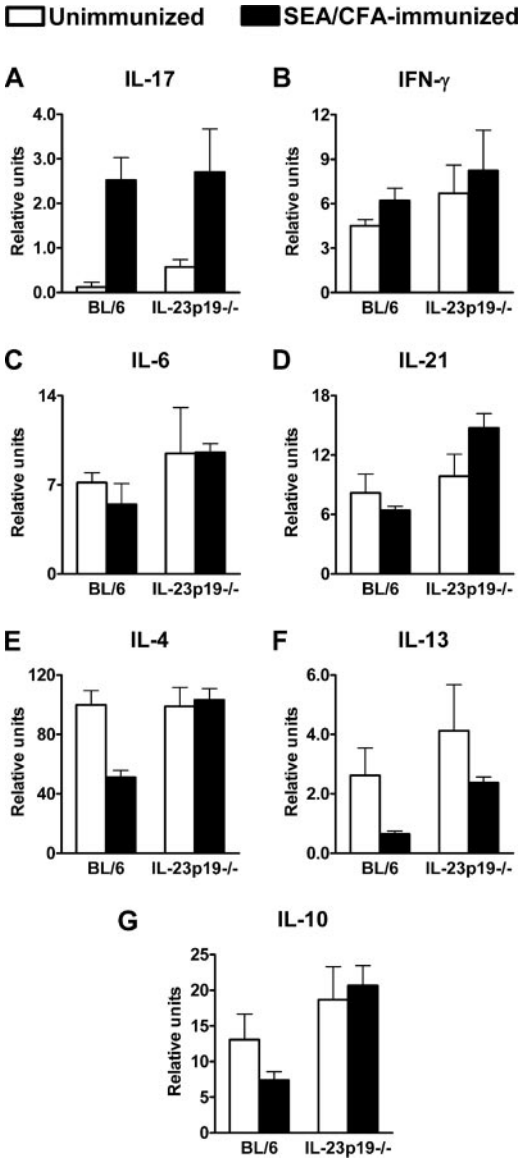

FIGURE 4. Cytokine mRNA transcripts from MLN of infected BL/6 and IL-23p19$^{-/-}$ mice. mRNA was measured by real-time RT-PCR as described in *Materials and Methods*. *A*, Elevated IL-17 levels in both SEA/CFA-immunized BL/6 and IL-23p19$^{-/-}$ mice are not significantly different from one another. *B*, IFN-γ is higher in SEA/CFA-immunized IL-23p19$^{-/-}$ mice, but the difference is not statistically significant. *C*, IL-6 is not significantly different in all four mouse groups. *D*, IL-21 is significantly higher in SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice ($p < 0.001$). *E*, IL-4 is significantly higher in SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice ($p < 0.001$). *F*, IL-13 is significantly higher in SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice ($p < 0.01$). *G*, IL-10 is significantly higher in the SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice ($p < 0.001$). PCR data for all seven transcripts were normalized to ubiquitin. Each bar represents the mean mRNA level ± SD of 5–12 mice from two to three independent experiments.

was ample transcription of IL-17 in the SEA/CFA-immunized IL-23p19$^{-/-}$ mice comparable to that seen in the BL/6 mice (Fig. 4*A*). IFN-γ (Fig. 4*B*) and IL-6 (Fig. 4*C*) were not significantly different in all four groups, but IL-21 (Fig. 4*D*) was significantly higher in the SEA/CFA-immunized IL-23p19$^{-/-}$ mice. IL-4 (Fig. 4*E*) and IL-13 (Fig. 4*F*) values were similar to those seen in the livers, and IL-10 was significantly higher in the SEA/CFA-immunized IL-23p19$^{-/-}$ mice (Fig. 4*G*).

In infected, SEA/CFA-immunized IL-23p19$^{-/-}$ mice, there is marked inhibition of IL-17 production in hepatic egg granulomas, but not in MLN, as well as general suppression of IFN-γ with a marked increase in IL-10

To correlate the observed differential gene transcription with secreted proteins, we measured the cytokines produced in vitro by SEA-stimulated GC and MLNC from the same mouse groups by ELISA. In agreement with the mRNA levels, there was a sharp

