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Helminth Antigens Modulate Immune Responses in Cells from Multiple Sclerosis Patients through TLR2-Dependent Mechanisms

Jorge Correale and Mauricio Farez

To better understand the link between parasite infections and the course of multiple sclerosis (MS), we studied the role of TLRs in helminth product recognition by dendritic cells (DCs) and B cells. Baseline expression of TLR2 was significantly higher in infected-MS patients compared with uninfected MS subjects or healthy controls. Moreover, cells exposed to TLR2 agonists or to soluble egg Ag (SEA) from Schistosoma mansoni resulted in significant TLR2 up-regulation. SEA suppressed the LPS-induced DCs production of IL-1β, IL-6, IL-12, and TNF-α and enhanced TGF-β as well as IL-10 production. Similarly, after exposure to SEA, anti-CD40-activated B cells increased IL-10 production. Both processes were MyD88 dependent. In addition, SEA down-regulated the expression of LPS-induced costimulatory molecules on DCs in a MyD88-independent manner. DCs stimulation by SEA and TLR2 agonists induced increasing phosphorylation of the MAPK ERK1/2. Neither stimulus showed an effect on p38 and JNK1/2 phosphorylation, however. Addition of the ERK1/2 inhibitor U0126 was associated with dose-dependent inhibition of IL-10 and reciprocal enhancement of IL-12. Finally, cytokine effects and changes observed in DCs costimulatory molecule expression after SEA exposure were lost when TLR2 expression was silenced. Overall, these findings indicate that helminth molecules exert potent regulatory effects on both DCs and B cells through TLR2 regulation conducted via different signaling pathways. This knowledge could prove critical in developing novel therapeutic approaches for the treatment of autoimmune diseases such as MS. The Journal of Immunology, 2009, 183: 5999–6012.

MUltiple sclerosis (MS) is an inflammatory demyelinating disease affecting the CNS. Autoimmunity plays a major role in both the susceptibility as well as the development of the disease. It is generally accepted that both MS and experimental autoimmune encephalomyelitis (EAE), an animal model resembling MS, involve Th1 and Th17 cells recognizing certain myelin sheath components (1). Autoimmune diseases such as MS are currently considered to be caused by the combination of genetic susceptibility and environmental factors (2–4). The genetic component of MS is thought to result from the action of common allelic variants in several genes. However, discordance of MS among monzygotic twins suggests that additional factors, such as environmental modulators, could also be involved (5).

In recent decades, epidemiological evidence has accumulated indicating a steady increase in autoimmune disease incidence in developed countries (6–8). Both improvement in sanitary conditions in general and reduction in infectious disease prevalence in particular have been proposed as the cause (9). Following this premise, we recently demonstrated that helminth-infected MS patients showed significantly lower number of relapses, reduced disability-evaluating test scores, and significantly lower magnetic resonance imaging activity compared with uninfected MS subjects. Parasite-driven protection was associated with the induction of regulatory T cells secreting suppressive cytokines such as IL-10 and TGF-β, as well as with the presence of CD4+CD25+FoxP3+ T cells displaying important suppressive function (10). Furthermore, helminth infections in MS patients also induced regulatory B cells capable of dampening the immune response through the production of IL-10 (11). These findings provide evidence to support autoimmune down-regulation secondary to parasite infection in MS patients through the action of regulatory cells, the effects of which extend beyond the classical response to a pathogen.

TLRs are type I transmembrane glycoproteins composed of extracellular transmembrane and intracellular signaling domains (12). They are expressed in different combinations on many cells of the immune system, at the cell surface and along endosomal cell membranes. Extracellular TLR domains have reiterated leucine-rich repeat modules bearing pathogen-associated molecular patterns able to recognize a wide range of microbial products, thus providing a link between innate and adaptive immunity (13–14). TLR-dependent proinflammatory cascades triggered by microbial agents must be tightly regulated to avoid severe pathology. Once activated by microbial pathogen-associated molecular patterns, TLRs trigger the recruitment of adaptor proteins to intracellular TIR (Toll/IL-1R) domains and initiate signaling (15). MyD88 is the universal adaptor, used by all TLRs except TLR3, and acts to recruit the IRAK (IL-1R-associated kinase) family of kinases.

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Abbreviations used in this paper: MS, multiple sclerosis; DC, dendritic cell; MBP, myelin basic protein; MFI, mean fluorescence intensity; MOG, myelin oligodendrocyte glycoprotein; PPD, purified protein derivative; rh, recombinant human; SEA, soluble egg Ag; siRNA, small interfering RNA.

