A systemic granulomatous response to *Schistosoma mansoni* eggs alters responsiveness of bone marrow-derived macrophages to Toll-like receptor agonists

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Abstract: Macrophages play a pivotal role in innate and acquired immune responses to *Schistosoma mansoni*. Classical (M1) or alternative (M2) activation states of these cells further delineate their roles in tissue damage through innate immunity or fibrotic remodeling, respectively. In the present study, we addressed the following question: Does systemic Th2-type cytokine polarization evoked by *S. mansoni* affect macrophage differentiation and activation? To this end, we analyzed bone marrow-derived macrophages from mice with *S. mansoni* egg-induced pulmonary granulomas and unchallenged (or naïve) mice to determine their activation state and their response to specific TLR agonists, including *S. mansoni* egg antigens. Unlike naïve macrophages, macrophages from Th2-polarized mice constitutively expressed significantly higher "found in inflammatory zone-1" (FIZZ1) and ST2 (M2 markers) and significantly lower NO synthase 2, CCL3, MIP-2, TNF-α, and IL-12 (M1 markers). Also, compared with naïve macrophages, Th2-polarized macrophages exhibited enhanced responses to the presence of specific TLR agonists, which consistently induced significantly higher levels of gene and protein levels for M2 and M1 markers in these cells. Together, these data show that signals received by bone marrow precursors during *S. mansoni* egg-induced granuloma responses dynamically alter the development of macrophages and enhance the TLR responsiveness of these cells, which may ultimately have a significant effect on the pulmonary granulomatous response. *J. Leukoc. Biol.* 83: 314–324; 2008.

Key Words: *alternative activation · classical activation · soluble egg antigen*

INTRODUCTION

Helminth worms such as *Schistosoma mansoni* possess potent immunomodulatory factors, which allow for successful parasitism, despite various host innate and adaptive immune responses [1]. Immune deviation during *S. mansoni* infection is characterized by profound alterations in the numbers and activation state of CD4+ and T regulatory cells, B cells, eosinophils, mast cells, dendritic cells (DC), and macrophages [2]. The culmination of this immune modulation ensures the reproductive success of *S. mansoni*. Additionally, after the release of eggs by this parasite, a profound granulomatous remodeling response is observed driven by Th2 cytokines, most notably, IL-4 and IL-13 [3]. The complex carbohydrates [4] expressed on the soluble egg antigen (SEA) secreted by *S. mansoni* ova appear to be the primary inducers of the Th2 polarization of the immune response that characterizes chronic schistosomiasis [5, 6].

SEA-induced granuloma formation involves intricate regulation by cytokines and chemokines [7]. In a series of experiments involving IL-4-, IL-10-, and IL-12-deficient mice, it has been shown that these cytokines are essential for host survival and play a key regulatory role in the pathology of the disease [8–10]. Chemokines such as CCL2, CCL17, and CCL22 have been shown to be up-regulated during SEA-induced granulomatous response [7, 11]. Immune neutralization of CCL17 results in increased matrix deposition in *S. mansoni* egg-associated granuloma, suggesting a regulatory role for this chemokine in the overt Th2 response during schistosomiasis [12]. CCL17 and IL-10 treatment of macrophages has been reported to inhibit classical activation and enhance alternative activation of macrophages [13].

During various phases of schistosomiasis, tissue resident macrophages are a major source of leukocyte chemotactic factors [14], cytokines [15], growth factors [16], and free radicals [17]. In response to exogenous signals provided by pathogen byproducts and/or soluble immune mediators, macrophages exhibit distinct phenotypes that reflect rather diverse functions (reviewed in ref. [18]). Classically activated macrophages (M1) result from exposure to IFN-γ, LPS, and TNF-α, and as high IL-12 and NO generators, they effectively kill a variety of microbial pathogens. However, upon prolonged exposure to IL-4, IL-13, IL-10, and other Th2-associated factors during *S. mansoni*-induced immunopathology [2], a subset of macrophages also undergoes an alternative or M2 activation [19], which ultimately leads to their overt involvement in the tissue-destructive fibrotic response [1]. These alternatively ac-

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tivated macrophages were shown recently to be essential for host survival during chronic responses to *S. mansoni*, as in their absence, the egg-induced, inflammatory response is lethal [10]. These findings highlight the complex role of the M2-activated macrophages and warrant study of their genesis during chronic inflammatory responses to *S. mansoni*.

Bone marrow myeloid progenitor cells differentiate into monocytes in the presence of cytokines such as M-CSF, GM-CSF, IL-3, c-kit ligand, and TNF-α [20]. Entrance into the circulation then permits appropriately activated monocytes to emigrate into infected and/or damaged tissues, where they respond to the cytokine microenvironment and differentiate into macrophages. It is currently thought that the polarization of tissue-resident macrophages toward the M1 or M2 activation state occurs in their local environment [19], although it has not been unequivocally established whether macrophage polarization is stable [21, 22].

Given that previous studies have suggested that Th2 inflammatory [23] or neoplastic [24] events in the pulmonary system and s.c. exposure of nematode antigen [25] affect the maturation of bone-marrow macrophage progenitor cells, we extended these studies to examine whether a systemic Th2 polarization elicited by *S. mansoni* egg sensitization and i.v. egg challenge [26, 27] impacted the TLR ligand responsiveness of bone marrow-derived macrophages. We show that bone marrow-derived macrophages from mice with a Th2-polarized immune response to *S. mansoni* eggs stimulated with cytokines/chemokines and TLR agonists are hyper-responsive to TLR stimulation and produced higher amounts of inflammatory mediators compared with macrophages from naïve animals. In particular, exposure of these cells to specific TLR agonists induced significantly higher levels of M1 and M2 gene transcript and protein levels of inflammatory genes. These data suggest that signals received by bone marrow precursors can dramatically skew phenotypic and functional differentiation of macrophages, which may have an impact on the ongoing innate immune response.

MATERIALS AND METHODS

Animals

Specific pathogen-free, female C57BL6 and Swiss-Webster mice (6–8 weeks old) were purchased from Taconic (Hudson, NY, USA). *S. mansoni*-infected Swiss-Webster mice were provided by Dr. Fred Lewis of Biomedical Research Laboratories (Rockville, MD, USA). All mice were housed in the University Laboratory Animal Medicine Facility at the University of Michigan Medical School (Ann Arbor, MI, USA). The University Committee on Use and Care of Animals at the University of Michigan approved all procedures involving mice used in the following experiments.

Reagents

All recombinant cytokines and chemokines were purchased from R&D Systems (Rochester, MN, USA). The following TLR targets (and their corresponding vendors) were used in the present study: (S)-[2,3-bis (palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH, trihydrochloride (Pam3cys; EMC Microcollections GmbH, Tubingen, Germany), LPS (Sigma-Aldrich, St. Louis, MO, USA), polyinosinic:polycytidylic acid [poly(I:C); Amersham Biosciences, Piscataway, NJ, USA], and CpG DNA (Cell Sciences, Canton, MA, USA). Soluble SEA was prepared from *S. mansoni* eggs as described previously [28].