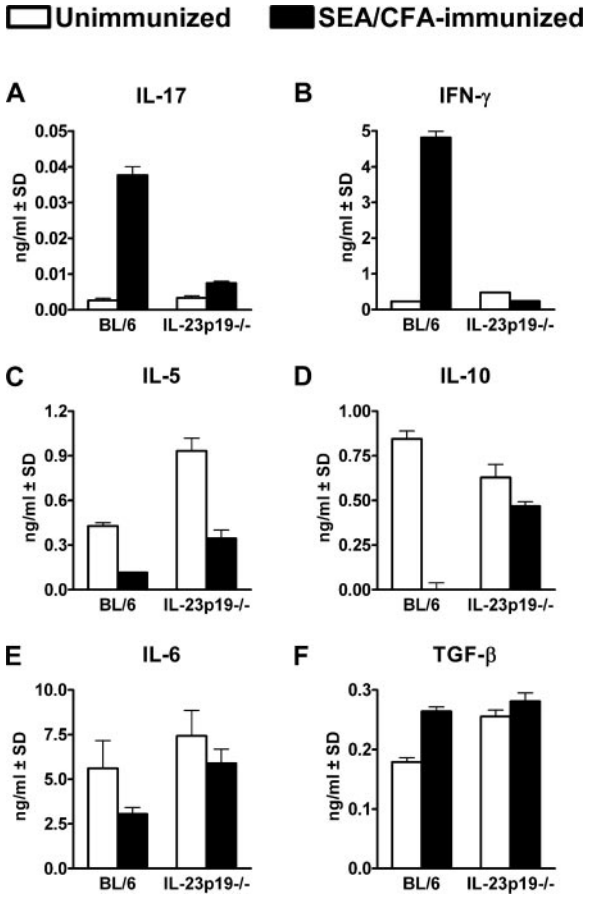

FIGURE 5. Cytokine production by SEA-stimulated GC from infected BL/6 and IL-23p19$^{-/-}$ mice. *A–F*, Cytokine levels in 48 h supernatants from SEA-stimulated GC were measured by ELISA. IL-17 (*A*) and IFN-γ (*B*) are significantly lower in SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice (both $p < 0.001$). IL-5 (*C*) and IL-10 (*D*) are reduced in both SEA/CFA-immunized groups but are significantly higher in the IL-23p19$^{-/-}$ mice ($p < 0.05$ and $p < 0.01$, respectively). IL-6 (*E*) is significantly higher in the SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice ($p < 0.05$). TGF-β (*F*) is not significantly different in both SEA/CFA-immunized mice. Cytokine levels are expressed as means of triplicate determinations ± SD; background cytokine levels from unstimulated GC were subtracted (except for TGF-β). Results shown are from one experiment representative of five.

increase in IL-17 production by GC from SEA/CFA-immunized BL/6 mice which completely failed to materialize in the IL-23p19$^{-/-}$ group (Fig. 5*A*). Interestingly, IFN-γ levels were also markedly inhibited in the IL-23p19$^{-/-}$ mice (Fig. 5*B*). This was most surprising given that these mice are capable of making IL-12, and that IFN-γ transcripts were as high or higher than in the BL/6 mice (Fig. 3*B*). Neither the unimmunized IL-23p19$^{-/-}$ nor BL/6 mice produced significant amounts of IL-17 or IFN-γ. In contrast to IL-17 and IFN-γ, as previously observed (10), IL-5 (Fig. 5*C*) and IL-10 (Fig. 5*D*) were down-regulated following immunization with SEA/CFA in both the IL-23p19$^{-/-}$ and BL/6 mice. However, interestingly, these cytokines were significantly higher in the IL-23p19$^{-/-}$ than in the BL/6 mice; this difference was striking in the case of IL-10. Lastly, IL-6 was lower in both SEA/CFA-immunized mice, but significantly higher in the IL-23p19$^{-/-}$ than the BL/6 mice (Fig. 5*E*), whereas TGF-β was higher in the SEA/CFA-immunized mice, but there was no significant difference between both immunized groups (Fig. 5*F*). No significant amounts of SEA-induced IL-21 were detected (data not shown). Given that both IL-6 and TGF-β have been implicated in the induction of Th17 cells (22–24, 29), our data fail to show an obvious correlation between their levels and the cells' ability to produce IL-17 (Fig. 5*A*).