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These kinases ultimately trigger NF-κB as well as MAPK, p38, ERK, and JNK activation (16).

In the present study we evaluate the role of TLRs in the recognition of helminth products by dendritic cells (DCs) and B cells in parasite-infected MS patients. We examine costimulatory molecule expression as well as IL-10, IL-12, and TNF-α production by DCs and B cells stimulated with the TLR2 ligand Pam3Cys or exposed to a soluble egg Ag (SEA) obtained from *Schistosoma mansoni*. In addition, effects of both stimuli on MAPK activity were also evaluated.

### Materials and Methods

#### Patients

Twelve patients (eight females and four males) with clinical diagnosis of MS according to Poser’s criteria and presenting eosinophilia were assessed in a prospective double-cohort study as previously reported elsewhere (10). Intestinal parasites were identified as the cause of eosinophilia and were present in stool samples from all patients. Mean patient age was 34 ± 0.8 years, the Kurtzke Expanded Disability Status Scale score was 2.8 ± 0.6, and mean disease duration was 7.3 ± 1.0 years. The presence of other endemic parasite infections including trypanosomiasis, leishmaniasis, amebiasis, giardiasis, and toxoplasmosis was ruled out in the study population by both microscopic stool exam and serum testing. MS diagnosis preceded parasite gut infection by 31.7 ± 5.6 mo (range 24–41 mo). Twelve healthy subjects and 12 uninfected MS patients in remission matched for age, sex, and disease duration served as controls. Thorough clinical and neurological examinations as well as standard chemical and hematological laboratory examinations failed to disclose other underlying disorders. None of the MS patients were under immunomodulatory or immunosuppressive therapy had been indicated. The study was approved by the Institutional Ethics Committee of the Dr. Raúl Carrea Institute for Neurological Research. Buenos Aires, Argentina.

Venous blood was drawn after obtaining informed consent from all subjects.

#### Ag and TLR ligand preparation

Myelin basic protein (MBP)3-102, MBP141-168, and myelin oligodendrocyte glycoprotein (MOG)3-8 peptides were synthesized using an automated peptide synthesizer, and purity was assessed through HPLC analysis.

B cells and DCs activation was induced using the following TLR ligands: 5 μg/ml Pam3Cys (EMC Microcollections); 100 ng/ml LPS (Escherichia coli serotype 0111:B4; Sigma-Aldrich); 100 ng/ml flagellin from *Escherichia coli* serotype 0111:B4; Sigma-Aldrich); 100 ng/ml GM-CSF (Gemini), 100 μg/ml streptomycin, and 150 μg/ml penicillin (Gemini) containing 10% FCS (Gemini Bioproducts), 100 U/ml penicillin (Gemini), 100 μg/ml streptomycin (Gemini), 100 ng/ml GM-CSF (R&D Systems), and 50 ng/ml recombinant human (rh)IL-4 (R&D Systems). On days 2 and 4, half of the culture medium was replaced, maintaining the same IL-4 and GM-CSF concentrations. After 5 days of culture, 2 μg/ml CD40L (Amgen) was added to stimulate DCs maturation. Cells were collected on day 7, extensively washed in PBS to remove exogenously added cytokines or residual CD40 ligand, and used in the experiments. DCs contamination with CD3+ T lymphocytes was analyzed using flow cytometry and found to be <0.2%.

#### Flow cytometry analysis

TLR and costimulatory molecule expression on DCs and B cells was evaluated using flow cytometry. Cells were washed in PBS with 0.1% BSA buffer and stained using PE-labeled anti-human TLR2, TLR4, TLR9, CD86, and CD54, as well as FITC-labeled CD40 and CD80 Abs (all from eBioscience). Anti-human-PE-labeled TLR5 Ab was purchased from Abcam. Isotype-matched murine mAbs were used as negative controls (DakoCytomation). For TLR9 staining, cells were washed in staining buffer and then fixed with 4% paraformaldehyde and kept in the dark for 20 min. Cells were washed twice, resuspended in FACS permeabilizing solution (BD Biosciences), and incubated for 30 min using PE-labeled anti-human TLR9 mAb. For phosphorylated c-Fos and c-Myc expression analysis, DCs were collected on day 7, stimulated with different stimuli, fixed, and permeabilized as described above. Cells were washed twice in staining buffer, labeled with a 1/50 dilution of phosphorylated c-Fos and phosphorylated c-Myc mAbs (Abcam) for 30 min, and then washed in staining buffer and labeled using FITC-conjugated anti-rabbit or antimouse Ig (BD Biosciences). All samples were acquired on FACScan using CellQuest software (BD Biosciences). Results were expressed as mean fluorescence intensity (MFI) of positively stained cells.

