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Dendritic Cell IL-23 and IL-1 Production in Response to Schistosome Eggs Induces Th17 Cells in a Mouse Strain Prone to Severe Immunopathology

Mara G. Shainheit, Patrick M. Smith, Lindsey E. Bazzone, Andrew C. Wang, Laura I. Rutitzky, and Miguel J. Stadecker

Infection with schistosomes results in a CD4 T cell-mediated inflammatory reaction against parasite eggs that varies greatly in magnitude both in humans as well as in mice. In the murine disease, the severe form of immunopathology correlates with high levels of IL-17. We now report that live schistosome eggs stimulate dendritic cells from high pathology-prone CBA mice to produce IL-12p40, IL-6, and TGF-β, whereas those from low pathology-prone BL/6 mice only make TGF-β. Moreover, egg-stimulated dendritic cells plus naïve CD4 T cells from CBA mice resulted in increased levels of IL-6, IL-23, IL-1β, as well as IL-17 and the chemokines CXCL1, CXCL2, and CCL2, whereas similarly treated BL/6 cell cocultures instead expressed higher IL-4, IL-5, IL-10, and the transcription factor Foxp3. Neutralization of IL-23 and IL-1, but not of IL-6 or IL-21, profoundly inhibited egg-induced IL-17 production in the CBA cocultures. Conversely, stimulation with schistosome eggs in the presence of exogenous IL-23 and IL-1β induced BL/6 cells to make IL-17. These findings identify IL-23 and IL-1 as critical host factors that drive IL-17 production, and suggest that parasite recognition followed by a genetically determined innate proinflammatory response induces the development of Th17 cells and thus controls the outcome of immunopathology in schistosomiasis.

School of Medicine Animal Facility in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. For some experiments, CBA and BL/6 mice were infected by i.p. injection with 80 cercariae of *Schistosoma mansoni* (Puerto Rico strain), which were shed from infected *Biomphalaria glabrata* snails provided to us by Dr. F. Lewis (Biomedical Research Institute, Rockville, MD), under National Institutes of Health/National Institute of Allergy and Infectious Diseases Contract NOI-AI-55270. All Swiss Webster mice were infected in an identical fashion for the purpose of isolating eggs and worms.

**Cell preparations**

Bone marrow-derived DC (BMDC). Bone marrow cells were flushed from the femurs and tibias of normal CBA and BL/6 mice. RBC were lysed with Tris ammonium chloride buffer, and the remaining cells were cultured at a concentration of $2 \times 10^5$ cells/ml in 10 ml of complete RPMI 1640 medium containing 10% FCS (Axygen Biologicals) and GM-CSF. GM-CSF-containing supernatants from the transfectant B cell hybridoma J558L (provided to us by Dr. N. Hacohen, Broad Institute, Cambridge, MA) were added to the bone marrow cells at an optimized concentration of 1/30. Three days after seeding, an additional 10 ml of GM-CSF-containing medium was added to the cultures. At day 10, nonadherent cells, which were $>85\%$ CD11c$^+$, were harvested and used for experiments.

**CD4 T cells.** Single-cell suspensions were prepared from the spleens of normal CBA and BL/6 mice. RBC were lysed, and CD4 T cells were purified by negative selection using CD4 MACS columns (Milltenyi Biotec) in accordance with the manufacturer's instructions. CD4 T cell purity was $>95\%$ by FACS analysis.

**Parasite preparations**

Schistosome eggs and worms were isolated under sterile conditions from 7- to 8-week infected mice. For eggs, livers were blended and eggs were isolated from the tissues using a series of sieves and washes with saline solution. For worms, the circulatory system was perfused via the aorta with 20 ml of sterile PBS supplemented with 25 nM sodium citrate (Fisher Scientific). Worms were collected in a sieve and transferred to a sterile petri dish containing medium.