The cytokine secretion profile of MLNC generally mimicked that of the GC although in the case of the T cell-derived cytokines, the absolute levels were usually higher. One notable exception was that SEA-stimulated, MLNC from schistosome-infected SEA/CFA-immunized IL-23p19$^{-/-}$ mice consistently produced low but significant amounts of IL-17, despite the absence of IL-23 (Fig. 6*A*). By comparison, IFN-γ production by the same cells was again exceedingly low (Fig. 6*B*). IL-5 levels (Fig. 6*C*) were similar to those seen in the GC and IL-10 was again markedly higher in the SEA/CFA-immunized IL-23p19$^{-/-}$ mice than in the corresponding BL/6 mice (Fig. 6*D*). Lastly, in contrast to the GC, IL-6 was higher in both SEA/CFA-immunized mouse groups (Fig. 6*E*) while TGF-β was not significantly different (Fig. 6*F*) and IL-21 was higher in the IL-23p19$^{-/-}$ mice (Fig. 6*G*). Importantly, IL-17 (Fig. 6*H*) and IFN-γ (Fig. 6*I*) production by purified CD4 T cells in the presence of normal splenic APC was similar to that from bulk MLNC (Fig. 6, *A* and *B*), confirming that this is largely a function of the T cells.

Neutralization of IL-10 results in sharp up-regulation of IFN-γ, but only weakly increases IL-17 production

The exceedingly low to absent IFN-γ production by GC and MLNC from schistosome-infected SEA/CFA-immunized IL-23p19$^{-/-}$ mice was most intriguing in view of the detected messages for IL-12p40, IL-12p35 and IFN-γ in these cells (Figs. 2–4), and suggested a posttranslational regulation. An obvious candidate for this activity was IL-10, which is known to inhibit proinflammatory responses and immunopathology in schistosomiasis (30, 31). Importantly, IL-10 was significantly higher in infected, SEA/CFA-immunized IL-23p19$^{-/-}$ mice than in the corresponding BL/6 mice (Figs. 5*D* and 6*D*). To further explore the impact of IL-10 on the production of IFN-γ and IL-17, neutralizing anti-IL-10 mAb were included in cultures from SEA-stimulated MLNC from infected, SEA/CFA-immunized, and unimmunized IL-23p19$^{-/-}$ and BL/6 mice. As shown in Fig. 7*A*, the treatment with anti-IL-10 mAb reconstituted strong IFN-γ production, which was significantly higher in SEA/CFA-immunized than unimmunized mice, but no different in IL-23p19$^{-/-}$ vs BL/6 mice. In contrast, blocking IL-10 had a very weak if any effect on the vigorous IL-17 production by MLNC from SEA/CFA-immunized mice, which was consistently lower in the IL-23p19$^{-/-}$ group; only a minor increase in IL-17 was observed in cells from unimmunized BL/6 mice (Fig. 7*B*). Lastly, as expected, IL-10 production was neutralized in the presence of the corresponding mAb (Fig. 7*C*). These findings indicate that IL-10 effectively suppresses potential IFN-γ production by T cells, but does not significantly affect IL-17.

Deficient recruitment of Gr-1$^+$ cells into hepatic egg granulomas of SEA/CFA-immunized IL-23p19$^{-/-}$ mice

A most likely mechanism by which the lack of IL-23 translates to reduced granulomatous inflammation is the overall down-regulation of Th17 cells capable of recruiting leukocytes to the lesions (32–34). Flow cytometric analysis demonstrated that SEA/CFA immunization in IL-23p19$^{-/-}$ mice fails to stimulate the recruitment of Gr-1$^+$ cells to the granulomas to the same extent as is the case in BL/6 mice (Fig. 8*A*). Given the relative expression of the CCL11 (eotaxin) receptor CCR3 on the Gr-1$^+$ cells, this shortfall is mostly at the expense of Gr-1$^+$CCR3$^-$ neutrophils rather than Gr-1$^+$CCR3$^+$ eosinophils (Fig. 8*B*) (35–37). In support of this interpretation is the marked decrease in the neutrophil attracting and activating chemokines CXCL1 (Gro-α, KC) (Fig. 8*C*) and