S. mansoni egg-induced pulmonary granuloma model

Purification of live *S. mansoni* eggs from livers of Swiss-Webster mice was carried out as described previously [29]. A secondary, synchronous, pulmonary, granulomatous response was induced in mice by the i.p. injection of 3000 *S. mansoni* eggs and a subsequent i.v. challenge with 5000 eggs 14 days later [12].

Isolation and culture of bone marrow-derived macrophages

At Day 8 after the i.v. egg challenge (when sensitized and challenged mice display a polarized Th2 cytokine phenotype in the lung [26]), macrophages were cultured from bone marrow derived by flushing femur and tibia bones with cold RPMI 1640. The recovered bone marrow progenitor cells were cultured in L929 cell-conditioned medium as described previously [30, 31]. Bone marrow cells were also isolated and cultured from unchallenged mice. For clarity, we subsequently refer to macrophages grown from unchallenged mice as "naïve" and those from *S. mansoni*-challenged bone marrow as "Th2-polarized". On Day 7 after initial bone marrow culture, bone marrow-derived macrophages were transferred to 24-well plates at a cell density of 2×10^5 cells/well, and 0.5 ml RPMI 1640 containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin was added to each well. Flow cytometric analysis of bone marrow-derived macrophages from naïve and Th2-polarized mice revealed that these cells were >98% Cd11b+ve and >95% F4/80+ve (data not shown). In separate experiments, exogenous murine IL-10 (20 ng/ml) and CCL17 (50 ng/ml) were added to triplicate wells. Twenty-four hours later, one of the following was added to triplicate tissue-culture plate wells: Pam3cys (2.5 μg/ml [32]), poly(I:C) (50 μg/ml [33]), LPS (1 μg/ml [32]), CpG (50 μg/ml [34]), and SEA (22.5 μg/ml [35]). At 24 and 48 h after media alone or TLR agonist addition, cell-free supernatants were removed for ELISA, and RNA was isolated from adherent macrophages (see below).

Real-time TaqMan analysis

To analyze the expression of various transcripts relevant to classically (M1) and alternatively (M2) activated macrophages, total RNA was isolated from cells using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was reverse-transcribed into cDNA using Muloney murine leukemia virus RT (Invitrogen Life Technologies), according to the manufacturer's instructions. The cDNA was then analyzed by real-time quantitative TaqMan PCR using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. All values are expressed as fold-change overexpression by macrophages incubated in medium alone. TaqMan reagents for IL-10, arginase-1, CCL3, NO synthase 2 (NOS-2), and GAPDH were bought from Applied Biosystems. SYBR Green Master PCR mix (Applied Biosystems) was used to amplify ST2, NOS-2, TLR3, TLR4, and TLR9. Primers for ST2 were GCAATTCTGACACTTCCCATGTAT and CCAGACCCCAGGACGATTTA; for NOS-2, CGCAGCTGGGCTGTACCAA and TGATGTTTGCTTCGGACATCA; for TLR3, CCCAGCTCGATCTTTCCTACA and AGGCTTGGGAGATAGGAGAAG; for TLR4, TTGAATCCCTGCATAGAGGTAGTTC and AAGAAGGAATGTCATCAGGGACTT; for TLR9, AGCTGAACATGAACGGCATCT and TGAGCGTGTACTTGTTGAGCG. Primers used for found in inflammatory zone-1 (FIZZ-1) real-time TaqMan analysis were TCCAGCTAACTATCCCTCCACTGT and GGCCCATCTGTTCATAGTCTTGA, and the probe was 6FAMCGAAGACTCTCTCTTGCTTAMRA. Primers for TLR2 were GCCACCATTTCCACGGACT and GGCTTCCTCTTGGCCTGG, and the probe used was 6FAMTGGTACCTGAGAATGATGTGGGCGTGTTAMRA.

ELISA analysis of chemokine and cytokine expression

Bone marrow macrophage supernatants were stored at –20°C until analyzed by sandwich ELISA. Supernatants were thawed on ice, and 50 μl samples were loaded onto individual wells of a flat-bottom, 96-well plate (Nunc, Roskilde, Denmark). Sandwich ELISA was performed as described before [36]. Recombinant cytokines and chemokines used to generate standard curves were purchased from R&D Systems. For studies involving *S. mansoni*-infected and naïve bone marrow macrophages from Swiss-Webster mice, cell-free supernatants were analyzed using a Multiplex Bead Immunoassay from BioSource

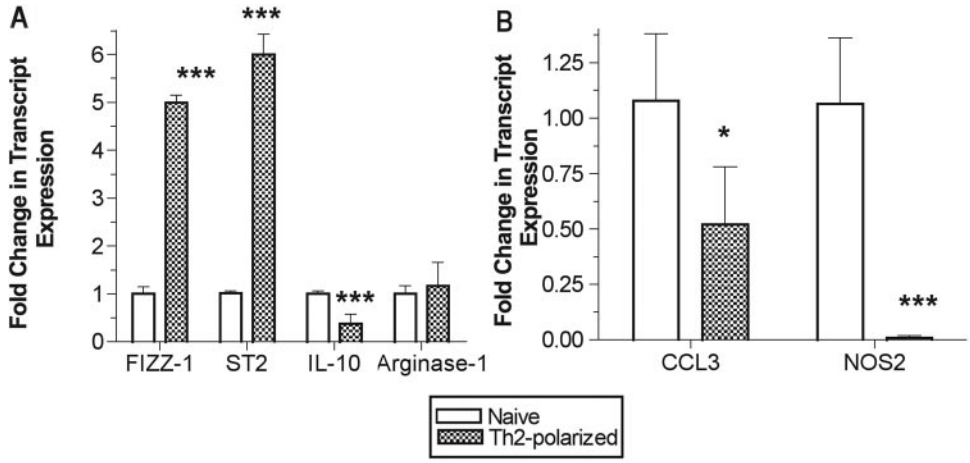

Fig. 1. Quantitative TaqMan PCR analysis of constitutive M2 (FIZZ-1, ST2, IL-10, and arginase-1; A) and M1 (CCL3 and NOS-2; B) transcript levels in naïve and Th2-polarized, bone marrow-derived macrophages. Transcript levels for each mediator in Th2-polarized macrophages are expressed as fold change over transcript levels in naïve macrophages cultured in media alone. Data shown are mean ± SEM, and these data are representative of three separate experiments. *, $P \leq 0.05$; ***, $P \leq 0.001$.

(Camarillo, CA, USA). The limit of detection for each cytokine and chemokine analyzed was 10 pg/ml.

Statistical analysis

Three separate experiments were performed, and results are expressed as mean ± SEM of values obtained from triplicate samples from a representative experiment. Statistical significance was calculated by one-way ANOVA followed by the Newman-Keuls test. P values less than 0.05 were considered significant. Figures depict significant differences between naïve and similarly treated, Th2-polarized macrophages only.

