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TLR3 modulates immunopathology during a *Schistosoma mansoni* egg-driven Th2 response in the lung

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We examined the role of TLR3 in Th2-driven pulmonary granulomatous disease, using wildtype ($TLR3^{+/+}$) and TLR3 gene-deficient ($TLR3^{-/-}$) mice in a well-established model of *Schistosoma mansoni* egg-induced pulmonary granuloma. The intravenous bolus injection of *S. mansoni* eggs into *S. mansoni*-sensitized $TLR3^{+/+}$ mice was associated with an increase in TLR3 transcript expression in alveolar macrophages and *ex vivo* spleen and lung cultures at day 8 after egg injection. Lungs from $TLR3^{-/-}$ mice showed an increase in granuloma size, greater collagen deposition around the granuloma, and increased Th2 cytokine and chemokine levels compared with similarly sensitized and challenged $TLR3^{+/+}$ mice. Macrophages from $TLR3^{-/-}$ mice exhibited an M2 phenotype characterized by increased arginase and CCL2 expression. Significantly greater numbers of $CD4^+CD25^+$ T cells were present in the lungs of $TLR3^{-/-}$ mice compared with $TLR3^{+/+}$ mice at day 8 after egg embolization. Cells derived from granulomatous lung and lung draining lymph nodes of $TLR3^{-/-}$ mice released significantly higher levels of IL-17 levels relative to $TLR3^{+/+}$ cells. Thus, our data suggest that TLR3 has a major regulatory role during a Th2-driven granulomatous response as its absence enhanced immunopathology.

Key words: *Schistosoma mansoni* granuloma · Th2 · Th17 · TLR3

Introduction

Schistosoma mansoni is a helminth parasite, which causes significant morbidity and mortality in the developing world [1]. It is estimated that more than 200 million people worldwide are affected by Schistosomiasis [2]. The egg from this parasite contributes largely to the profound immunopathology associated with *S. mansoni* infection as the chronic granulomatous response is clearly deleterious to any tissue containing these eggs [3]. *S. mansoni* eggs release highly antigenic glycoproteins collectively referred to as Schistosoma egg antigen (SEA) that promote dominant Th2 responses [4–7]. In its chronic phase, the granulomatous response to *S. mansoni* eggs is driven almost entirely by Th2 cytokines and chemokines, and this response involves the recruitment and activation of eosinophils, alternatively activated macrophages (or M2 macrophages), DC, and $CD4^+$ Th2 cells [8]. Although the egg-induced Th2 granulomatous response is required for host survival [9], the Th2 response is highly tissue destructive, due, in part, to fibrotic scarring around these granulomas. More recently, it was shown that IL-17-producing T cells (Th17) also contribute to the severity of the egg-induced granulomatous response [10]. Th17 are $CD4^+$ T cells that have been implicated in exacerbating autoimmune diseases such as experimental autoimmune encephalomyelitis and collagen-induced arthritis indicating that Th17 cells are highly pro-inflammatory [11, 12].

The manner in which TLR contribute to innate and adaptive immune responses associated with *S. mansoni*-induced immunopathology continues to garner research attention. TLR such as TLR2, TLR4, TLR9, and the TLR adaptor protein MyD88 have been shown to play a significant role in cytokine and cellular composition of granulomas induced by *S. mansoni* [13–15]

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(S. L. K., unpublished observations). TLR3 is an example of a TLR that has been traditionally viewed as a sensor of dsRNA, which is usually of viral origin. Activation of TLR3 engages an MyD88-independent pathway involving toll/IL-1 receptor domain-containing adaptor inducing type I interferon beta (also known as TRIF). More recently, studies have highlighted that endogenous RNA from necrotic mammalian cells activates TLR3 as does dsRNA from the helminth parasite *S. mansoni* [16–18]. The latter observation was of interest to us since we recently reported that bone-marrow-derived macrophages from mice sensitized and challenged with *S. mansoni* eggs exhibited markedly increased TLR3 expression and heightened responses to the synthetic TLR3 ligand, poly I:C [19].

In the present study, we examined the role of TLR3 in a model of secondary synchronous pulmonary granulomatous disease by sensitizing TLR3$^{+/+}$ and TLR3$^{-/-}$ mice with *S. mansoni* eggs and later challenging both groups of sensitized mice with an intravenous bolus of eggs. The intravenous introduction of *S. mansoni* eggs induced TLR3 transcript expression in alveolar macrophages and *ex vivo* spleen and lung cultures of wildtype mice. Histologically, the granulomas in the TLR3$^{-/-}$ group were significantly larger and more fibrotic at days 8 and 16 after challenge when compared with the TLR3$^{+/+}$ group. The host immune response in the gene knockout group was distinctly skewed toward Th2, and this was observed in SEA recall responses in isolated cells from spleen, lung, lymph node, and bone marrow macrophages. Interestingly, significantly increased IL-17 was detected in cultures of isolated lung and draining lymph node cultures following SEA stimulation. Together, our results indicated that TLR3 is a key modulator of *S. mansoni* egg-induced pulmonary granulomatous response, and its absence promoted increased Th2 and Th17 responses.

Results

TLR3 transcript and protein expression in granulomatous lungs and dispersed cells

We first examined the relative expression of TLR3 transcript and protein in whole lung samples taken from naïve mice, and *S. mansoni*-sensitized mice prior to and at days 8 and 16 after *S. mansoni* egg embolization. As shown in Fig. 1A, the whole lung transcript levels of TLR3 were lower at all times examined prior to and after egg embolization compared with naïve controls. The whole lung protein levels of TLR3 in *S. mansoni*-naïve, -sensitized and egg-challenged mice are shown in Fig. 1B (top panel of Fig. 1B shows Western blot and bottom panel shows normalization of TLR3 band to GAPDH band). Among these groups of mice, the greatest whole lung expression of TLR3 was observed in naïve mice. Prior to and at days 8 and 16 after egg embolization in sensitized mice, the whole lung levels of TLR3 were markedly lower (Fig. 1B).

Because the decrease in whole lung TLR3 expression during the lung granulomatous response might have been the consequence of increased cell recruitment of cells unable to express TLR3, we next investigated TLR3 expression in specific cell populations prior to and after egg embolization. We analyzed TLR3 expression in alveolar macrophages isolated from BALF (Fig. 1C). Alveolar macrophages isolated from mice 8 days after *S. mansoni* egg challenge exhibited significantly higher TLR3 transcript levels compared with macrophages from naïve mice. These data demonstrated increase in TLR3 expression by alveolar macrophages after *S. mansoni* egg sensitization and challenge.

We also analyzed TLR3 transcript expression in *in vitro* cultures of dispersed spleen cells and bone-marrow-derived macrophages both prior to and at day 8 after egg embolization. As shown in Fig. 1D, transcript levels of TLR3 were increased at day 8 after egg embolization in dispersed spleen cells in media alone relative to the levels of this TLR immediately prior to egg embolization at day 0. The addition of SEA to cultures of dispersed spleen cells from the day 8 time point significantly enhanced TLR3 transcript expression relative to the media alone group at day 0. Enhanced TLR3 transcript expression was observed in SEA-activated bone-marrow-derived macrophages regardless of the source of these cells (*i.e. S. mansoni* egg-sensitized mice and *S. mansoni* egg-challenged mice) but greater induction of TLR3 was observed in bone-marrow-derived macrophages from *S. mansoni*-sensitized mice (day 0, Fig. 1E). Thus, these data showed that TLR3 gene expression by alveolar macrophages, dispersed spleen cells, and bone-marrow-derived macrophages was up-regulated by the presence of *S. mansoni* egg antigens.

Augmented Th2 cytokine generation by splenocytes from sensitized TLR3$^{-/-}$ mice

Evidence that the systemic immune response in *S. mansoni*-sensitized TLR3$^{-/-}$ mice prior to egg embolization was markedly different from that in similarly sensitized TLR3$^{+/+}$ mice was derived from an analysis of antigen recall responses in dispersed spleen cultures. Protein levels in cell-free supernatants were examined using a multiplex immunobead assay and as shown in Fig. 2, constitutive levels of Th2 cytokines including IL-4, IL-5, and IL-13 were similar in cultures of TLR3$^{+/+}$ and TLR3$^{-/-}$ splenocytes incubated in media alone. However, media-alone cultures of TLR3$^{-/-}$ splenocytes contained significantly greater levels of IL-17 (Fig. 2D). The addition of SEA to cultured splenocytes from both groups of mice significantly augmented the levels of all four cytokines but cultures of TLR3$^{-/-}$ splenocytes contained significantly greater levels of the Th2 cytokines compared with SEA-stimulated TLR3$^{+/+}$ splenocytes. Thus, the antigen recall responses in cultured splenocytes from TLR3$^{-/-}$ suggested that these mice were more heavily skewed toward Th2 cytokine production, but these data also suggested that IL-17 levels in *S. mansoni*-sensitized splenocytes from the knockout group were also augmented.