**BMDC-parasite cocultures**

BMDC (1 x $10^5$ cells/ml) were cultured in the presence of 100 or 500 live eggs, 2 live worms (1 male, 1 female), or LPS (10 ng/ml, Sigma-Aldrich). After 24 h, culture supernatants were collected, filtered, and assayed by ELISA for IL-12p40 and TGF-β using Ab, standards, and protocols from BD Pharmingen, and for IL-6, using Ab, standards, and protocols from R&D Systems. Potential contamination of schistosome-containing cultures with LPS was carefully ruled out using the *limulus* amoebocyte lysate assay (Cambrex) and by testing for possible TNF-α production by thioglycollate-elicited indicator peritoneal macrophages (28, 29). Furthermore, similar results were obtained in selected experiments in which culture medium was supplemented with 50 µg/ml polymyxin B (Sigma-Aldrich, data not shown).

**BMDC-T cell-parasite cocultures**

Purified CD4 T cells (1 x $10^5$) from normal spleens plus syngeneic BMDC (2.5 x $10^5$) were cultured in 1 ml of medium together with 100 eggs and anti-CD3/anti-CD28 coated beads (3 x $10^2$, Dynal). After 4 days, the culture supernatants were removed and assayed by ELISA for IL-17 (6, 7), we examined the ability of BMDC to produce the mediators of Th17 cell differentiation in response to schistosomes. For this, we assessed cytokine production by DC following a 24-h incubation with live eggs or worms, which are the stages of the schistosome life cycle present in the mammalian host. DC derived from CBA mice secreted significant amounts of IL-12p40 and IL-6 upon stimulation with eggs, while the worms elicited a considerably lower response (Fig. 1, A and B). In striking contrast, the parasites induced only marginal amounts of either cytokine in DC derived from BL/6 mice (Fig. 1, A and B) although both strains produced similar amounts of TGF-β (Fig. 1C), and of all three cytokines in response to control LPS (Fig. 1, A–C). There were no detectable amounts of IL-12p70, IL-23, or IL-10 by ELISA in either strain (data not shown). These results suggest that DC from mice that develop high pathology have the ability to react to schistosomes with a proinflammatory cytokine response.

**Results**

CBA, but not BL/6 DC produce proinflammatory cytokines in response to live schistosomes

In view of the strong association of severe egg-induced immunopathology with high levels of IL-17 (6, 7), we examined the ability of BMDC to produce the mediators of Th17 cell differentiation in response to schistosomes. For this, we assessed cytokine production by DC following a 24-h incubation with live eggs or worms, which are the stages of the schistosome life cycle present in the mammalian host. DC derived from CBA mice secreted significant amounts of IL-12p40 and IL-6 upon stimulation with eggs, while the worms elicited a considerably lower response (Fig. 1, A and B). In striking contrast, the parasites induced only marginal amounts of either cytokine in DC derived from BL/6 mice (Fig. 1, A and B) although both strains produced similar amounts of TGF-β (Fig. 1C), and of all three cytokines in response to control LPS (Fig. 1, A–C). There were no detectable amounts of IL-12p70, IL-23, or IL-10 by ELISA in either strain (data not shown). These results suggest that DC from mice that develop high pathology have the ability to react to schistosomes with a proinflammatory cytokine response.

**Schistosome eggs induce Th17 cell differentiation in CBA, but not in BL/6, DC-CD4 T cell cocultures**

To examine the ability of schistosome-stimulated DC to instruct differential T cell development, CBA and BL/6 BMDC were incubated together with syngeneic naïve CD4 T cells in the presence of live schistosome eggs and anti-CD3/anti-CD28 coated beads, and cytokine production was assessed after 4 days of culture. CBA DC-CD4 T cell cocultures produced abundant IL-6 and IL-23 in response to the eggs, while identically stimulated BL/6 cocultures secreted significantly lower amounts of these cytokines (Fig. 2, A and B). Furthermore, consistent with the notion that IL-6 and IL-23 are involved in the development and expansion of Th17 cells (16–18), there was robust IL-17 production in the egg-stimulated CBA DC-CD4 T cell cocultures, while production in their BL/6 counterparts was negligible (Fig. 2C). In contrast, IL-5 production was higher in the egg-stimulated BL/6 DC-CD4 T cell cocultures in comparison to cultures comprised of CBA cells (Fig. 2D). In each case, both DC and anti-CD3/anti-CD28 stimulation were necessary for egg-induced cytokine production. Schistosome eggs did not...
stimulate IFN-γ or IL-10 production above that induced by anti-CD3/anti-CD28 alone, and adult schistosome worms elicited little to no cytokine production (data not shown).