RESULTS

Bone marrow-derived macrophages from Th2-polarized mice were alternatively activated

Bone marrow-derived macrophages from *S. mansoni* egg i.p.-sensitized and i.v.-challenged mice exhibited many features at the transcript and protein level that were consistent with M2 activation [19]. Specifically, it was apparent that constitutive transcript expression of M2 markers, such as FIZZ-1 [19] and ST2 [37], were present in significantly greater abundance in the Th2-polarized macrophages (derived from *S. mansoni* egg-challenged mice) compared with the naïve macrophages (derived from unchallenged mice; **Fig. 1A**). However, other markers of M2 activation in Th2-polarized macrophages were significantly lower (i.e., IL-10) or unchanged (i.e., arginase-1). The Th2-polarized macrophages constitutively expressed significantly less transcripts for CCL3 and NOS-2, both of which are M1 or classically activated macrophage markers [19] (Fig. 1B).

Further confirmation of M2 activation in Th2-polarized macrophages is summarized in **Figure 2.** These macrophages constitutively expressed significantly less CCL3, IL-12, TNF-α, and MIP-2 classical M1 protein markers in cell-free supernatants compared with naïve macrophages (Fig. 2A). CCL17 and IL-10 have been shown to inhibit classical activation and promote alternative activation of macrophages [17]. Interestingly, many of the protein differences between the naïve and Th2-polarized macrophages were eliminated when cultures of both types of macrophages were stimulated with CCL17 and IL-10 (Fig. 2B). Thus, the bone marrow macrophages derived from mice with a Th2-polarized, pulmonary, granulomatous response exhibited a profile more consistent with that of M2 activation than with M1 activation.

Differential response by Th2-polarized and naïve bone marrow macrophages following exposure to TLR agonists

TLRs play a crucial role in the ability of macrophages to respond to microbial byproducts released during active infections and provide an important bridge between innate and adaptive immunity [38]. However, little is known about the

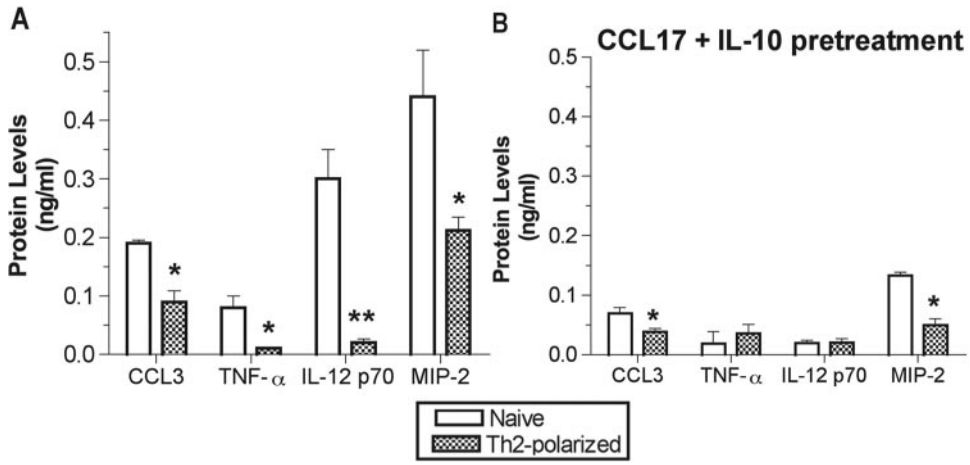

Fig. 2. Protein analysis of immunoreactive levels for cytokines and chemokines in cultures of naïve and Th2-polarized, bone marrow-derived macrophages. Cell-free supernatants from both groups of macrophages were collected and analyzed by ELISA (see Materials and Methods). (A) Summarizes the protein levels in both groups of cells in culture for 48 h. (B) Summarizes the 48-h protein levels in both groups of cells that were pretreated with IL-10 (20 ng/ml) and CCL17 (50 ng/ml) for 24 h. Data shown are mean ± SEM, and these data are representative of three separate experiments. *, $P \leq 0.05$; **, $P \leq 0.01$.

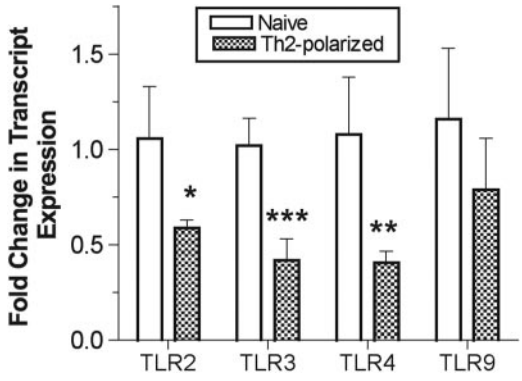

Fig. 3. Quantitative TaqMan PCR analysis of constitutive TLR2, TLR3, TLR4, and TLR9 transcript levels in naïve and Th2-polarized, bone marrow-derived macrophages. Transcript levels for each TLR in unstimulated, Th2-polarized macrophages are expressed as fold change over transcript levels in naïve macrophages cultured in media alone. Data shown are mean ± SEM, and these data are representative of three separate experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

regulation of TLR expression in M1 and M2 macrophages, and therefore, we next examined whether naïve and Th2-polarized macrophages differed in their transcript expression of TLR2, TLR3, TLR4, and TLR9. In addition, we analyzed the response of macrophages following in vitro incubation with SEA, which is a complex mixture of highly antigenic molecules, inducing strong Th2 responses. Data obtained after macrophage stimulation for 24 h showed trends similar to the 48 h stimulation; hence, to be consistent with the protein expression data, only the 48 h data are shown. The constitutive transcript expression level for each TLR was lower (reaching statistical significance for TLR2, TLR3, and TLR4) in the Th2-polarized bone marrow macrophages compared with the naïve macrophages (**Fig. 3**). However, the addition of TLR agonists or SEA had a marked effect on the expression of TLRs by naïve and Th2-polarized macrophages, particularly for TLR3 and TLR9 (**Fig. 4**). Specifically, TLR3 transcript expression was increased significantly by poly(I:C) or SEA addition compared with medium alone in cultures of naïve and Th2-polarized macrophages, and a greater increase in TLR3 expression was observed in Th2-polarized macrophages (Fig. 4). In contrast, CpG or SEA stimulation reduced TLR9 transcript expression significantly in cultures of naïve macrophages, and CpG had a similar effect on this transcript in Th2-polarized macrophage cultures. Taken together, these data demonstrated that M2 activation alters TLR transcript expression under constitutive and stimulated conditions in bone marrow-derived macrophages.