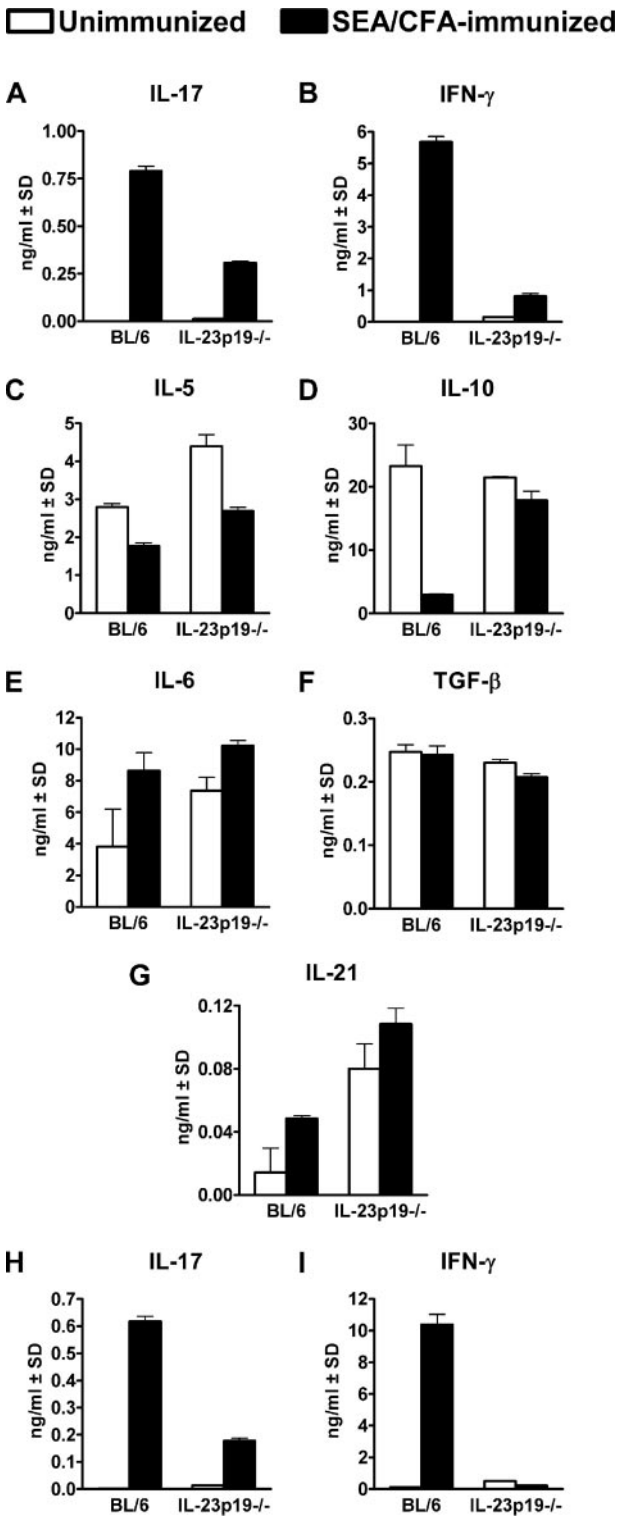

FIGURE 6. Cytokine production by SEA-stimulated MLN and CD4 T cells from infected BL/6 and IL-23p19$^{-/-}$ mice. *A–G*, Cytokine levels in 48 h supernatants from SEA-stimulated bulk MLNC or, *H* and *I*, purified CD4 T cells plus APC (as described in *Materials and Methods*) measured by ELISA. IL-17 (*A*) and IFN-γ (*B*) are significantly lower in SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice (both $p < 0.001$). IL-5 (*C*) and IL-10 (*D*) are reduced in both SEA/CFA-immunized mice but are significantly higher in the SEA/CFA-immunized IL-23p19$^{-/-}$ mice ($p < 0.05$ and $p < 0.001$, respectively). IL-6 (*E*) is increased in the SEA/CFA-immunized mice but is not significantly different in the IL-23p19$^{-/-}$ vs BL/6 groups. TGF-β (*F*) is not significantly different in all groups. IL-21 (*G*) is increased in the SEA/CFA-immunized mice and is significantly higher in the IL-23p19$^{-/-}$ vs BL/6 groups ($p < 0.01$). IL-17 (*H*) and IFN-γ (*I*) secreted by CD4 T cells are significantly lower in the SEA/CFA-immunized IL-23p19$^{-/-}$ mice (both $p < 0.001$). Cytokine levels are expressed as means of triplicate determinations ± SD; background cytokine levels from unstimulated MLNC were subtracted (except for TGF-β). Results shown are from one experiment representative of five.