Because the schistosome eggs for these experiments were isolated from the livers of infected outbred Swiss Webster mice, there existed the possibility that egg-induced cytokine production in the DC-CD4 T cell cocultures was afforded by residual, contaminating allogeneic liver tissue. However, this was not the case as similar levels of IL-17 were produced in CBA DC-CD4 T cell cocultures stimulated with eggs obtained from either infected Swiss Webster, CBA or BL/6 mice (Fig. 2, C and E).

Naive CD4 T cells from CBA and BL/6 mice are equally capable of developing into Th17 cells in the presence of IL-6, TGF-β and IL-23

In view of the consistent inability of egg-stimulated BL/6 DC-CD4 T cell cocultures to activate a proinflammatory response resulting in the production of IL-17, we asked whether the BL/6 T cells were inherently capable of making IL-17 under the present culture conditions. For this, CBA and BL/6 DC-CD4 T cell cocultures were stimulated with anti-CD3/anti-CD28 coated beads in the presence of rIL-6, TGF-β, and IL-23; a combination of cytokines known to promote optimal Th17 cell differentiation and expansion (16–18). As shown in Fig. 2F, and in contrast to egg-induced IL-17 secretion, the cytokines equally stimulated IL-17 production in both the CBA and BL/6 cocultures, with maximal IL-17 output dependent on DC and anti-CD3/anti-CD28 stimulation. These findings clearly indicate that BL/6 T cells are intrinsically capable of differentiating into Th17 cells, but unlike the CBA T cells, fail to do so when in the presence of syngeneic DC and schistosome eggs.

Schistosome eggs induce contrasting immune profiles in CBA vs BL/6 DC-T cell cocultures

To more broadly explore and clearly define the immune profiles that develop in the CBA vs BL/6 DC-T cell cocultures following...
FIGURE 3. Immune profile of egg-stimulated CBA vs BL/6 DC-T cell cocultures. A–C, mRNA expression was measured by real-time quantitative RT-PCR as detailed in Materials and Methods. A, Eggs induced a significant increase in the expression of IL-6 (p < 0.001), IL-12p40 (p < 0.05), IL-23p19 (p < 0.001), and IL-1β (p < 0.05), but not of IL-12p35 in CBA cocultures. B, Transcript levels for IL-17 (p < 0.001), CXCL1 (p < 0.001), CXCL2 (p < 0.001), and CCL2 (p < 0.001) were markedly enhanced by egg stimulation in CBA cocultures; there were no increases in the BL/6 cocultures. C, IL-4 (p < 0.05), IL-10 (p < 0.001), and Foxp3 (p < 0.05) were significantly increased in egg-stimulated BL/6 cocultures, and IFN-γ was not significantly different between egg-stimulated CBA and BL/6 cocultures. p values compare egg-stimulated vs unstimulated cocultures within the CBA or BL/6 groups. Data from real-time quantitative RT-PCR analyses are normalized to GAPDH. Each bar represents the mean mRNA level from duplicate determinations ± SD from one of three independent experiments with similar results.

Contrasting immune profiles induced by schistosome eggs in vitro CBA vs BL/6 DC-CD4 T cell cocultures parallel those induced during schistosome infection in vivo

To lend relevance to the contrasting immune responses induced by schistosome eggs in CBA vs BL/6 DC-CD4 T cell cocultures in vitro, we investigated the expression of some of these molecules in 7-wk infected mice. Real-time RT-PCR analyses of livers from infected high pathology CBA mice revealed a proinflammatory environment characterized by significantly elevated levels of IL-6, IL-23p19, IL-1β, and IL-17, whereas IL-10 expression was higher in the low pathology BL/6 strain; there were no significant differences in IL-12p40 or IL-12p35 (Fig. 5). These data clearly demonstrate that the immune profile induced by schistosome eggs in DC-CD4 T cell cocultures from these two strains in vitro is similar to that induced during the schistosome infection in vivo.