Functional responses to TLR agonists differ between naïve and Th2-polarized macrophages

Given that the expression of TLR transcripts differed between the two bone marrow macrophage phenotypes, we next compared the MI and M2 marker responsiveness of these cells to Pam3cys (a TLR2 agonist), poly(I:C) (a TLR3 agonist), LPS (a TLR4 agonist), CpG (a TLR9 agonist), or SEA. Generally, naïve macrophages from bone marrow showed markedly smaller (i.e., two- to tenfold) increases in M1 and M2 markers compared with Th2-polarized macrophages, which exhibited two- to 50,000-fold increases in transcripts for both types of markers (**Fig. 5, A** and **B**). TLR agonist stimulation of Th2-

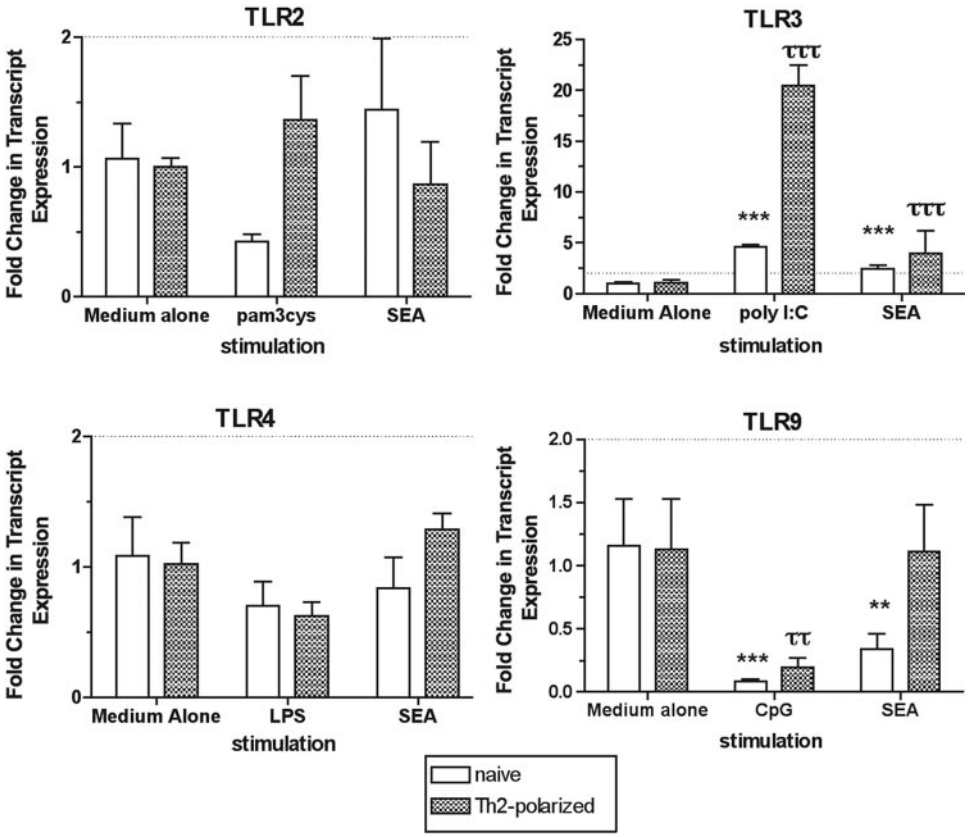

Fig. 4. Quantitative TaqMan PCR analysis of specific TLR agonist or SEA-modulated TLR2, TLR3, TLR4, and TLR9 transcript levels in naïve and Th2-polarized, bone marrow-derived macrophages. TLR agonists or SEA (see Materials and Methods for concentrations) were added to naïve and Th2-polarized macrophages for 48 h prior to RNA isolation and analysis. Transcript levels for each mediator in naïve and Th2-polarized macrophages are expressed as fold change over transcript levels in naïve or Th2-polarized macrophages cultured in media alone. Data shown are mean ± SEM, and these data are representative of three separate experiments. **, $P \leq 0.01$; ***, $P \leq 0.001$, compared with naïve macrophages exposed to medium alone. ττ, $P \leq 0.01$; τττ, $P \leq 0.001$, compared with Th2-polarized macrophages exposed to media alone.

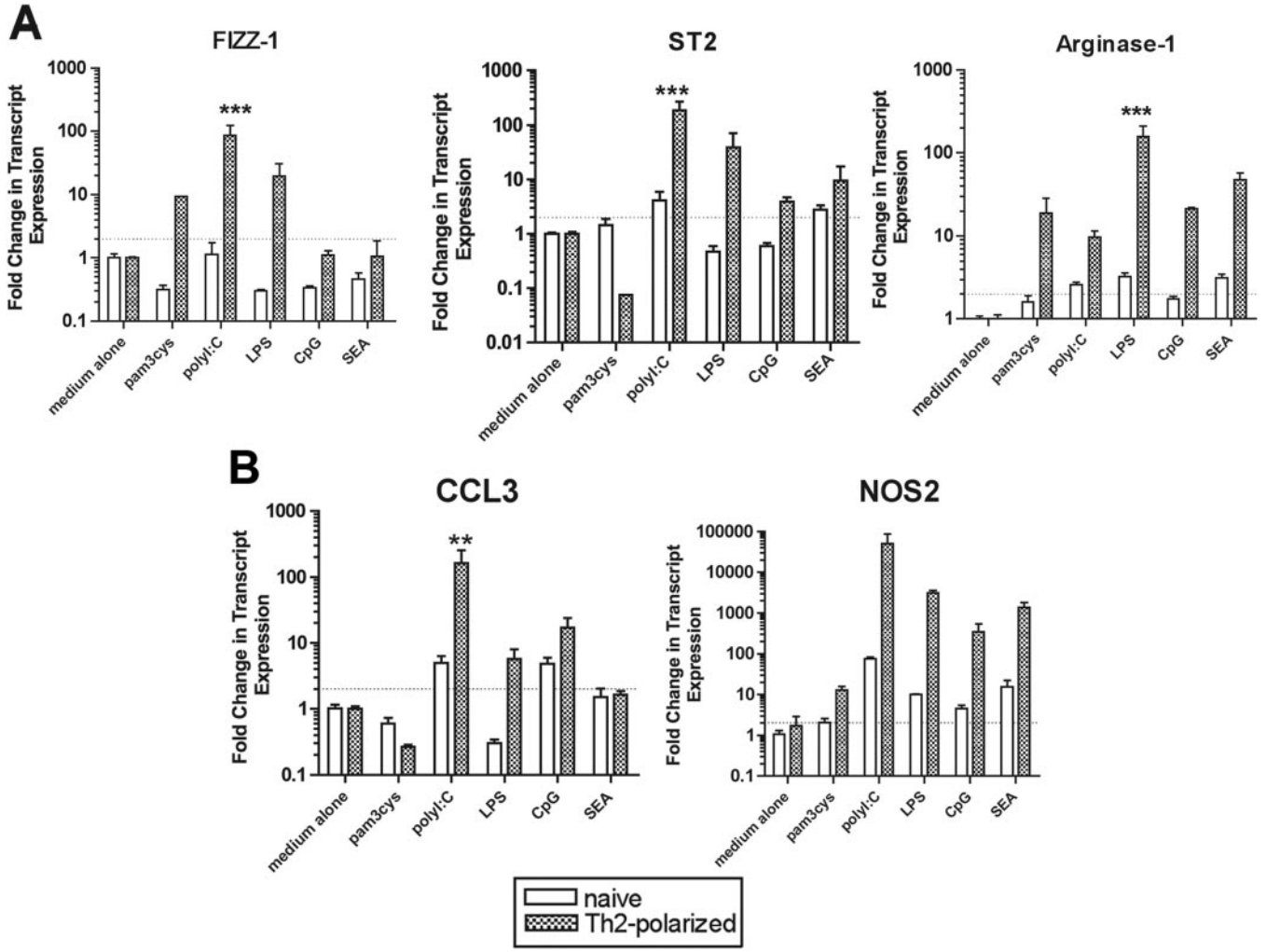

Fig. 5. Quantitative TaqMan PCR analysis of M2 (A) and M1 (B) transcript levels in naïve and Th2-polarized, bone marrow-derived macrophages. TLR agonists or SEA (see Materials and Methods for concentrations) were added to naïve and Th2-polarized macrophages for 48 h prior to RNA isolation and analysis. Transcript levels for each mediator in naïve and Th2-polarized macrophages are expressed as fold change over transcript levels in naïve or Th2-polarized macrophages cultured in media alone. The dashed line in each panel depicts a twofold increase in transcript expression relative to the medium-alone treatment for both groups of macrophages. Data shown are mean ± SEM, and these data are representative of three separate experiments. **, $P \leq 0.01$; ***, $P \leq 0.001$, compared with similarly treated, naive macrophages.