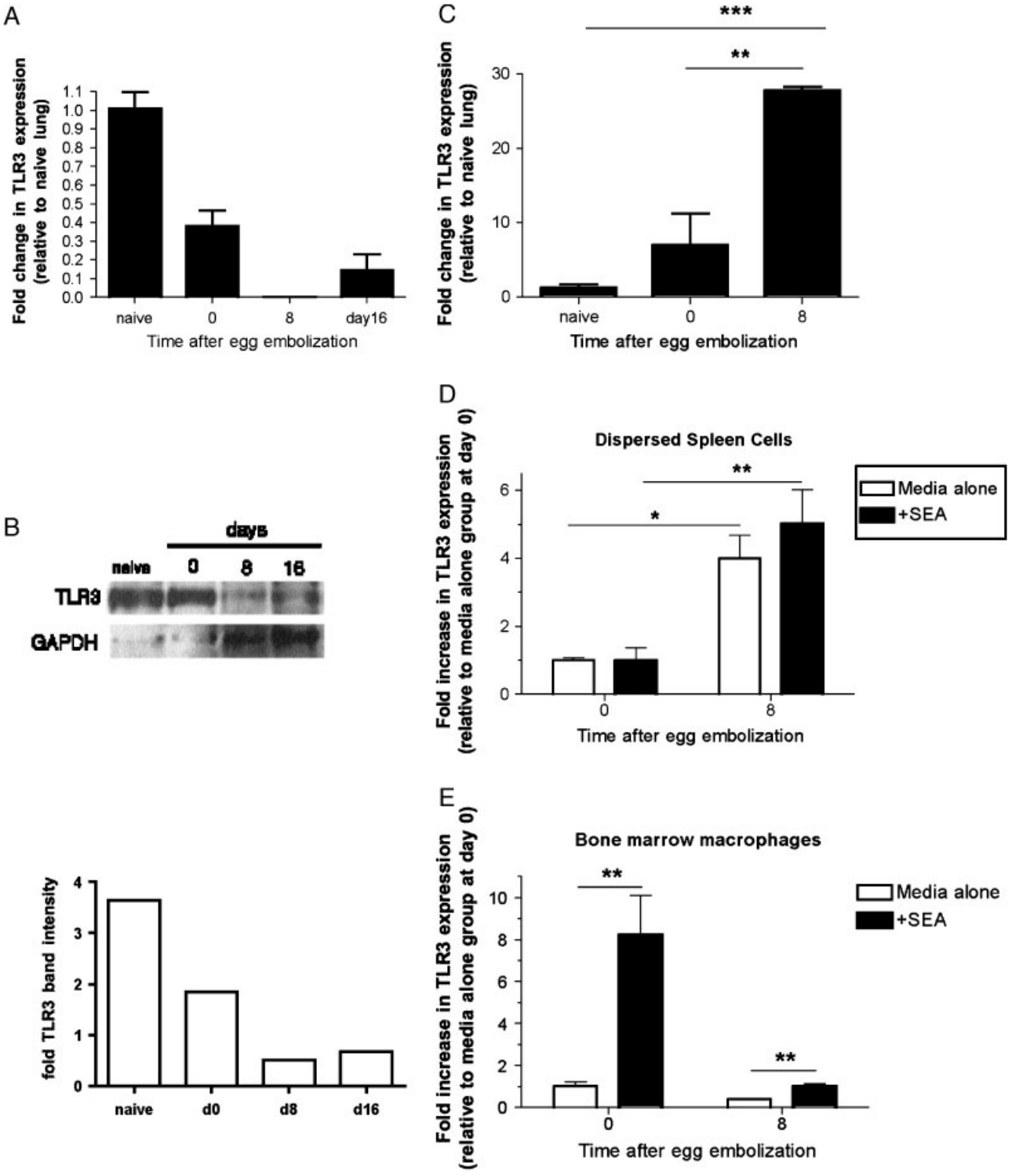

Figure 1. Transcript and protein expressions of TLR3 prior to and after egg embolization in *S. mansoni*-sensitized TLR3$^{+/+}$ mice. (A) Whole lung cells were analyzed by TAQMAN for TLR3 gene expression. Data are expressed as fold change over TLR3 transcript level in naïve lungs. (B) Membrane fractions derived from whole lungs were subjected to Western blot analysis. Also shown (bottom panel, B) are results of the normalization of TLR3 to GAPDH. Alveolar macrophages obtained from BALF were analyzed by TAQMAN for TLR3 gene expression (C). Data are expressed as fold change over transcript levels in naïve alveolar macrophages. Spleen cells (D) and bone-marrow-derived macrophages (E) harvested from *S. mansoni*-sensitized TLR3$^{+/+}$ mice were incubated in media alone or SEA for 24 h before RNA extraction for TLR3 gene expression analysis. Data shown are mean ± SE and are from a representative experiment of two independent experiments. Each time point had at least three to five mice *per* group. One-way ANOVA and Newman–Keuls post-test were used to analyze significance. Significant differences are shown as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

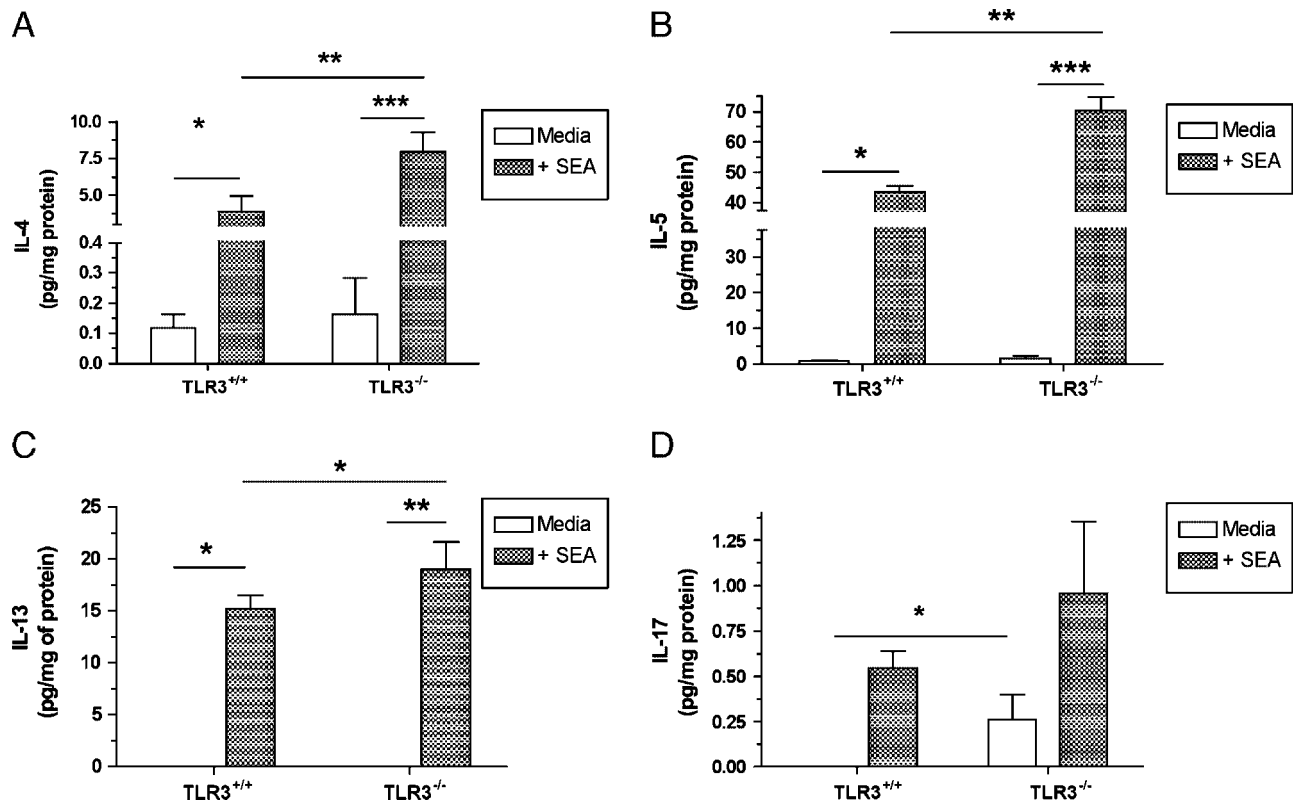

Figure 2. IL-4, IL-5, IL-13, and IL-17 levels in cultures of dispersed spleen cells from sensitized TLR3$^{+/+}$ and TLR3$^{-/-}$ mice. Spleen cells pooled from three to five mice *per* group were cultured *in vitro* in the presence of SEA for 48 h. Cell-free supernatant was then collected and analyzed by multiplex bead immunoassay. Data shown are mean ± SE of triplicate cultures from a representative of three separate experiments. Significance was determined by using one-way ANOVA and Newman–Keuls post-test (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