Egg-stimulated IL-17 production in CBA DC-CD4 T cell cocultures is dependent on IL-23 and IL-1

Given that both CBA and BL/6 CD4 T cells were equally capable of developing into Th17 cells in the presence of rIL-6, TGF-β, and IL-23 (Fig. 2F), but only CBA CD4 T cells did so following stimulation with schistosome eggs (Fig. 2C), we investigated the factor(s) that facilitated Th17 cell differentiation in the CBA DC-CD4 T cell cocultures, but were absent in the BL/6. In the first approach, we neutralized candidate Th17 cell-inducing cytokines in CBA cocultures, and in the second, we supplemented these cytokines into the corresponding BL/6 cocultures, which typically do not produce IL-17. The addition of anti-IL-12p40 or anti-IL-23p19 neutralizing Ab effectively blocked IL-17 production by egg-stimulated CBA cells, whereas, surprisingly, anti-IL-6 or anti-IL-21 Ab, individually or in combination, had no effect (Fig. 6A). IL-1 blockade with...
IL-1Ra, an endogenous inhibitor of IL-1 (36), also significantly inhibited IL-17 production (Fig. 6B), which is in agreement with observations from other groups demonstrating the importance of IL-1/H9252 for Th17 cell development (21, 22, 37).

Exogenous IL-23 and IL-1/H9252 enhance egg-induced IL-17 production in BL/6 DC-T cell cocultures

The reciprocal experiments using exogenous cytokines demonstrated that IL-23 significantly enhanced IL-17 production in egg-stimulated BL/6 cocultures, and to a lesser extent did so by itself, whereas IL-12p40, IL-12p70, IL-6, or IL-21, either individually or in combination, had no effect (Fig. 7A). Exogenous IL-1/H9252 similarly facilitated significant IL-17 production, albeit less than IL-23, while both cytokines together had no additive effect (Fig. 7B). Moreover, only the addition of exogenous IL-12p70 was capable of inducing robust IFN-γ production, independent of the presence of schistosome eggs (Fig. 7C). However, none of the added cytokines induced significant additional IL-4, IL-5, or IL-10 production (data not shown). Taken together, these findings demonstrate that IL-23 and IL-1 produced by DC in response to schistosome eggs represents a plausible mechanism by which Th17 cell differentiation is induced in the CBA strain.

Discussion

Th17 cells have been shown to mediate chronic inflammation in several autoimmune diseases including autoimmune encephalomyelitis, collagen induced arthritis, and inflammatory bowel disease (8–11). More recently, they were also shown to play a role in host defense against a variety of extracellular pathogens (38–40). Our laboratory has demonstrated that severe egg-induced immunopathology associated with the schistosome infection in CBA mice, or SEA/CFA-immunized BL/6 mice, strongly correlates with a proinflammatory cytokine environment characterized by high levels of IL-17, and that in the absence of immunization, BL/6 mice develop a significantly milder immunopathology and IL-17 levels are typically low or absent (6).