polarized macrophages exhibited markedly increased M2 marker transcript expression (between ten- and 100-fold compared) with cultures that received no stimuli (Fig. 5A). The greatest increase in FIZZ-1 and ST2 was observed in cultures of Th2-polarized macrophages challenged with poly(I:C), whereas LPS was the strongest stimuli for arginase-1 transcript expression in these cells. Significantly higher transcript levels of CCL3 above medium-alone levels were observed when Th2 macrophages were exposed to poly(I:C) (Fig. 5B). This TLR agonist also increased NOS-2 gene expression by the greatest amount relative to the other TLR and SEA stimuli applied to Th2 bone marrow macrophages (Fig. 5B). Taken together, these results suggested that bone marrow from Th2-polarized animals appears to give rise to macrophages that are of a hybrid M1/M2 phenotype, as these cells exhibited marked up-regulation in the expression of transcripts for M1 and M2 markers after TLR activation, relative to naïve macrophages.

Activation of TLRs leads to increased production of proinflammatory chemokines and cytokines by macrophages from Th2-polarized mice

As TLR activation leads to increased production of key inflammatory chemokines and cytokines from M1 macrophages [39], bone marrow macrophages from naïve and Th2-polarized mice were analyzed for changes in these soluble mediators following stimulation with the indicated TLR agonists (**Fig. 6**). Following Pam3cys stimulation, Th2-polarized macrophages produced ten- to 100-fold more CCL3, TNF-α, IL-12, and MIP-2 compared with Pam3cys-activated, naïve macrophages (Fig. 6). All of these increases reached statistical significance (Fig. 6). Poly(I:C) treatment promoted significantly higher CCL3 and TNF-α release in cultures of bone marrow macrophages from Th2-polarized mice compared with poly(I:C)-treated macrophages from naïve mice (Fig. 6). LPS activation led to significantly higher levels of IL-12, TNF-α, and MIP-2 in superna-

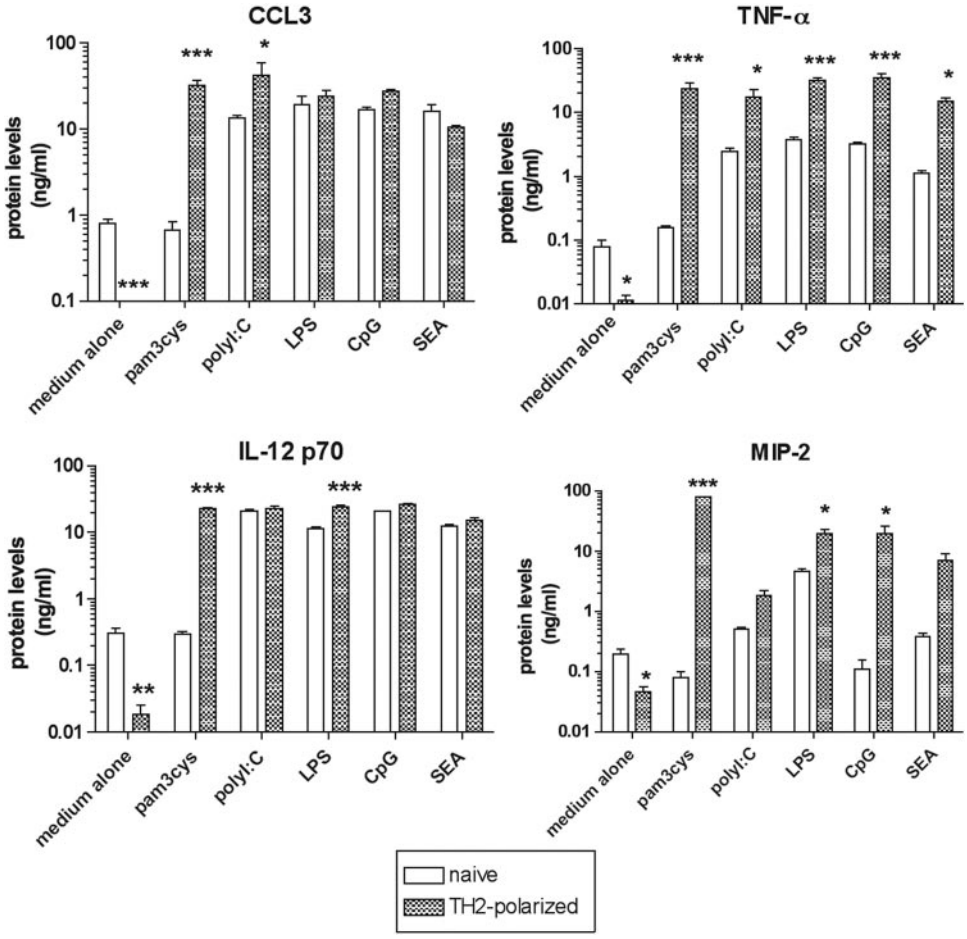

Fig. 6. Protein analysis of immunoreactive levels for cytokines and chemokines in cultures of naïve and Th2-polarized, bone marrow-derived macrophages. TLR agonists or SEA (see Materials and Methods for concentrations) were added to naïve and Th2-polarized macrophages for 48 h prior to the removal of cell-free supernatants from both groups of macrophage for ELISA analysis (see Materials and Methods). Data shown are mean ± SEM, and these data are representative of three separate experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, compared with similarly treated, naïve, bone marrow-derived macrophages.

tants of Th2-polarized macrophages compared with LPS-activated, naïve macrophages (Fig. 6). Similarly, CpG treatment significantly increased the expression of TNF-α and MIP-2 from Th2-polarized macrophages, again in comparison with similarly treated, naïve macrophages (Fig. 6). Finally, SEA-stimulated, Th2-polarized macrophages also released significantly greater amounts of TNF-α compared with SEA-stimulated, naïve macrophages (Fig. 6). The apparent discrepancy of results between Figure 4 and Figure 6 appears because the former shows gene expression of TLR at the 48-h time-point, whereas the latter represents protein levels accumulated over 48 h after TLR stimulation. Altogether, it was surprising that Th2-polarized macrophages produced significantly higher amounts of proinflammatory or M1-associated mediators than naïve macrophages, suggesting that these cells maintain the ability to generate M1 mediators in response to TLR activation, despite evidence of a pre-existing M2 phenotype. Ongoing studies in the laboratory are aimed toward understanding signaling events that lead to hyper-responsiveness of Th2-polarized macrophages after TLR ligation.