Sensitized and challenged TLR3$^{-/-}$ mice exhibit larger, fibrotic granulomas

To determine whether TLR3 is involved in Th2-type pulmonary granulomatous disease, we examined histological lung sections from *S. mansoni*-sensitized TLR3$^{+/+}$ and TLR3$^{-/-}$ mice at days 8 and 16 after egg embolization. Masson's Trichrome stained lung sections revealed that compared with TLR3$^{+/+}$ egg granulomas (Fig. 3A and C; days 8 and 16, respectively), the TLR3$^{-/-}$ granulomas had distinct fibrotic rims or caps surrounding the egg (Fig. 3B and D; days 8 and 16, respectively). Also apparent in the day 16 granulomas from TLR3$^{-/-}$ mice were macrophages filled with chitinase-like crystals, a feature that is associated with M2 activation (Fig. 3E and F; TLR3$^{+/+}$ and TLR3$^{-/-}$, respectively) [20]. Morphometric analysis of granulomas in both groups of mice revealed that the granuloma size (Fig. 3G) and percentage of collagen in granulomas (Fig. 3H) were significantly greater in the knockout groups compared with the wildtype groups at both times after egg embolization. Quantification of eosinophils in histological sections of lung granulomas at days 8 and 16 after egg embolization did not show significant difference between TLR3$^{+/+}$ and TLR3$^{-/-}$ mice (Table 1). Thus, the absence of TLR3 resulted in a morphologically distinct granulomatous response characterized by exaggerated Th2 features including increased granuloma size, collagen deposition, and the marked presence of chitinase-like crystals.

TLR3$^{-/-}$ lungs reveal increased Th2 and decreased Th1 cytokines and chemokines

The histological picture in TLR3$^{-/-}$ mice sensitized and challenged with *S. mansoni* eggs suggested that this knockout strain might have a major alteration in the lung cytokine and chemokine pattern. IL-4 and CCL11 have been shown to be elevated in Th2 skewed pulmonary granulomas whereas CXCL10 and CXCL9 have been shown to be highly expressed in Th1-type pulmonary granulomas [21]. We examined these prototypical factors in whole lung homogenates by ELISA. As shown in Fig. 4A and B, respectively, IL-4 and CCL11 were significantly elevated after egg embolization at the day 16 time point. Also, at day 16, whole lung CXCL10 and CXCL9 protein levels were significantly lower in the TLR3$^{-/-}$ group compared with the TLR3$^{+/+}$ group (Fig. 4C and D, respectively). There was no difference in the expression levels of IL-13 and IFN-γ in lung homogenates of TLR3$^{+/+}$and TLR3$^{-/-}$ mice (data not shown). Collectively, the analysis of whole lung samples from wildtype and knockout groups revealed a significant difference in the levels of cytokines

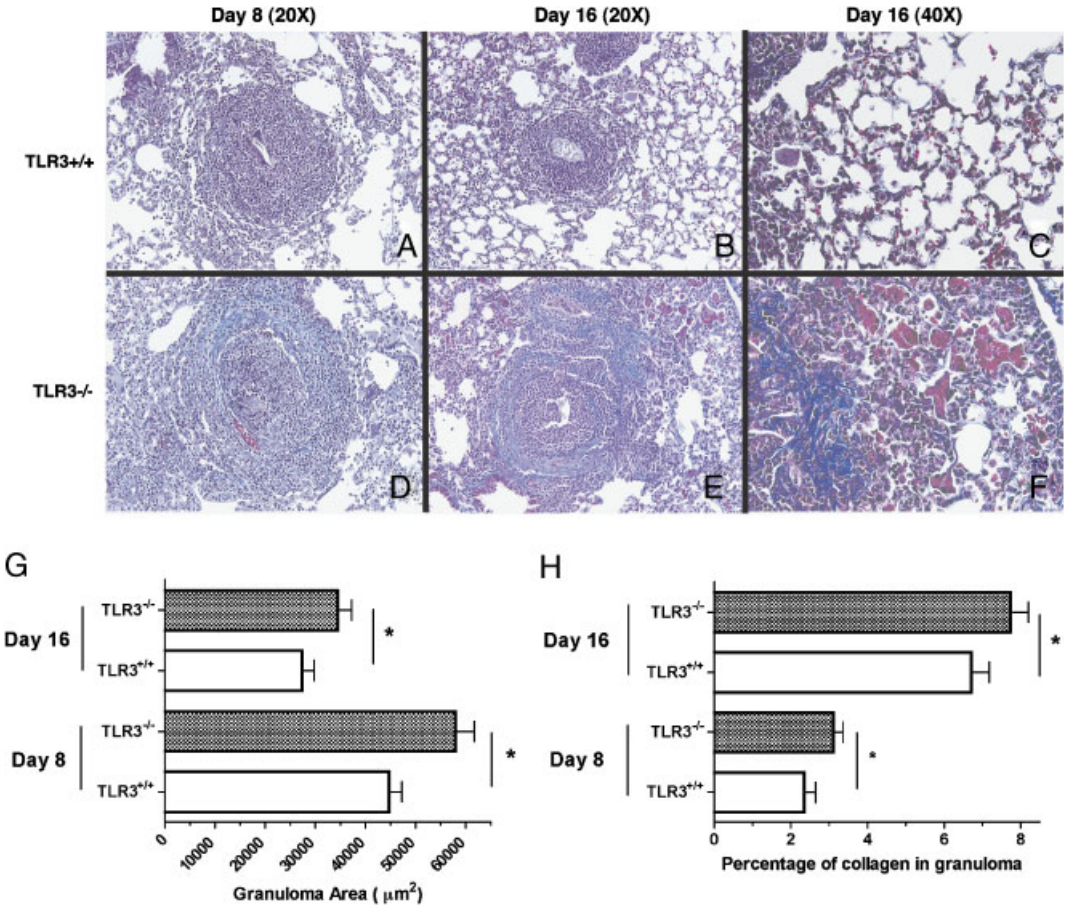

Figure 3. Histopathological appearance of egg granulomas in TLR3$^{+/+}$ and TLR3$^{-/-}$ mice at days 8 and 16 after egg embolization. Masson's trichrome staining (magnification, 200×) of TLR3$^{+/+}$ granulomas at day 8 (A) and day 16 (C) and of TLR3$^{-/-}$ granulomas at day 8 (B) and day 16 (D) is depicted. High power micrographs (magnification, 400×) of Masson's Trichrome staining of TLR3$^{-/-}$ (F) granulomas display chitinase-like crystals, which were absent in TLR3$^{+/+}$ granulomas (E). Granuloma area (G) and percentage of collagen in each granuloma (H) was quantified using IP lab software. Data shown are mean ± SE and are from one representative experiment of three independent experiments. Each time point had at least three to five mice *per* group. Student's *t*-test was used to analyze significance. Significant differences are depicted as $^{*}p \leq 0.05$.

and chemokines further suggestive of a Th2 skewing in *S. mansoni* egg-sensitized and -challenged TLR3$^{-/-}$ mice.