To further dissect the mechanisms leading to differential Th17 cell development in schistosomiasis, we used an in vitro model in which naive lymphoid cells were directly stimulated with live parasites. CBA DC significantly responded to this stimulation by producing IL-12p40, IL-6, and TGF-β; neither IL-12p70 nor IL-23 were demonstrable by ELISA. Although the sole detection of IL-12p40 could denote monomeric or dimeric forms of this molecule, similar egg-stimulated DC in the presence of CD4 T cells clearly...
In CBA cell cultures, stimulation with schistosomes led to the production of IL-6, TGF-β, IL-23, and IL-1, a cytokine configuration known to promote Th17 cell differentiation (16–18, 21, 22). The increase in IL-17 was accompanied by a rise in the leukocyte chemotactants CXCL1, CXCL2, and CCL2 and by an enhanced state of APC activation reflected by the increase of CD86 costimulatory molecule expression. Of all these mediators, the BL/6 cells only produced TGF-β, an immunomodulatory cytokine linked to the development of T regulatory cells (16). Moreover, the BL/6 cells responded to egg stimulation with an increase in IL-4, IL-5, IL-10, and Foxp3, further suggesting the induction of Th2 (25, 31) and T regulatory cells (26, 27, 32, 33). The striking Th17 cell response induced by schistosome eggs in CBA cell cocultures starkly contrasts with the weaker and largely anti-inflammatory response seen in the BL/6 cells, a finding that is supported by similar cytokine profiles observed in the lesional environment of schistosome-infected CBA vs BL/6 mice in vivo (7). Of note, our observations in the BL/6 strain are in agreement with previous studies using SEA as the stimulus (23, 24), but are at odds with a report suggesting that schistosome eggs instruct proinflammatory responses in DC from this strain (42). Such dissenting findings, which may result from the use of up to a hundredfold higher egg to cell ratios, still need to be reconciled.

Our findings indicate that IL-23 and/or IL-1 produced by schistosome ligand-activated DC are capable of inducing the development of Th17 cells in genetically susceptible hosts. This observation contrasts with the notion that Th17 cell induction is largely a function of IL-6 and TGF-β (16–18); in fact, neutralization of IL-6 did not abolish IL-17 production induced by eggs. Additionally, neutralization of IL-21, which has been implicated as an IL-6-independent Th17-inducing cytokine (19, 20), also failed to abrogate egg-stimulated IL-17 production, individually or in combination with anti-IL-6 Ab. On the other hand, IL-23 has been widely recognized as an important factor in Th17 cell development, either by itself or together with IL-6 and TGF-β (16–18). IL-1 has also been shown to play a critical role in the induction of pathogenic Th17 cells as demonstrated in experimental models of autoimmune encephalomyelitis and arthritis (22, 43, 44). Importantly, IL-1 alone or together with, IL-23, IL-6, and TGF-β is an essential cytokine for Th17 cell development in humans (37, 45, 46). IL-23

produced IL-23. This was supported by the marked increase in mRNA transcripts for IL-12p40 in conjunction with IL-23p19. These findings are consistent with the observation that significant levels of IL-23 are only attained following interaction with, and feedback from T cells; a process that has been linked to CD40 ligation (41). However, the addition of soluble CD40L (CD40L, CD154) to egg-stimulated DC cultures consistently failed to substitute for T cells in the production of measurable IL-23 protein or message (data not shown).

FIGURE 6. Neutralization of IL-23 and IL-1 inhibits egg-induced IL-17 production in CBA DC-T cell cocultures. A and B, DC-T cell cocultures were established in the presence or absence of eggs, anti-CD3/anti-CD28 coated beads, and the indicated blocking reagents as described in Materials and Methods. Cytokine levels in 4-day supernatants were measured by ELISA. A, Egg-induced IL-17 production in CBA cocultures was significantly blocked by anti-IL-12p40 and anti-IL-23p19 neutralizing Ab (both p < 0.001), while Ab against IL-6 or IL-21, individually or in combination, had no significant effect. B, Egg-induced IL-17 production in CBA cocultures was significantly inhibited by IL-1Ra at all tested concentrations (all p < 0.001). p values provided compare cocultures that received blocking Ab or IL-1Ra with those that did not receive either reagent. Cytokine levels are expressed as means of triplicate ELISA determinations ± SD. Results shown are from one experiment representative of two to three with similar results.
and IL-1 appear to be equally capable of eliciting schistosome egg-induced Th17 cell development in CBA cell cocultures although the addition of exogenous cytokines to the BL-6 cocultures suggests IL-23 to be more potent than IL-1; no synergistic effect between the two cytokines was observed. Regardless, the requirement of anti-CD3/anti-CD28 stimulation for optimal IL-17 production suggests that T cell feedback through TCR engagement further enhances IL-23 and IL-1 production, thus sparking an amplification loop for maximal Th17 cell differentiation (22, 47). These scenarios are consistent with the notion that expression of the Th17-specific transcription factor RORγt can be induced by a variety of cytokine combinations involving IL-6, TGF-β, IL-23, IL-1β, and IL-21 (20, 37, 48, 49). The considerable variations of cytokine requirements observed in the different systems may ultimately depend on the nature of the pathogen-specific ligands, as well as the engaged pattern recognition receptors and downstream signaling pathways.