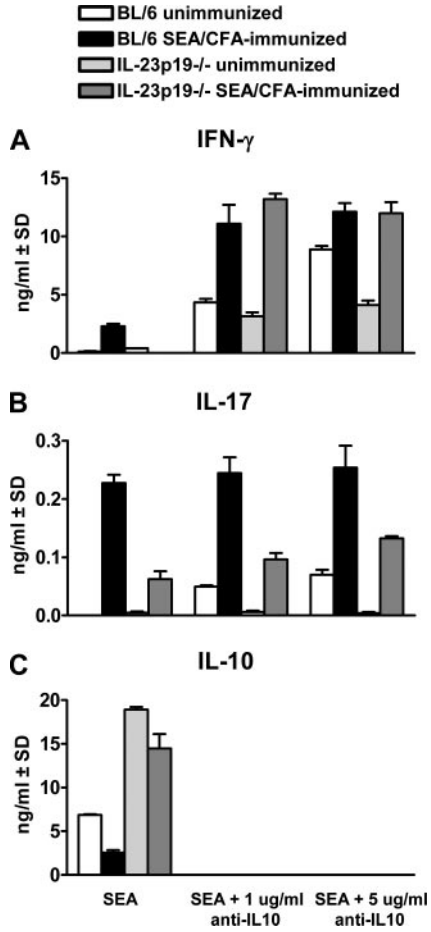

FIGURE 7. IFN-γ and IL-17 production by MLNC in the presence of anti-IL-10 mAb. MLNC were cultured for 48 h in the presence or absence of anti-IL-10 mAb as described in *Materials and Methods*. *A*, IFN-γ levels produced by the MLNC are significantly increased in the presence of neutralizing anti-IL-10 mAb in all four groups (all $p < 0.001$) whereas IL-17 levels (*B*) are not significantly modified by the addition of anti-IL-10 mAb. *C*, IL-10 is not detected in cultures receiving anti-IL-10 mAb. Cytokine levels are expressed as means of triplicate determinations ± SD; background cytokine levels from unstimulated MLNC were subtracted. Results shown are from one experiment representative of two.

CXCL2 (Gro-β, MIP-2) (Fig. 8*D*) (33, 38) in the SEA/CFA-immunized IL-23p19$^{-/-}$ mice, which contrasted with an increased expression of the eosinophil chemoattractant CCL11 (eotaxin) (Fig. 8*E*) (39).

In infected, SEA/CFA-immunized IL-23p19$^{-/-}$ mice, macrophages preferentially display alternative activation

The severity of inflammation in a given lesional environment typically is reflected in, if not dictated by, the state of activation of its macrophage population. "Classically" activated macrophages are typically induced after phagocytosis of microorganisms or exposure to IFN-γ, whereas "alternatively" activated macrophages arise