Naïve and sensitized macrophages from TLR3$^{-/-}$ mice exhibit an M2 phenotype

We next addressed how this TLR deficiency affected the responsiveness of macrophages to by-products from *S. mansoni* eggs, specifically dsRNA and SEA. We first examined bone-marrow-derived macrophages, which we previously have shown to up-regulate TLR3 expression during the course of the pulmonary granulomatous response induced by *S. mansoni* eggs. In the present study, we extended these observations to examine the role of TLR3 in macrophage responses to *S. mansoni* dsRNA. Macrophages from naïve TLR3$^{+/+}$ mice generated both IL-12 and CXCL10 following the addition of *S. mansoni* dsRNA or poly I:C for 48 h (Fig. 5). Bone-marrow-derived macrophages from naïve TLR3$^{-/-}$ mice generated markedly less IL-12 and CXCL10 following exposure to *S. mansoni* dsRNA compared with wildtype macrophages (Fig. 5). TLR3$^{-/-}$ and TLR3$^{+/+}$ macrophages generated nearly equivalent amounts of CXLC10 following exposure to poly I:C, but IL-12 production by TLR3$^{-/-}$ macrophages was significantly lower than that observed in cultures of TLR3$^{+/+}$ macrophages in the presence of this TLR3 agonist (Fig. 5). Thus, these data suggested that the

Table 1. Eosinophil numbers in granulomas from TLR3$^{+/+}$ and TLR3$^{-/-}$ mice at days 8 and 16 after egg embolization

Day	Strain	Number of eosinophils *per* 100 μm^2 granuloma area
8	TLR3$^{+/+}$	0.223 ± 0.013
	TLR3$^{-/-}$	0.201 ± 0.038
16	TLR3$^{+/+}$	0.232 ± 0.018
	TLR3$^{-/-}$	0.197 ± 0.015

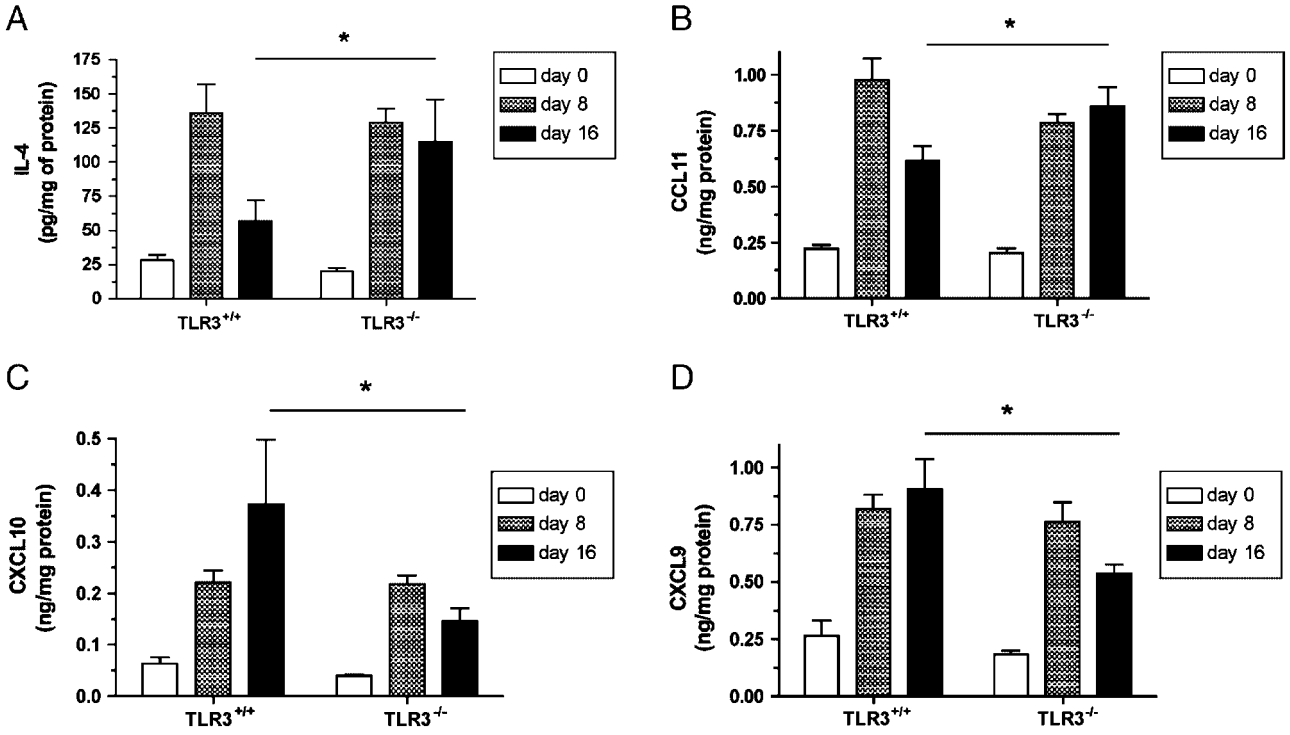

Figure 4. IL-4, CCL11, CXCL10, and CXCL9 levels in whole lung samples from TLR3$^{+/+}$ and TLR3$^{-/-}$ mice prior to and at days 8 and 16 after egg embolization. Whole lung lobes were homogenized in Triton X-100 containing buffer and centrifuged to yield membrane-free supernatants, which were analyzed by ELISA (see *Materials and methods*). Data shown are mean ± SE from one representative of two separate experiments. Each time point had three to five mice *per* group. Significance was determined by using one-way ANOVA and Newman–Keuls post-test (*$p \leq 0.05$).

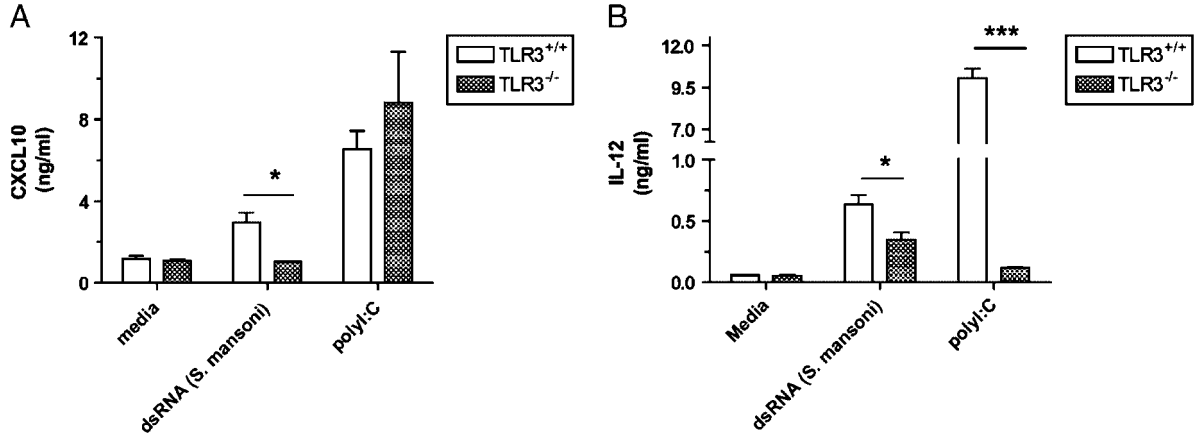

Figure 5. CXCL10 and IL-12 generation by TLR3$^{+/+}$ and TLR3$^{-/-}$ bone marrow macrophages derived from naïve mice following either no activation or exposure to dsRNA or poly I:C for 48 h. Cell-free supernatants were removed and analyzed by ELISA. Data shown are mean ± SE from triplicate cultures. These data are representative of three separate experiments. One-way ANOVA and Newman–Keuls post-test were used to analyze significance (*$p \leq 0.05$ and ***$p \leq 0.001$).

absence of TLR3 impaired the production of *S. mansoni* dsRNA-stimulated IL-12 and CXCL10 and poly I:C-stimulated IL-12 by macrophages.

Additional studies were conducted with bone-marrow-derived macrophages from *S. mansoni*-sensitized TLR3$^{+/+}$ and TLR3$^{-/-}$ mice immediately prior to the egg challenge. As shown in Fig. 6A, SEA stimulation of macrophages isolated from TLR3$^{+/+}$ mice resulted in expression of NOS2 and CCL3, genes associated with classical activation (M1), at higher levels when compared with similarly treated TLR3$^{-/-}$ macrophages. Macrophages isolated from *S. mansoni*-sensitized TLR3$^{-/-}$ mice displayed an M2 phenotype as evidenced by the marked expression of arginase and CCL2 transcripts (Fig. 6B). The addition of SEA to cultures of sensitized macrophages markedly augmented arginase transcript

expression in both TLR3$^{+/+}$ and TLR3$^{-/-}$ macrophages but the increase was greater in TLR3$^{-/-}$ cultures. SEA treatment similarly increased CCL2 transcript expression in both groups of macrophages (Fig. 6B). Analysis of protein levels in cultures of macrophages from both groups of mice revealed that TLR3$^{-/-}$ macrophages constitutively released significantly greater amounts of vascular endothelial growth factor (VEGF) compared with similar numbers of wildtype macrophages (Fig. 6C). VEGF is an important mediator of angiogenesis and is crucial for development of a fibrotic response [22]. SEA-augmented CCL2,