Proinflammatory mediator release by TLR agonist-activated, Th2-polarized macrophage was muted in the presence of IL-10 and CCL17

CCL17 and IL-10 are important regulators of the Th2 reponse during *S. mansoni* egg-induced granuloma. Addition of these two mediators to macrophage cultures inhibits M1 or classical activation and enhances M2 or alternative activation of macrophages [13]. We thus asked whether TLR agonist-induced M1 mediator production by Th2-polarized macrophages was modified by a pretreatment with CCL17 and IL-10 [13]. As summarized in **Figure 7**, the transcript level for M1 markers CCL3 and NOS-2 remained higher in Pam3cys- and poly(I:C)-treated, Th2-polarized macrophages compared with naïve macrophages, but the transcript expression in both macrophage types was dramatically lower than that observed in cultures of these cells not pretreated with CCL17 and IL-10 (Fig. 5B). Thus, macrophages from Th2-polarized animals remained responsive to CCL17 and IL-10, which dramatically decreased the expression of M1 marker transcripts in these cells, but these levels were still enhanced when compared with similarly treated, naïve macrophages.

ELISA analysis of chemokines and cytokines produced by CCL17 and IL-10-pretreated, naïve, and Th2-polarized macrophages exposed to TLR agonists is summarized in **Figure 8.** Overall, the TLR agonist-induced release of CCL3, TNF-α, IL-12, and MIP-2 from both groups of CCL17 and IL-10-pretreated, naïve, and Th2-polarized macrophages was markedly lower than levels of each mediator measured in cultures of these macrophages not pretreated with CCL17 and IL-10 (Fig. 8 vs. Fig. 6). However, cultures of poly(I:C)-, LPS-, CpG-, and SEA-treated, Th2-polarized macrophages contained significantly greater levels of TNF-α and MIP-2 relative to cultures of similarly treated, naïve macrophages (Fig. 8). Therefore, these

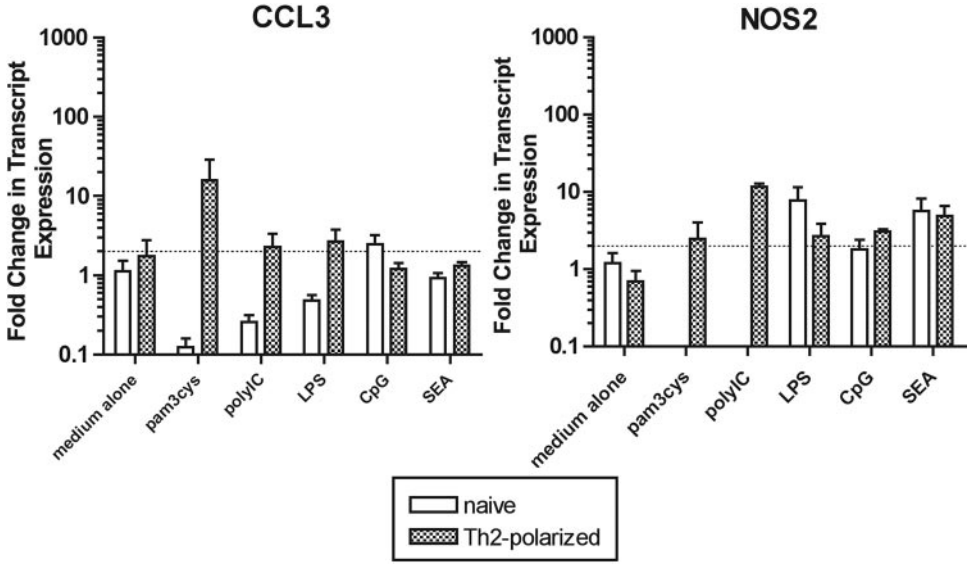

Fig. 7. Quantitative TaqMan PCR analysis of M1 transcript levels in naïve and Th2-polarized, bone marrow-derived macrophages. Both groups of macrophages were pretreated with IL-10 (20 ng/ml) and CCL17 (50 ng/ml) prior to the addition of TLR agonists or SEA (see Materials and Methods for concentrations). At 48 h, RNA was isolated from both groups of macrophages and analyzed using TaqMan (see Materials and Methods). Transcript levels for each mediator in naïve and Th2-polarized macrophages are expressed as fold change over transcript levels in naïve or Th2-polarized macrophages cultured in media alone. Data shown are mean ± SEM, and these data are representative of three separate experiments.

data confirmed that CCL17 and IL-10 pretreatment markedly affected the generation of M1 mediators by bone marrow macrophages, but this modulatory effect was greater in cultures of naïve rather than Th2-polarized macrophages.

Bone marrow-derived macrophages from *S. mansoni*-infected mice have altered responses to TLR agonists

Mice infected with *S. mansoni* parasites mount a vigorous type 2 response, which is required for the host survival [10, 40]. Next, we asked the question of whether infection of mice with live parasites affected development of bone marrow-derived macrophages in a manner similar to a *S. mansoni* egg-induced granulomatous response. Mice were killed 8 weeks after exposure to live cercariae, at which time, the host immune response is primarily Th2 [2]. Bone marrow-derived macrophages from naive and *S. mansoni*-infected mice were cultured and then stimulated with poly(I:C) and SEA, as these stimulants resulted consistently in increased M1 and M2 mediator expression as shown in Figures 5–8. Macrophages from infected mice ex-