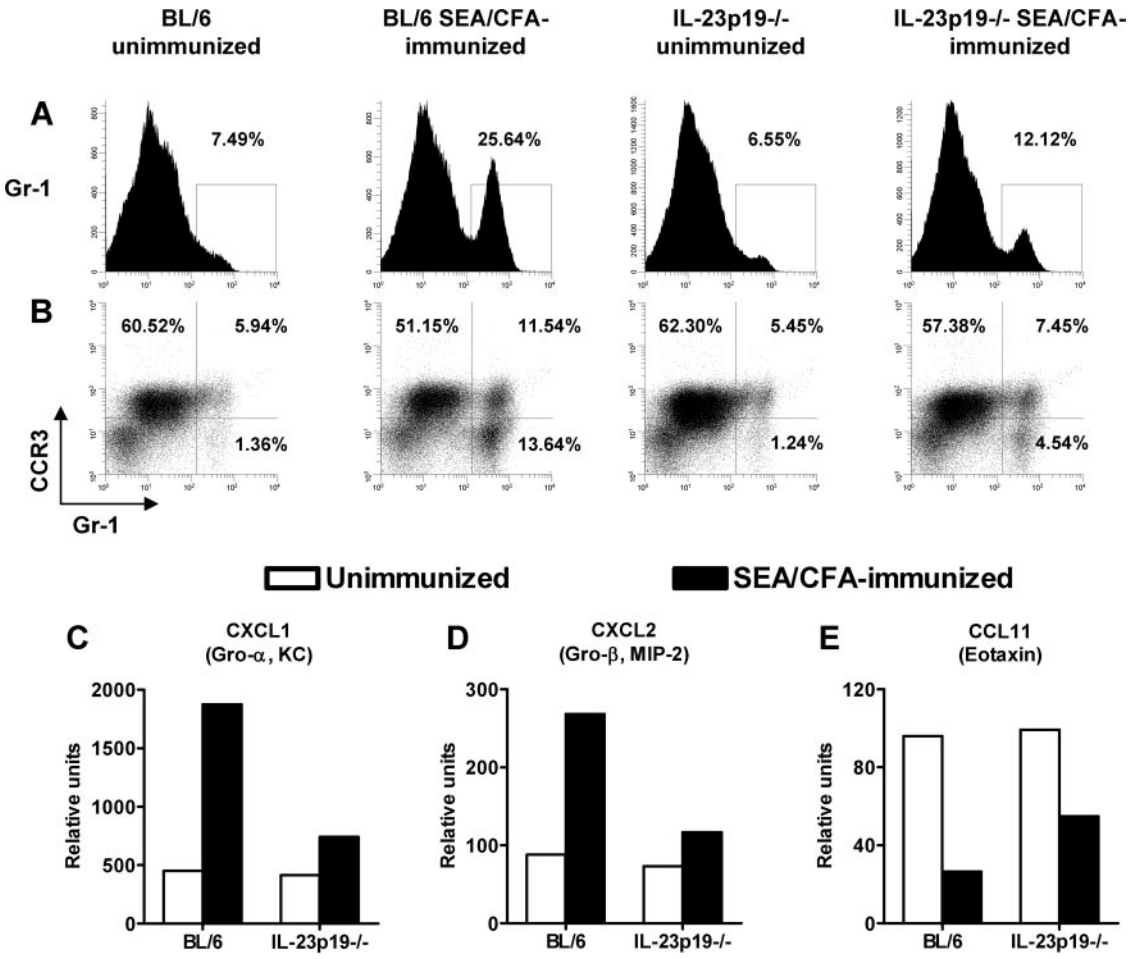

FIGURE 8. Characterization of GC from infected BL/6 and IL-23p19$^{-/-}$ mice. *A* and *B*, Liver GC were isolated and stained ex vivo with anti-Gr-1 and -CCR3 mAb as described in *Materials and Methods*. *A*, Gr-1^{+} cells are markedly reduced in the granulomas from SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice. *B*, There is a greater decrease in Gr-1^{+}CCR3^{-} cells than Gr-1^{+} CCR3^{+} cells in granulomas from the SEA/CFA-immunized IL-23p19$^{-/-}$ mice. *C–E*, Chemokine mRNA transcripts from livers were measured by real-time RT-PCR as described in *Materials and Methods*. (*C*) CXCL1 (Gro-α, KC) and (*D*) CXCL2 (Gro-β, MIP-2) are lower whereas (*E*) CCL11 (eotaxin) is higher in the SEA/CFA-immunized IL-23p19$^{-/-}$ compared with SEA/CFA-immunized BL/6 mice. PCR data for all three transcripts were normalized to ubiquitin. Results shown are from one experiment representative of three with similar results, using pooled GC or pooled liver mRNA from three to eight mice per group.

in context with a Th2-dominated immune response under the influence of the cytokines IL-4, IL-13, and IL-10 (40–43). Importantly, in schistosomiasis, alternatively activated macrophages were shown to be critical in protecting the host from severe pathology and death (44). We examined whether the observed differences in immunopathology and cytokine configuration between IL-23p19$^{-/-}$ and BL/6 mice preferentially associate with a particular state of macrophage activation. Indeed, SEA/CFA-immunized IL-23p19$^{-/-}$ mice displayed a phenotype consistent with alternative macrophage activation, as measured by the increased