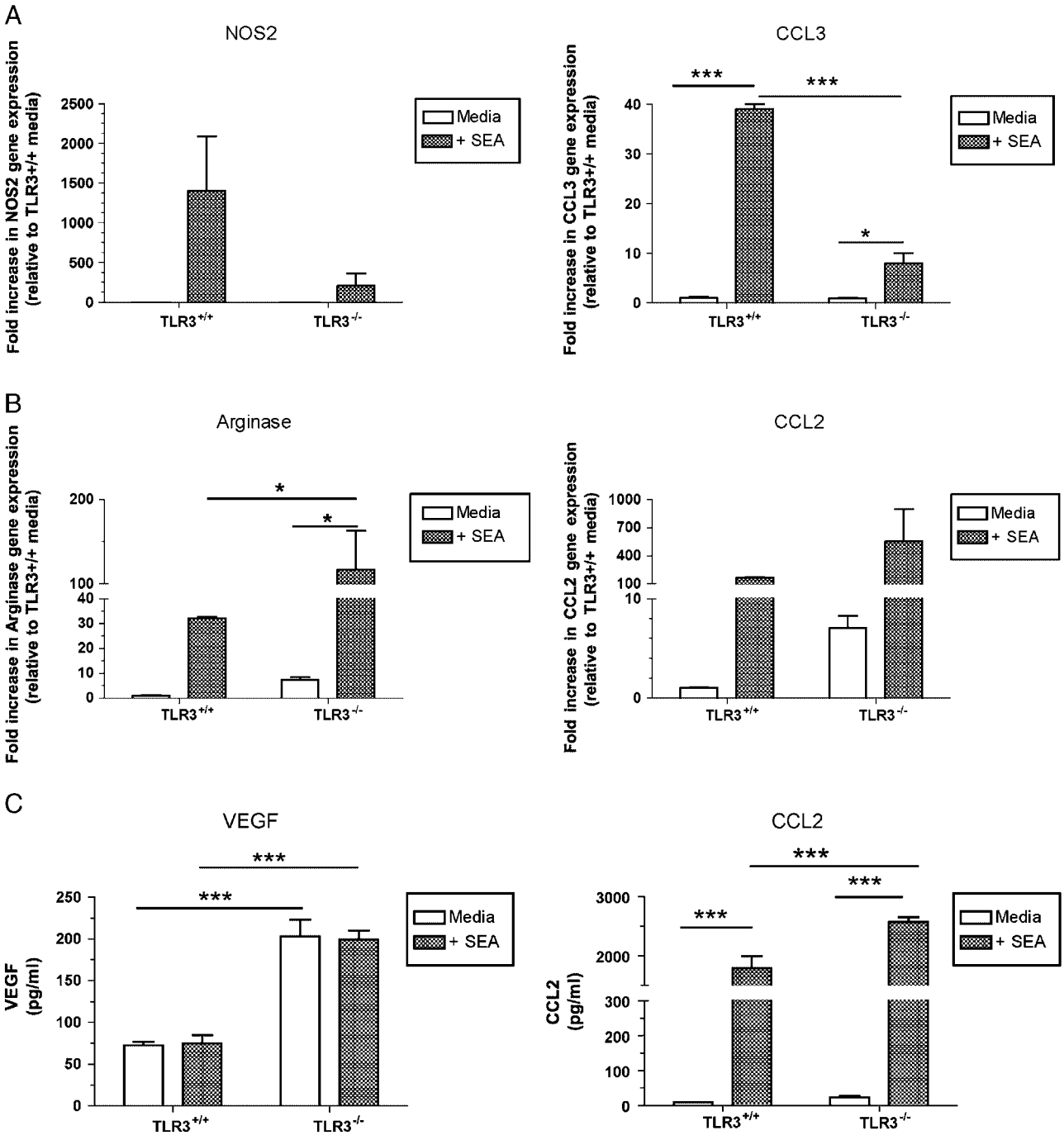

Figure 6. Gene expression analysis of M1 (A) and M2 (B) macrophage marker expression by bone-marrow-derived macrophages from *S. mansoni*-sensitized TLR3$^{+/+}$ and TLR3$^{-/-}$ mice. Bone-marrow-derived macrophages were treated with SEA for 24 h before RNA extraction and analysis. Transcript level of each gene, under each condition, is expressed as fold over transcript levels in macrophages from sensitized TLR3$^{+/+}$ mice, incubated in media alone. ELISA analysis of cell-free supernatants collected after 24 h stimulation of macrophages with SEA or media alone is shown in (C). Data are representative of two independent experiments and expressed as mean ± SE from triplicate wells. One-way ANOVA and Newman–Keuls post-test were used to analyze significance (*$p \leq 0.05$, ***$p \leq 0.001$).

a pro-fibrotic chemokine but not VEGF in TLR3$^{+/+}$ and TLR3$^{-/-}$ macrophages, but the increase in CCL2 production was significantly greater in the knockout cultures compared with the wildtype cultures (Fig. 6C). Similar results were obtained after analyzing bone-marrow-derived macrophages from TLR3$^{+/+}$ and TLR3$^{-/-}$ sensitized mice 8 and 16 days after egg embolization (data not shown). Together, these data suggested that *S. mansoni*-sensitized TLR3$^{-/-}$ mice contained macrophages skewed toward an M2 phenotype, which can promote fibrosis.

Granulomatous TLR3-deficient mice exhibited increased CD4$^+$CD25^{hi} T cells

Lymphocytes exert a prominent role in regulating and determining the cytokine environment within the granulomatous lung. Flow cytometric analysis of T-cell subsets in the lung at day 8 after egg embolization revealed major differences in the numbers of CD4$^+$ T cells in TLR3$^{+/+}$ compared with TLR3$^{-/-}$ mice. Granulomatous lungs from TLR3$^{-/-}$ mice contained significantly greater numbers of CD4$^+$, CD8$^+$, and CD4$^+$CD25^{hi} cells compared with granulomatous lungs from TLR3$^{+/+}$ mice (Fig. 7A, representative dot plot; Fig. 7B) *per* 100 000 cells indicating greater numbers of effector T cells in the gene-deficient mice. No difference in Treg cells as quantified by number of CD4$^+$CD25^{hi} FoxP3$^+$ cells was observed between the two groups of mice at this time point (Fig. 7A). In addition, no difference in numbers between percentage of CD11b$^+$ and CD11c$^+$ cells was observed between TLR3$^{+/+}$and TLR3$^{-/-}$ mice (Table 2). Taken together, these data suggest that the absence of TLR3 enhanced the relative numbers of activated or effector CD4$^+$ T cells in the granulomatous lung.

Dispersed cell cultures from lung and MLN showed increased IL-17

Given that we observed significantly greater numbers of CD4$^+$ CD25^{hi} T cells in granulomatous lungs from TLR3$^{-/-}$ mice at day 8 after egg embolization, we further explored the cytokine phenotype of these cells *via* antigen recall responses *in vitro*. Lung and draining lymph nodes in the vicinity of the lung were isolated from both groups of mice at day 8 after egg embolization and identical numbers of dispersed cells from both compartments were restimulated *in vitro* with SEA. Constitutive and SEA stimulated levels of IL-4 and IFN-γ in dispersed lung cultures were similar between the two groups (data not shown). The presence of SEA in dispersed lung cultures significantly increased levels of IL-17 above those detected in cultures with media alone and the level in TLR3$^{-/-}$ cells was significantly higher than in TLR3$^{+/+}$ cells (Fig. 8A). A similar pattern was observed in dispersed cultures of draining lymph node cells from both groups of mice (Fig. 8B). Again, the addition of SEA significantly increased immunoreactive levels of IL-17 in cultures containing lymph node cells from TLR3$^{-/-}$ mice compared with SEA-activated lymph node cells from TLR3$^{+/+}$ mice. Together, these data indicated that the absence of TLR3 significantly enhanced T-cell responses to SEA, and that increased Th17 activation characterized these responses.

To investigate the cellular source of IL-17, we quantified CD4$^+$ IL-17$^+$ lymphocytes at day 8 after egg embolization in wildtype and knockout mice. As shown in Fig. 8C, TLR3$^{-/-}$ lungs had significantly greater numbers of CD4$^+$ IL-17$^+$ lymphocytes compared with TLR3$^{+/+}$ lungs although the numbers were lower than expected. We examined the presence of CD25$^+$ IL-17$^+$ cells and CD8$^+$ IL-17$^+$ cells in the lungs of both types of mice and found no difference between TLR3$^{-/-}$ and TLR3$^{+/+}$ mice. In addition, the numbers of CD25$^+$ IL-17$^+$ cells and CD8$^+$ IL-17$^+$ cells were lower than those of CD4$^+$ IL-17$^+$ cells. Collectively these data suggest that CD4$^+$ lymphocytes are one of the contributing sources of IL-17 in TLR3$^{-/-}$ granulomatous lung.