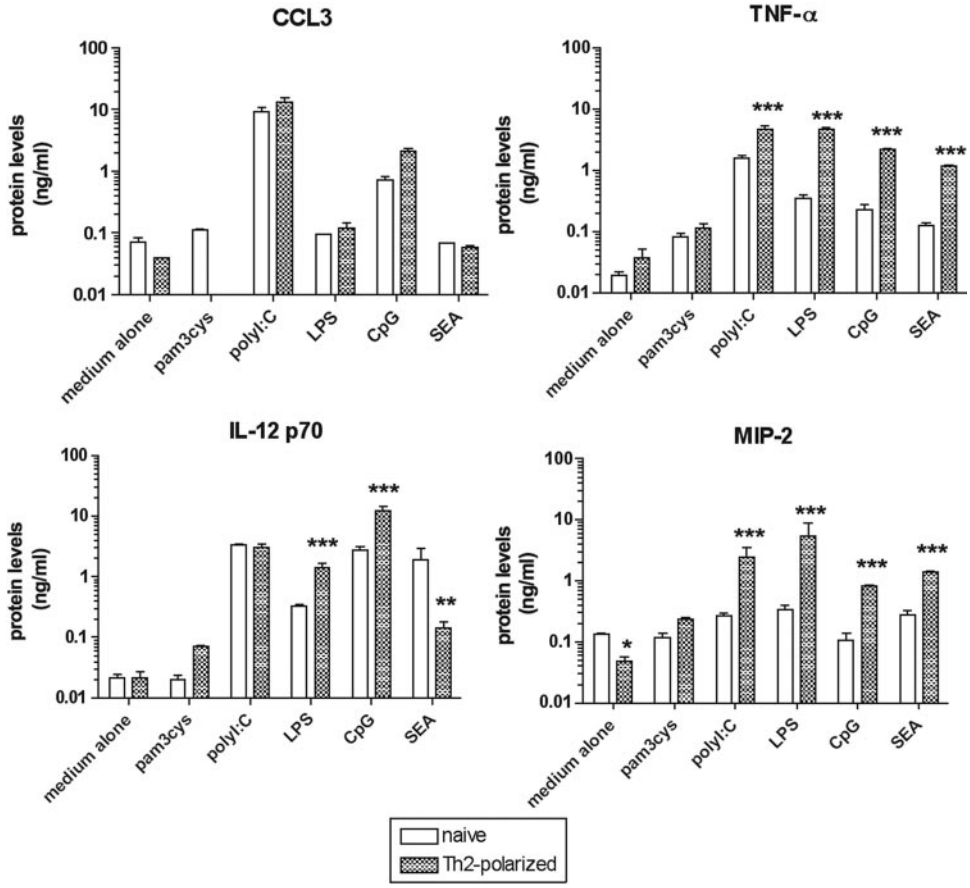

Fig. 8. Protein analysis of immunoreactive levels for cytokines and chemokines in cultures of naïve and Th2-polarized, bone marrow-derived macrophages. Both groups of macrophages were pretreated with 20 ng/ml IL-10 and 50 ng/ml CCL17 prior to the addition of TLR agonists or SEA (see Materials and Methods for concentrations). At 48 h, cell-free supernatants were removed from both groups of macrophages and analyzed using ELISA (see Materials and Methods). Data shown are mean ± SEM, and these data are representative of three separate experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, compared with similarly treated, naïve, bone marrow-derived macrophages.

pressed higher levels of gene transcripts of M2 mediators, arginase-1 and IL-10 (**Fig. 9A**), compared with similarly treated macrophages from naïve mice. As shown in Figure 9B and as expected, NOS-2 gene expression was up-regulated significantly in poly(I:C)-stimulated macrophages from infected mice compared with poly(I:C)-treated macrophages from naïve mice. In contrast, activation by poly(I:C) resulted in significantly lower CCL3 gene expression by macrophages from infected mice compared with similarly treated macrophages from naïve mice. Consistent with gene expression analysis, CCL3 protein was detected at significantly lower amounts in supernatants from poly(I:C)-treated macrophages from infected mice compared with similarly treated macrophages from naïve mice (Fig. 9C). On the other hand, significantly higher levels of TNF-α, a M1 mediator, and CCL2, a M2 mediator, were detected in cultures of poly(I:C)-stimulated macrophages from infected mice compared with similarly treated macrophages from naïve mice. In summary, infection with live parasites, which leads to a systemic Th2 response, yields bone marrow-derived macrophages, which are hyper-responsive to poly(I:C).

DISCUSSION

There is substantial evidence that helminth infections modulate the adaptive immune response through various mechanisms including their inherent ability to induce polarized Th2-type cytokine responses [41]. Nevertheless, more recent stud-

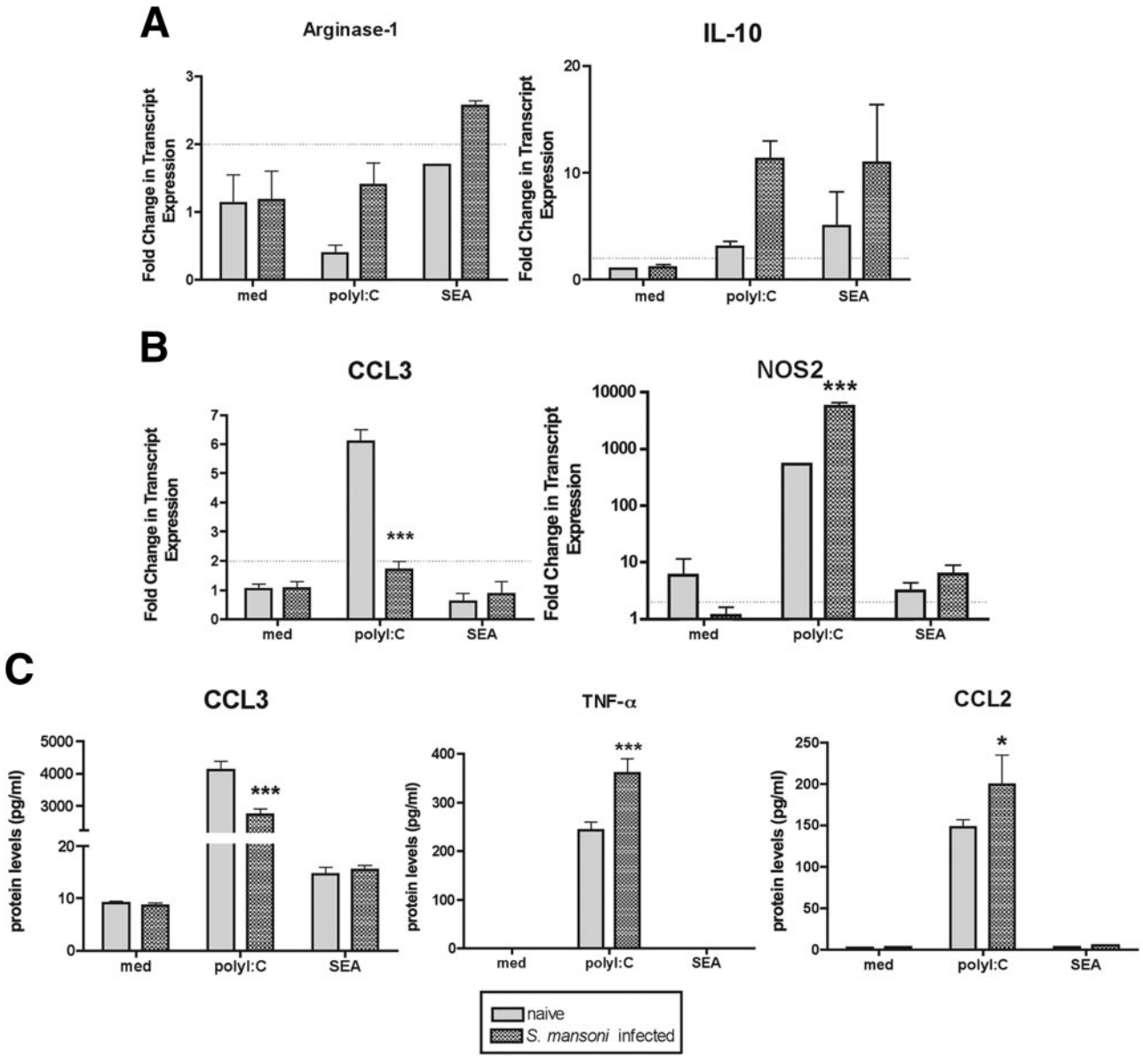

Fig. 9. Quantitative TaqMan PCR analysis of M2 (A) and M1 (B) transcript levels in bone marrow macrophages cultured from naïve and *S. mansoni*-infected Swiss-Webster mice. Macrophages were treated with poly(I:C) or SEA for 48 h before isolating RNA. Transcript levels for each mediator are expressed as fold change over level in macrophages from naïve or infected mice cultured in media alone. (C) Protein analysis of cell culture supernatants by Multiplex Bead Immunoassay is depicted. Data shown are mean ± SEM from a representative of three separate experiments. *, $P \leq 0.05$; ***, $P \leq 0.001$, compared with similarly treated, bone marrow-derived macrophages from naïve animals.