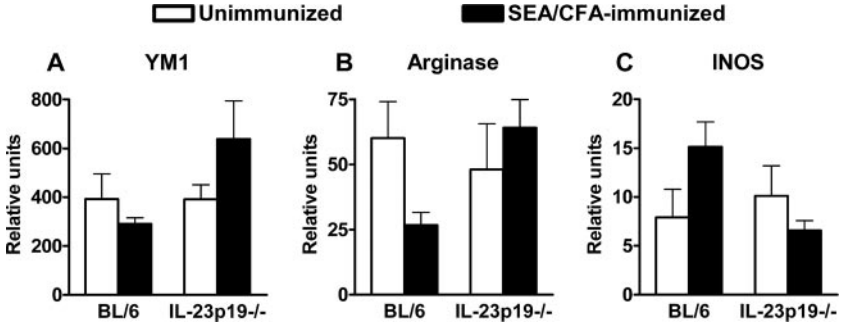

FIGURE 9. Macrophage activation in infected BL/6 and IL-23p19$^{-/-}$ mice. mRNA transcripts for YM1, arginase, and INOS were measured by real-time RT-PCR as described in *Materials and Methods*. (*A*) YM1 and (*B*) arginase are significantly higher, whereas (*C*) INOS is significantly lower in the SEA/CFA-immunized IL-23p19$^{-/-}$ vs BL/6 mice (all $p < 0.01$). mRNA for YM1 and INOS are from livers and arginase from MLN, given the high endogenous expression of arginase in hepatocytes. PCR data for all three transcripts were normalized to ubiquitin. Each bar represents the mean mRNA level ± SD of 5–12 mice per group from two independent experiments.

expression of the markers YM1 and arginase (45–47), whereas classical activation, measured by higher expression of inducible NO synthase (INOS) (47), was dominant in the BL/6 mice (Fig. 9). In other words, in IL-23p19$^{-/-}$ mice, SEA/CFA-immunization solidified alternative macrophage activation similar to that seen in the unimmunized groups, whereas in BL/6 mice it induced classically activated macrophages, which correlates well with the magnitude of immunopathology and the prevailing cytokine milieu.

Lastly, although there were no significant differences in the percentage of CD4 T cells in the granulomas of SEA/CFA-immunized IL-23p19$^{-/-}$ mice, the T cells were less activated than in their BL/6 counterparts as judged by a drop in the expression of CD69 (data not shown). Overall, the above parameters are indicative of a diminished cellular inflammatory response in the IL-23p19$^{-/-}$ mice, which is consistent with the reduced size of the granulomatous lesions.

Discussion

Our previous work has pointed out the critical role of IL-12p40 in the development of severe schistosomiasis associated with the secretion of IL-17 by SEA-stimulated CD4 T cells (10). Although IL-12 per se was ruled out for this activity, this observation did not strictly implicate IL-23 instead. To address this issue, the present study was conducted with schistosome-infected IL-23p19$^{-/-}$ mice specifically unable to make IL-23. Because they are on the naturally low pathology background H-2^{b}, some IL-23p19$^{-/-}$ mice were concomitantly immunized with SEA/CFA as a means to enhance their hepatic lesions and levels of IL-17 (10).

A most significant observation in our study was that in IL-23p19$^{-/-}$ mice, SEA/CFA immunization elicited a small but significant increase in pathology with respect to the unimmunized controls, which contrasted with IL-12p40$^{-/-}$ mice, that completely fail to respond to this treatment (10). Furthermore, in comparison with their BL/6 counterparts, the SEA/CFA-immunized IL-23p19$^{-/-}$ mice displayed a diminished IL-17 response, a profoundly inhibited IFN-γ response, and a rise in IL-4, IL-5, IL-13, IL-21, but mainly in IL-10. Taken together, our findings, in agreement with others (19, 48), demonstrate that the absence of IL-23 does not result in an absolute loss of IL-17, thus implying an IL-23-independent pathway for its production; they also show that IL-23 is critical to attain full development of high pathology in response to immunization with SEA/CFA.

In the infected SEA/CFA-immunized IL-23p19$^{-/-}$ mice, there was ample IL-17 expression and production in the MLN, which contrasted with a profound suppression of IL-17 in the lesional environment, despite the presence of IL-6, TGF-β, and IL-21. These findings confirm and emphasize the critical role of IL-23 in sustaining Th17 cells in diseased peripheral organs and tissues, as previously demonstrated in the nervous system, intestine and joints (15–19, 49). Remarkably, there was no obvious regional correlation between the levels of IL-17 with those of IL-6, TGF-β, or IL-21; however, this observation is not inconsistent with the currently held view that ultimate control over the generation and maintenance of Th17 cells is exerted by the combined effect of IL-6, TGF-β, and IL-21, in concert with IL-23 (22, 24–26, 29). Direct support for this contention comes from our observation that in IL-6$^{-/-}$ mice there is no exacerbation of egg-induced immunopathology or production of IL-17 in response to immunization with SEA/CFA (L. I. Rutitzky and M. J. Stadecker, unpublished results).