Discussion

TLR serve as a critical bridge between the innate and adaptive arms of the host immune response *via* their ability to recognize PAMP [23]. PAMP activation of antigen-presenting cells such as DC and macrophages subsequently facilitates T-cell activation. Depending upon the nature, amount, and location of antigen, CD4$^+$ T cells differentiate into Th1, Th2, or Th17 subsets and mount corresponding immune responses. While TLR are known to provide the link between the innate and adaptive immunity, the relative contribution of each TLR to this process remains poorly described. Herein, we show that TLR3 has a major influence on the level of activation and the phenotype of effector CD4$^+$ T cells. We observed that TLR3 gene and protein expression in the lung decreased in a time-dependent manner following the pulmonary embolization of *S. mansoni* eggs but TLR3 transcript levels increased over time in alveolar macrophages. These contrasting data suggest that a large number of cells that are recruited in the lung during a granulomatous response do not express TLR3, thus decreasing the overall level of expression in this tissue. Nevertheless, key contributors to the granulomatous response, namely alveolar macrophages, exhibited elevated levels of TLR3. The complete absence of TLR3 in the knockout mice had a profound effect on the cytokine and histological phenotype of the egg granulomatous response. Specifically, the cytokine profile in the lungs of TLR3$^{-/-}$ mice with *S. mansoni* eggs was skewed more toward Th2 cytokines and chemokines than their wildtype counterparts. Accordingly, the egg granulomas in the TLR3$^{-/-}$ groups when compared with the TLR3$^{+/+}$ groups were significantly larger, contained significantly greater amounts of collagen, and were surrounded by chitinase-like crystals usually associated with M2 macrophages. A significantly greater percentage of CD4$^+$ CD25^{hi} cells were present in the lungs of *S. mansoni* egg-challenged TLR3$^{-/-}$ mice compared with TLR3$^{+/+}$ mice. Interestingly, antigen activation of T cells from whole lung and draining lymph nodes from TLR3$^{-/-}$ and TLR3$^{+/+}$ mice revealed that T cells from the former group of mice expressed significantly higher amounts of IL-17 compared with the latter group of

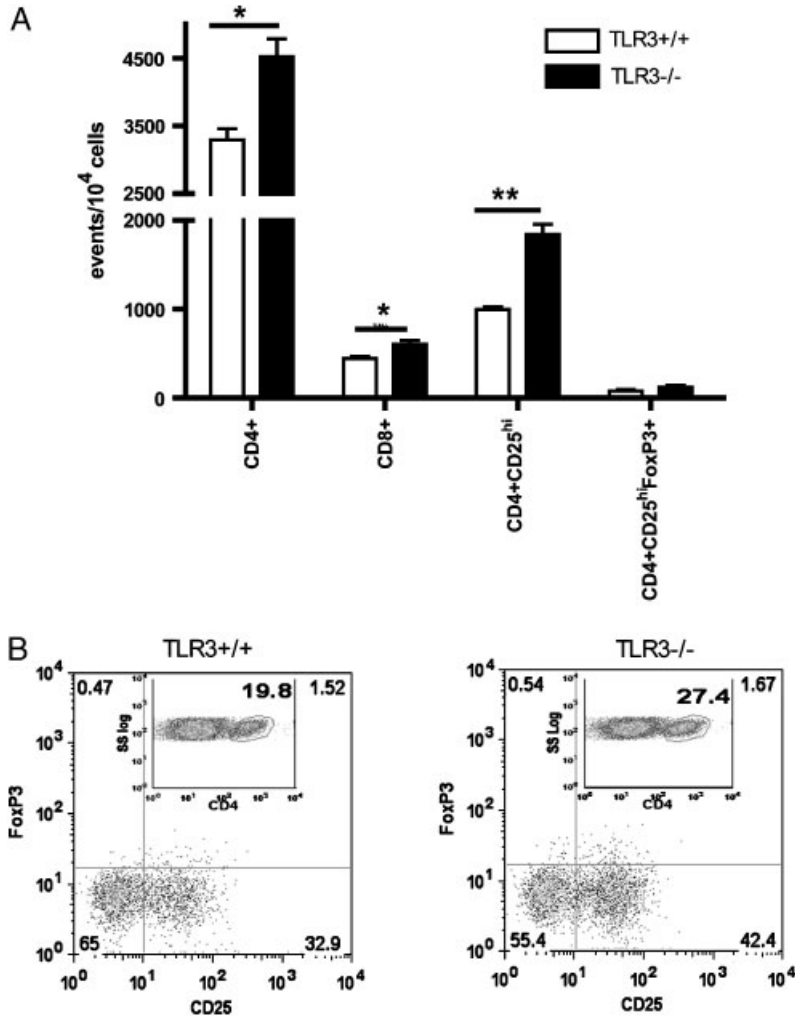

Figure 7. Phenotype of lymphocytes derived from whole lung samples of TLR3$^{+/+}$ and TLR3$^{-/-}$ mice at day 8 after egg embolization in *S. mansoni*-sensitized mice. Lung cells were dispersed and stained with anti-CD4, anti-CD8, anti-CD25, and anti-foxP3 as described in *Materials and methods*. A representative dot plot (B) shows gating of CD4$^+$ cells as an inset. The percentage of CD25$^+$ and foxP3$^+$ cells in the CD4$^+$ gate is shown. The bar graph (A) shows CD4$^+$ and CD8$^+$ cells based on lymphocyte gate. The events shown for CD25$^+$ and CD25$^+$foxP3$^+$ cells are based on CD4$^+$ lymphocyte gate. Data shown are representative of three separate experiments using three to five mice *per* group. Significance was determined by using one-way ANOVA and Newman–Keuls post-test. Significant differences are shown as $^*p \leq 0.05$ and $^{**}p \leq 0.01$

Table 2. CD11b$^+$ and CD11c$^+$ cells in macrophage-dendritic cells gate in TLR3$^{+/+}$ and TLR3$^{-/-}$ mice at day 8 after egg embolization

	TLR3$^{+/+}$	TLR3$^{-/-}$
% CD11b$^+$	86.14±0.57	85.42±0.79
% CD11c$^+$	7.8±0.53	8.22±0.57

mice. Together, these data show that the presence of TLR3 during *S. mansoni* granulomatous responses dampens the immunopathology directed by Th2 and Th17 cells in the lung.

Previous studies have shown that clinical helminth infections are associated with profound alterations in the expression and activation of various TLR on immune cells. Lymphatic filariasis is an example of a disease characterized by a dramatic decrease in the expression of TLR1, 2, 4, and 9 due to the direct actions of filarial antigen on B cell, T cell, and monocytes [24, 25]. Schistosomiasis is also associated with diminished TLR4 expression in circulating mononuclear cells, presumably due to the chronic exposure of the immune system to helminth antigens, such as SEA [26]. SEA directly inhibits the ability of DC to respond to LPS (a TLR4 ligand) and CpG (a TLR9 ligand) [27, 28], and consequently adversely impacts Th1 and Th2 responses [29]. Paradoxically, RNA and protein components from *S. mansoni* directly activate DC *via* TLR3 [16]. The role of TLR3 has been examined previously in the context of *S. mansoni* infection and its absence in gene-deficient mice had no impact on the host resistance to this parasite [30]. However, the absence of TLR3 had a major impact on the Th1/Th2 balance in *S. mansoni*-infected mice, and the presence of this TLR was responsible for the reduced Th2 response induced by SEA-stimulated DC.