An unresolved issue concerns the schistosome products that elicit differential host innate immune and consequent immunopathological responses. Our current observations suggest that these are most strongly expressed by the eggs, as the worms elicited little or no responses despite their larger mass; in fact, their activity may be attributable to egg laying during the culture. Of the ligands that have received the most attention are the schistosome glycans, which are expressed on these parasites while residing in their vertebrate hosts (50, 51). For example, egg glycans terminating in Lewis (Le)α or pseudo-Lewisα have been proposed to respectively signal via TLR-4 (52) and the C-type lectin receptor (CLR) DC-specific intracellular adhesion molecule grabbing nonintegrin (DC-SIGN, CD209) (53, 54), while LacdiNAc is recognized by the S-type lectin, galectin-3 (51, 55). Additionally, a schistosome-specific lipid, lyso-phosphatidylserine, has also been identified as a putative TLR-2 agonist (56). Although these ligands have been a subject of considerable scientific scrutiny, a consensus about one or more distinct schistosome ligand-innate receptor interactions that significantly impacts the host immune response has yet to be reached. Nevertheless, because schistosome surfaces are heavily glycosylated, pattern-recognition receptors such as the CLR remain attractive host receptor candidates (51, 57), particularly because the yeast zymosan (β glucan)-binding CLR, Dectin-1, was capable of inducing Th17 cell development through a TLR-independent pathway involving the Syk-CARD9 signaling cascade (40). Not only did this study demonstrate that CLR were capable of linking the innate and adaptive immune responses, but relevant to our present report, it also highlighted the requirement of IL-23 for the differentiation of Th17 cells in a pathogen-based system (40).

Although a significant increase in Th17 cells is clearly linked with the exacerbation of inflammation in autoimmune and infectious disease including schistosomiasis, recent work indicates that IL-17 secretion per se does not automatically afford pathogenicity to these cells. In fact, McGeachy et al. have described two types of Th17 cells of markedly diverging pathogenicity based on the circumstances of their induction. Both cell types depended on IL-6 and TGF-β, but the pathogenic form was induced in the presence of IL-23, whereas the absence of IL-23 gave rise to a nonpathogenic form characterized by the coproduction of IL-10 (58). These findings are in agreement with our previous observations in schistosomiasis that IL-23 is not only required for the severe form of the disease but also profoundly inhibits IL-10 (7). They also suggest that the production of IL-23 and IL-17, but not IL-10, in egg-stimulated CBA DC-T cell cocultures reflects the generation of Th17 cells of the pathogenic type. IL-27, another member of the IL-12 family of heterodimeric cytokines, has been proposed as a likely candidate that confers to Th17 cells the ability to coproduce IL-10, thereby reducing their pathogenicity (58–60). However, in our experience with schistosomiasis, IL-27 signaling did neither significantly affect the magnitude of immunopathology nor the levels of IL-17 or IL-10 (61).
Taken together, our results demonstrate that genetically diverse hosts differentially interpret and respond to schistosome products resulting in the development of distinct innate and adaptive immune profiles leading to contrasting immunopathologies. Although the full extent of the underlying pathways remains to be elucidated, Th17 cells driven by IL-23 and IL-1-producing APCs promote pathology exacerbation, whereas IL-10-, TGF-β-, and Foxp3-expressing T regulatory cells (33, 62), likely in conjunction with Th2 cytokine-driven alternatively activated macrophages (63), serve to curtail the severity of disease. Although the bases of immunopathology are likely to differ among the various pathogens and autoimmune conditions, an increased understanding of the underlying mechanisms and their regulation will make it possible to envisage strategies to prevent or treat the most severe forms of disease.

Disclosures
The authors have no financial conflict of interest.

References