ies show that helminth parasites have potent effects on the innate immune response. Accordingly, the present study addressed the effect of a Th2-polarized *S. mansoni* egg-induced pulmonary granuloma response on the phenotype of bone marrow-derived macrophages. Herein, we show that macrophages from Th2-polarized mice showed transcript and protein evidence of alternative or M2 activation. It was also apparent that these Th2-polarized macrophages exhibit markedly enhanced responsiveness to TLR agonists compared with naïve macrophages. However, the responses by these cells were not consistent with M1 or M2 activation, as levels of both markers were increased significantly by the addition of specific TLR agonists. These findings suggest that Th2-polarized macrophages retain their ability to recognize pathogen-associated molecular patterns present on invading pathogens, thereby allowing these cells to initiate a signal transduction cascade that leads to inflammation and the subsequent killing of the pathogen, which is characteristic of M1 activation. However, as these cells also express M2 markers, it is possible that these cells contribute to the abnormal remodeling that characterize granulomatous responses to *S. mansoni*.

The alternative activation state of macrophages has been shown in the context of murine nematode infections, and these cells were shown to express several specific markers such as chitinases and carbohydrate receptors [42, 43]. These previous studies identified that M2 activation was prominent in macrophages. The present study extends these studies through the demonstration that the precursors of macrophages in the bone marrow are affected by the presence of *S. mansoni* eggs and leads to their constitutive expression of M2 and not M1 markers. Putative pathways that lead to the distinctive M2 activation state have been studied, and it has been shown that mice lacking *src* homology 2-containing 5′-phosphatase (SHIP) as a result of gene deletion display several abnormalities that relate, in part, to the role of this phosphatase in regulating PI-3K-driven, alternative activation of macrophages [44]. Interestingly, this alternative or M2 macrophage phenotype was observed in tissue-resident macrophages, but it could only be recapitulated in SHIP−/− bone marrow macrophages with the exogenous addition of murine TGF-β [45]. It was apparent from the present study that the exogenous addition of murine TGF-β in vitro was not required to promote the M2 activation state of cultured bone marrow macrophages, possibly reflecting the fact that the levels of this cytokine are increased dramatically during *S. mansoni* egg challenge in vivo [46]. Other cytokines and chemokines that are increased during this pulmonary granulomatous response are IL-10 and CCL17 [8, 12], and the exogenous addition of these soluble mediators to cultures of naïve and Th2-polarized macrophages appeared to decrease M1 markers in both groups of cells in our study. However, Th2-polarized macrophages remained more responsive to the presence of TLR agonists, particularly TLR3, compared with naïve macrophages, as evidenced by the fact that the former cells continued to produced significantly higher amounts of TNF-α, IL-12, and MIP-2 following TLR activation. It is possible that in addition to soluble mediators, the cellular composition of the bone marrow of Th2-polarized animals is altered and affects macrophage precursors and their expression of TLRs. This suggestion was highlighted in a previous study, in which it was shown that macrophages derived from the bone marrow of NOD mice highly expressed TLR3 and TLR5, an effect that appeared to be partly the result of the hyperglycemic environment [47]. Together, these data show that macrophage development from the bone marrow is markedly affected by the systemic effect of *S. mansoni* egg-induced granuloma.

Infective *S. mansoni* cercariae penetrate host skin and then migrate and develop into mature worms, which reside in the portal vasculature, where they can live for several years. Female worms release 200–300 eggs per day, which get trapped in the liver sinusoids and result in granuloma formation, liver fibrosis, portal hypertension, and ultimately, organ failure. The host elicits an initial Th1 response followed by a sustained Th2 response. Data presented in this study show that *S. mansoni* infection alters generation of bone marrow macrophage precursors. Consistent with a *S. mansoni* egg-induced, secondary, synchronous, granuloma model, poly(I:C) treatment results in higher gene and protein expression of M1 and M2 mediators, such as NOS-2, TNF-α, CCL2, arginase-1, and IL-10. Surprisingly, bone marrow macrophages from *S. mansoni*-infected mice showed lower CCL3 gene expression and protein release after stimulation with poly(I:C). CCL3 has been reported to play a pivotal role in a murine model of schistosomiasis, such that CCL3−/− mice had reduced hepatic granuloma size, collagen deposition, and reduced adult worm and egg burden [25]. Therefore, it is possible that decreased CCL3 expression by bone marrow-derived macrophages is a mechanism of host defense against the increased parasite burden. Together, these data show that consistent with egg-induced granuloma, infection with live parasites affects bone marrow precursors of macrophages.

To date, the majority of reports describes an inhibitory effect of helminth infection or persistence on TLR-mediated responsiveness by human and animal immune cells. For example, diminished TLR expression and function have been shown in the T cells of filarial-infected individuals [48], and the exposure of human DC to SEA severely limits their ability to respond to LPS and CpG [49, 50]. Likewise, a diminished up-regulation of TLR was observed in B cells and monocytes of infected individuals. Stimulation of B cells and monocytes with TLR agonists resulted in decreased B cell, CD4+ T cell, and monocyte activation/cytokine production upon stimulation with filarial antigen [51]. Finally, it has been shown that TLR2 and TLR4 expression by circulating immune cells are significantly lower in schistosome-infected children [52]. More recently, it was shown that the stimulation of DC by the egg stage of the helminth parasite *S. mansoni* activates a signaling pathway resulting in type I IFN and IFN-stimulated gene expression in a TLR3-dependent manner via *S. mansoni*-derived dsRNA [53]. Others have shown that schistosome larvae stimulate M1 and M2 macrophages to produce several cytokines including IL-6, IL-12p40, and IL-10, and this effect was dependent on MyD88, a TLR adaptor [54], possibly through a TLR4-dependent mechanism [55]. Thus, further investigation of the modulatory effect of parasite antigens on TLR responsiveness by innate and adaptive immune cells is certainly warranted.

In summary, the present study showed that progenitor cells from the bone marrow of mice with *S. mansoni* pulmonary granulomatous responses exhibit baseline characteristics of

M2-activated macrophages. However, the activation status of these cells is altered dramatically by the exogenous addition of various TLR agonists, most notably, poly(I:C). The consequence of this type of activation is consistent with these cells having the ability to participate in antimicrobial and tissue remodeling responses.

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