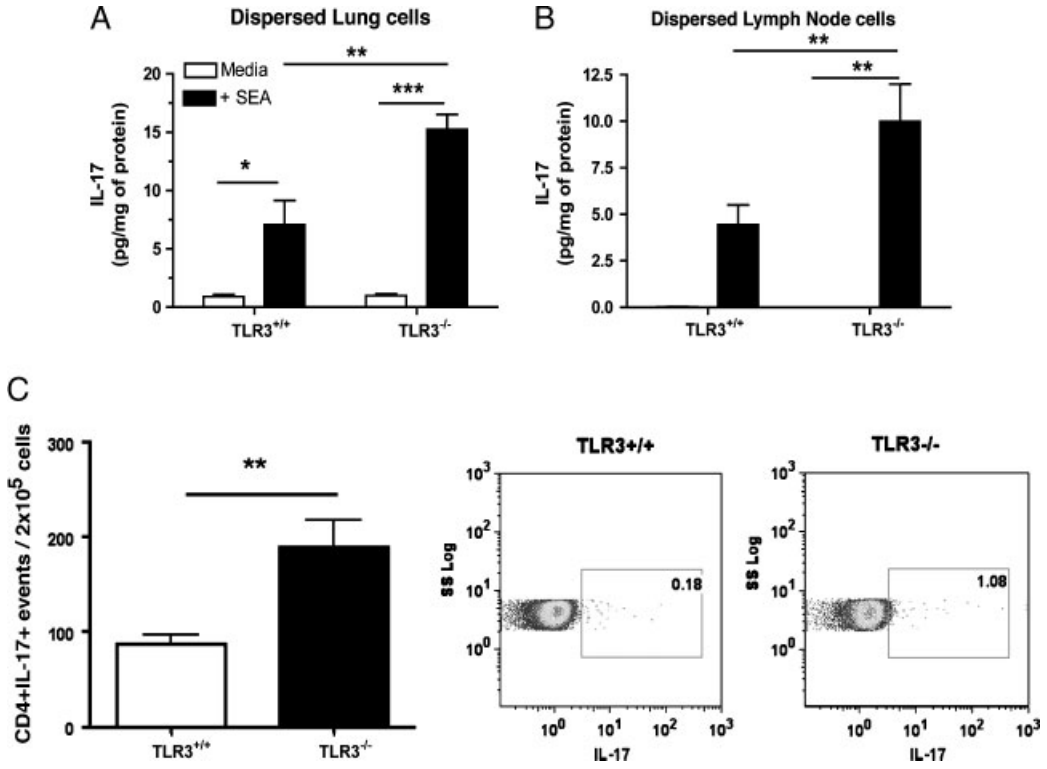

Figure 8. IL-17 levels in *ex vivo* cultures from lungs (A) and draining lymph node (B) cells from five to six *S. mansoni*-sensitized mice *per* group at day 8 after egg embolization. Cells were cultured with media alone or with SEA antigen for 48 h prior to Bioplex analysis for IL-17. Data shown are mean ± SEM from triplicate wells. One-way ANOVA and Newman–Keuls post-test were used to analyze significance. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ compared with media-alone conditions. Flow cytometric analysis of dispersed lung cells showing elevated levels of Th17 cells is shown in (C). The top panel indicates the number of IL-17$^+$cells in CD4$^+$ lymphocyte gate. Significance was determined using Student's *t*-test. Significant differences are shown as **$p \leq 0.01$. Representative dot plot is shown in the bottom panel of (C). Boxed region shows percentage of IL-17$^+$cells in CD4$^+$ lymphocyte gate.

These findings are entirely consistent with those of the present study in which the absence of TLR3 in gene-deficient mice correlated with significantly increased Th2 cytokines and chemokines. Also, the histopathological consequences on increased Th2 immunity in TLR3$^{-/-}$ mice were also apparent as the granulomas in these mice were significantly larger and contained significantly greater amounts of collagen compared with the TLR3$^{+/+}$ groups at days 8 and 16 after egg embolization. The manner in which TLR3 expression dampens the Th2 response is not readily apparent but we observed that Th1-associated factors such as IL-12, CXCL9, and CXCL10 were significantly lower in the knockout groups compared with the wildtype groups, suggesting that the absence of signaling through TLR3 affected the generation of these cytokines and chemokines. Of interest were results obtained from *in vitro* stimulation of bone-marrow-derived macrophages with dsRNA isolated from *S. mansoni* eggs and synthetic TLR3 ligand, poly I:C. Our data suggest that release of CXCL10 and IL-12 after stimulation of macrophages with *Schistosoma* RNA is TLR3 dependent, but release of IL-12 and not CXCL10 after stimulation with poly I:C is TLR3 dependent. This difference in dependence of CXCL10 on TLR3 signaling could be due to distinct secondary structures of the two types of RNA resulting in diverse signaling cascade.

Further evidence of a strongly skewed Th2 response in *S. mansoni* egg-challenged TLR3$^{-/-}$ mice was the presence of alternatively activated or M2 macrophages. Evidence for the polarization of these cells was provided by the presence of chitinase-like crystals, which are usually associated with M2 macrophages in histological tissue sections from these mice at days 8 and 16 after egg embolization. Further, examination of the phenotype of bone-marrow-derived macrophages from TLR3$^{+/+}$ and TLR3$^{-/-}$ mice confirmed that cells from the latter group of mice were more prone to express M2 markers (*i.e.* arginase and CCL2) rather than M1 markers (*i.e.* NOS2 and CCL3), either constitutively or after exposure to SEA for 24 h. Little is presently known about the manner in which TLR3 regulates the phenotype of macrophages but one study has shown that helminth antigens potently skew macrophages to the M2 phenotype through a TLR-dependent mechanism [31].

TLR3 activation leads to activation of macrophages and the maturation of DC resulting in a polarized Th1 immune response. During *in vivo* infection, *Schistosoma* eggs trapped in the liver cause an initial Th1 response followed by an overt Th2 response characterized by exuberant tissue fibrosis. In the absence of TLR3, the recognition of dsRNA from *Schistosoma* egg or the recognition of endogenous TLR3 ligands (*i.e.* by-products of cell necrosis) by

antigen-presenting cells would be impaired. Our data suggest that the robust Th2 environment observed in TLR3 gene-deficient mice leads to efficient sequestration of the egg, which can be beneficial to the host. On the other hand, excessive fibrotic responses such as greater collagen deposition around the granuloma can be detrimental to the host, suggesting that alterations in TLR3 signaling might be a double-edged sword.

Another surprising feature of the immune response in *S. mansoni* egg-challenged TLR3$^{-/-}$ mice was the presence of significantly elevated levels of IL-17. We observed increased IL-17 in spleen-cell preparations cultured in the absence or presence of SEA. Further, SEA activation of isolated cells from lung and lung-draining lymph node revealed significantly higher levels of this cytokine in the TLR3$^{-/-}$ groups compared with the TLR3$^{+/+}$ groups. CD4^{+} T cells can differentiate into at least four different subsets depending upon the interaction with an antigen-presenting cell and the cytokine milieu [32]. Th1 cells direct the killing of intracellular pathogens, produce IFN-γ, and proliferate in the presence of IL-12. Th2 cells respond to extracellular bacteria and parasites, participate in wound healing, and produce IL-4, IL-5, and IL-13. Treg arise in the presence of TGF-β and IL-10 and down-regulate proliferation of other T cells. Finally, T cells differentiate into the Th17 class in the presence of TGF-β, IL-6, and IL-23 and function in various innate immune responses. IL-17 is a potent inflammatory cytokine produced by Th17 cells, and this cytokine appears to contribute to immunopathology associated with autoimmunity models such as collagen-induced arthritis and encephalomyelitis. Cytokines cells, such as IL-21 and IL-23, involved in differentiation of Th17 cells have been shown to play an important role in schistosomiasis [10, 33, 34]. In this report we showed significantly greater number of Th17 cells in TLR3$^{-/-}$ lungs possibly contributing to the severity of pathology of the disease. Our data also showed that an effector type T cell (CD4^{+}CD25^{hi}) was present in greater abundance in the lungs of TLR3$^{-/-}$ mice compared with TLR3$^{+/+}$ mice, supporting the observation that both Th2 and Th17 responses were increased in the knockout group. Of interest was our finding that although Th17 cells were increased in TLR3$^{-/-}$ mice, the relative percentages of Treg did not differ between the two groups of mice. This is of note because a dichotomy between Treg and Th17 activation exists such that presence of IL-6 inhibits generation of Treg but enhances generation of Th17 [32, 35]. How IL-17 contributes to the immunopathology associated with TLR3-deficient mice is currently under investigation in our laboratory.

In conclusion, TLR3 is a critical regulator of pulmonary granulomatous disease orchestrating both innate and adaptive host responses. The expression of TLR3 in the lung is negatively regulated during the course of *S. mansoni*-induced egg granuloma formation. The presence of TLR3 during this response is required for the regulation of both Th2- and Th17-type responses, raising the possibility that enhancing the expression and/or activity of this TLR might provide effective regulation of deleterious immune responses. Further studies are required in order to elucidate the mechanism responsible for the regulatory effect of TLR3 activation on Th17 immune responses.

Materials and methods

Mice

Six to 8-wk-old female C57BL6 mice were purchased from Taconic (Hudson, NY, USA). Dr. R. A. Flavell (Yale University School of Medicine, New Haven, CT, USA) provided a breeding pair of TLR3$^{-/-}$ mice, and this pair was housed for breeding purposes in the University Laboratory Animal Medicine facility at the University of Michigan Medical School under specific pathogen free conditions. The animal care committee at the University of Michigan Medical School approved of all experiments conducted in this study.