An unexpected observation in our study was the posttranslational down-regulation of IFN-γ produced by SEA-stimulated GC and MLNC from the schistosome-infected, SEA/CFA-immunized IL-23p19$^{-/-}$ mice, given the fact that these mice can make IL-12 and produce normal levels of mRNA for IFN-γ. Our findings also demonstrated sharply higher levels of IL-10 in the IL-23p19$^{-/-}$ mice, which, when neutralized, resulted in a full restoration of IFN-γ production. Although cross-regulation between IL-10 and IFN-γ has been well-documented (50), a direct control by IL-23 over IL-10, and thus indirect regulation of IFN-γ, is novel and as yet not described. The loss of IFN-γ has been reported to cause an increase of IL-17 in other systems (14, 51); however, this differs from our results with schistosome-infected, SEA/CFA-immunized IL-23p19$^{-/-}$ mice, in which the overall IL-17 levels were low. Aside from markedly inhibiting IFN-γ, IL-10 only had a marginal direct effect on IL-17 production.

Analogous to IL-10, the SEA/CFA immunization caused a marked decrease in IL-4, IL-5, and IL-13; however, this decrease was significantly more pronounced in BL/6 mice than in IL-23p19$^{-/-}$ mice, in which levels of these cytokines remained close or were equal to those seen in the unimmunized counterparts. IL-21 production was also higher in the IL-23p19$^{-/-}$ mice, but increased after SEA/CFA immunization, thus reflecting its dual role as a Th2 cytokine and a stimulator of Th17 cells (25, 26, 52). Taken together, these findings are indicative of a severely impaired IL-17-mediated inflammatory response in the IL-23p19$^{-/-}$ mice, in which there remained a Th2-dominated state akin to that seen in the absence of immunization.

Th17 cells have now been shown to play an important pathogenic role in an increasing number of inflammatory and infectious conditions including schistosomiasis (10, 13, 18, 53, 54). Th17 cells mediate pathology by secreting IL-17 and thereby stimulating the production of additional proinflammatory and chemotactic molecules such as IL-1, TNF-α, IL-6, CXCL1 (Gro-α, KC), CXCL2 (Gro-β, MIP-2), and CCL2 (MCP-1)—and in the human, CXCL8 (IL-8)—by local lymphoid, vascular, and mesenchymal cells (33, 34, 38, 55). Enhanced neutrophil recruitment and activation is an important element in Th17 cell-mediated inflammation (56–58). A closer analysis of the reduced, IL-17-depleted egg granulomas in the SEA/CFA-immunized IL-23p19$^{-/-}$ mice indeed disclosed a greater reduction in Gr-1^{+}CCR3^{-} neutrophils than CCR3^{+} eosinophils (36), which correlates well with the selective loss of the neutrophil chemoattractants CXCL1 and CXCL2 (59). Neutrophils thus contribute more significantly to pathology exacerbation in response to SEA/CFA immunization than eosinophils, which are normal constituents of the schistosome egg granulomas.

Another significant finding in the SEA/CFA-immunized IL-23p19$^{-/-}$ mice was in their macrophage population, which, by virtue of expressing high YM1 and arginase, and low INOS, displayed a phenotype characteristic of alternative activation. This contrasted sharply with macrophages from BL/6 mice, which expressed low YM1 and arginase, and high INOS, typical of classical activation. The alternative macrophage activation in the IL-23p19$^{-/-}$ mice correlates well with, and is a likely consequence of, the prevalent Th2-polarized environment that explains the reduced immunopathology and enhanced survival of the infected mice (44).

In sum, IL-23 emerges as a critical factor that contributes to pathology exacerbation in schistosomiasis both by boosting predominantly lesional Th17 cell development and by restraining regulatory networks involving IL-10, which results in proinflammatory cytokine and chemokine release, neutrophil recruitment and activation, and phagocyte-associated NO release. The production of IL-23 by innate immunocytes such as dendritic cells and macrophages may be in direct response to specific schistosome egg-associated molecules recognized by pattern recognition receptors such as the TLRs or C-type lectins (60–62), although positive

feedback loops involving additional cells may amplify the initial pathogen stimulus. The absence of IL-23 therefore results in a significantly decreased immunopathology owed to the reduction of Th17 cells amid an anti-inflammatory cytokine environment marked by high levels of IL-10. How much of this IL-10 is associated with T regulatory cell function (63, 64) remains to be elucidated. In any case, severe schistosomiasis represents an ideal condition in which disruption of the IL-23/IL-17 axis could be further explored as a means to control excessive harmful inflammation.

Disclosures

The authors have no financial conflict of interest.

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