Th2-driven lung granuloma model

Swiss-Webster mice infected with *S. mansoni* were obtained from Dr. Fred Lewis (Biomedical Research Laboratory, Rockville, MD, USA). Eggs from infected livers were purified as described in detail previously [36]. To induce a synchronous pulmonary granulomatous response, age and sex-matched wildtype and TLR3$^{-/-}$ mice were sensitized by an intraperitoneal injection of 3000 live *S. mansoni* eggs. Fourteen days later the mice were challenged with 3000 eggs *via* an i.v. injection [37]. BALF, whole lungs, mediastinal lymph nodes, spleen, and bone marrow cells were collected prior to the egg challenge (*i.e.* day 0) and at days 8 and 16 after the i.v. egg challenge.

Preparation of *S. mansoni* egg antigen (SEA) and purified dsRNA from *S. mansoni* eggs

Grinding *S. mansoni* eggs on ice for 40 min and centrifuging the ground material at 60 000 rpm for 40 min extracted SEA. The supernatant was analyzed for protein content by Bradford assay, and the SEA was subsequently used for *in vitro* stimulation experiments. For isolation of RNA from *S. mansoni* eggs, 1×10^4 eggs were resuspended in 3 mL of TRIzol (Invitrogen Life Technologies; Carlsbad, CA, USA). Subsequently, each sample was homogenized for 1 min. Next, dsRNA was extracted according to the manufacturer's instructions and treated with DNAase I (Roche Diagnostics GmbH, Mannheim, Germany) before adding to macrophage cultures. The presence of dsRNA was confirmed using RNAase A (New England Biolabs, Ipswich, MA, USA) in low and high salt solutions (New England Biolabs) as described in detail previously [16].

Microscopic analysis of whole lung tissue sections

Prior to and at days 8 and 16 after an i.v. challenge of *S. mansoni* eggs, whole left lung lobes were excised, inflated by injecting 10% formalin, and fixed overnight before embedding in paraffin.

Routine histological staining of 5 μm tissue sections was performed and at least 20 granulomas *per* tissue section were analyzed for granuloma area using light microscopy and IP Lab Spectrum software. Morphometric analysis of collagen present in Masson's Trichrome-stained lung sections was also carried out using light microscopy and the same software as described in detail previously [38].

Western blot analysis

Lungs were dispersed in HBSS containing Triton X-100 and centrifuged to yield cell membranes. Proteins from cell membrane fractions were extracted using a method described by Slomianny *et al.* [39]. Briefly, membrane fractions were resuspended in a buffer containing 20 mmol/L HEPES pH 7, 160 mmol/L KCl, and complete MINI protease inhibitor. Next, solubilization buffer containing 1% Triton X-100 in 20 mmol/L Tris-HCl, pH 8.8, was added and the samples were incubated at 4°C overnight. The following day, samples were centrifuged at $10\,000 \times g$ for 30 min; 25 μg of supernatant was loaded onto 4–20% acrylamide gel (Pierce Biotechnology, Rockford, IL, USA) and subsequently transferred to nitrocellulose membrane. A primary antibody against TLR3 was purchased from Imgenex (San Diego, CA, USA), a primary antibody against GAPDH was purchased from Abcam (Cambridge, MA, USA) and a secondary antibody conjugated to horseradish peroxidase was purchased from Cell Signaling (Danvers, MA, USA). Bands were visualized using an enhanced chemi-luminescence detection kit (Pierce Biotechnology). The level of TLR3 expression was determined by normalization to respective GAPDH band intensity using Molecular Imager FX system and Quantity One software (Bio-Rad, Hercules, CA, USA).

Gene and protein expression profile in whole lung samples

Whole right lung lobes were excised from *S. mansoni*-sensitized mice prior to and at days 8 and 16 after egg embolization, snap-frozen in liquid nitrogen, and then stored at −20°C until further use. One right lung lobe was resuspended in 1 mL TRIzol and homogenized using a tissue homogenizer. RNA was extracted, reverse transcribed to cDNA, and analyzed using real-time TAQMAN analysis for expression of TLR3. TLR3 expression is expressed as fold increase or decrease in transcript levels prior to and after 8 and 16 days of egg embolization compared with expression in naïve lungs. The remaining right lung lobes were resuspended in 1 mL of HBSS containing 0.1% TritonX-100 and complete MINI protease inhibitor (F. Hoffmann-La Roche, Basel, Switzerland). These samples were homogenized and centrifuged to obtain cell-free supernatants. Fifty microliters of this homogenate was loaded into ELISA plates to determine protein content. Standardized sandwich ELISA technique was used to detect IL-4, CCL11, CCL3, MIP-2, CXCL9, and CXCL10 as described in detail [40]. Limit of detection for all proteins was approximately 10–50 pg/mL.

In vitro restimulation of lung, lymph node, and spleen cells

Left and right lung lobes were excised from *S. mansoni*-sensitized mice prior to and at day 8 after *S. mansoni* egg-challenge, minced, and digested in 0.22% collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 45 min at 37°C. Digested tissue was then passed through a 20-ga needle to aid in the release of cells. Spleens were cut into small sections and then mechanically dissociated by filtering through a 40 μM nylon cell strainer. Cell suspensions from lymph nodes were generated in a similar manner. Erythrocytes in lung, lymph node, and spleen cell suspensions were lysed in a hypotonic solution containing NH_4Cl; $2–6 \times 10^6$ cell/mL of the remaining cells were cultured in the presence of 22.5 μg/mL SEA in RPMI supplemented with 10% FBS for 24 or 48 h. Cell-free supernatants were stored at −20°C prior to analysis using a multiplex bead immunoassay from Invitrogen Life Technologies. Spleen cells were resuspended in TRIzol and RNA was extracted for TLR3 expression analysis by real-time TaqMan PCR. Data are expressed as fold change over dispersed cells obtained from spleens prior to embolization and incubated in medium alone.

Flow cytometric analysis of dispersed lung cells

Erythrocyte-free lung cell suspensions obtained as described above were incubated with anti-CD16/32 (eBioscience, San Diego, CA, USA) to block Fc receptors. These cell suspensions were stained with either FITC-conjugated anti-CD8 or with FITC-conjugated anti-CD25, PECy5-conjugated anti-foxP3 and PECy7-conjugated anti-CD4 (all conjugated antibodies were bought from BD Biosciences, San Jose, CA, USA). IL-17^+ cells were detected using PE-conjugated anti-IL-17 and BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD Biosciences). Cells were analyzed using a Beckman Coulter FC500 and FlowJo software (Tree Star, Ashland, OR, USA).

Alveolar and bone-marrow-derived macrophage culture

One milliliter of broncho-alveolar lavage buffer (HBSS containing 5 mM EDTA) was injected intratracheally into mice and the lavage fluid was aspirated. The lavage fluid was then centrifuged and the resulting cell pellet was washed with RPMI 1640. Cells were allowed to adhere to 24-well plates overnight in RPMI 1640 containing 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (RPMI-FBS). TRIzol was added to wells to isolate RNA. Bone marrow from femurs and tibias of mice was flushed with cold RPMI and these cells were grown in the presence of

L-cell conditioned media to yield macrophages as described before [41, 42]. The purity of culture was tested by flow cytometry. Cells cultured in this way were >98% $CD11b^+$ and >95% $F4/80^+$. On day 7, cells were replated in triplicate wells of a 24-well plate at a density of 2.5×10^5 cells *per* well in RPMI-FBS for *in vitro* SEA or *S. mansoni* egg RNA stimulation. Twenty-four hours later, cell-free supernatants were collected for ELISA analysis and TRIzol was added to cells to isolate RNA. RNA was converted into cDNA using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen Life Technologies) and then used for real-time quantitative TAQMAN PCR analysis using an ABIPRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). TAQMAN reagents for CCL2, CCL3, Arginase, and GAPDH were bought from Applied Biosystems. SYBR green PCR mix (Applied Biosystems) was used to amplify TLR3 and NOS-2. Primers for TLR3 were CCCAGCTC-GATCTTTCCTACA and AGGCTTGGGAGATAGGAGAAG, and for NOS-2 they were CGCAGCTGGGCTGTACCAA and TGAT GTTTGCTTCGGACATCA. GAPDH was used as internal control for samples.

Statistical analysis

Three to six mice were used *per* group *per* time point in each experiment. Student's *t*-test and one-way ANOVA were used to analyze significance. *p* values greater than 0.05 were considered significant.

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Abbreviations: **SEA:** schistosoma egg antigen · **VEGF:** vascular endothelial growth factor

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