#### Cytokine assay

IL-4, IL-5, IL-13, IL-17, and IFN-γ production by MBP peptide- and MOG peptide-reactive T cell lines were evaluated by ELISPOT assays using commercially available kits (R&D Systems) and following the manufacturer’s instructions. The specific cytokine-secreting cell number was calculated by subtracting the numbers of spots obtained in unaggregated background control cultures from the number of spots obtained in cultures exposed to the cognate Ag. Results given express number of spots per 106 PBMC.

To measure activated B cells and LPS- or SEA-stimulated DCs cytokine production, cell culture supernatants were collected between 48 and 72 h poststimulation, and TNF-α, TGF-β, IL-1β, IL-6, IL-10, IL-27 (all from R&D Systems), and IL-12p70 (eBioscience) assays were performed using commercially available ELISA kits following the manufacturer’s instructions.

#### Real-time PCR quantification

For quantitative assessment of relative mRNA levels, total RNA was prepared using TRIzol LS reagent (Invitrogen) following the manufacturer’s instructions. RNA was reverse transcribed using a Moloney murine leukemia virus real-time reverse transcription kit with random hexamer primers (Invitrogen). The relative levels of the IL-27p28 subunit were determined using real-time PCR on an ABI 7000 sequence detection system (Applied Biosystems). The values obtained were normalized to the human glyceraldehyde 3-phosphate dehydrogenase reference gene. The relative expression levels were calculated by the ΔΔCt method. All samples were run in duplicate with negative controls.

**Small interfering RNA (siRNA) technique**

In some experiments, MyD88 and TLR2 expression were silenced using siRNA. B cells and DCs were transfected with 25 nM siRNA for 48 h.
or TLR2 or with scrambled nonsilencing control oligonucleotides using TransIT-TKO siRNA transfection reagent (Mirus Bio) according to the manufacturer’s instructions. The sequence of sense strand siRNA used to silence the MyD88 gene was AAGGAGAUGGGCUUCGGAGUAC d(T)d(T), and for TLR2 the sequence was AAGCAGGGCCGACAAACCUUUdGdTd(T). Three days posttransfection, no MyD88 or TLR2 was found expressed on DCs or B cells as estimated by flow cytometry and immunoblotting analysis. The procedure did not affect the number of viable cells monitored by a trypsin blue exclusion test.

Evaluation of MAPK signaling

p38, ERK1/2, and JNK1/2 activities were evaluated using commercially available ELISA kits (Invitrogen). Briefly, DCs (1.5–2 × 10^6) were collected on day 7 and cultured for pre-established periods of time with different stimuli. ELISA tests were performed following the manufacturer’s (Invitrogen) instructions. For inhibition studies, DCs were incubated with the specific ERK1/2 U0126 inhibitor (Calbiochem) for 1 h before stimulation.

Statistical analysis

Differences observed between groups were evaluated using the Mann-Whitney U test. Values of p < 0.05 were considered statistically significant.

Results

Increased expression of TLR2 in DCs and B cells from helminth-infected MS patients

To determine whether helminth infections had any effect on TLR expression, DCs and B cells baseline expression of TLR2, TLR4, TLR5, and TLR9 from helminth-infected MS patients was compared with that of uninfected MS patients and healthy controls using flow cytometry. Surface expression of TLR2 on both B cells and DCs was significantly higher in helminth-infected MS patients (p < 0.001), compared with uninfected MS subjects and controls (Fig. 1, A–D). In contrast, MFI of TLR4, TLR5, and TLR9 did not differ between groups. Of interest, TLR2 was expressed at similar levels on DCs isolated from peripheral blood and monocyte-derived DCs (Fig. 1E), indicating that TLR2 expression on cultured DCs is not the result of in vitro culture manipulation.

To determine whether altered TLR2 expression in DCs and B cells of helminth-infected MS patients translated to functional changes in response to TLR ligands, we stimulated MBP peptide-specific T cell lines from helminth-infected MS patients, uninfected MS subjects, and healthy controls with the TLR2 ligand Pamp3Cys and examined T cell proliferation as well as IFN-γ and IL-10 secretion as readouts of T cell activation. TLR2 stimulation induced a significant inhibition of T cell proliferation and IFN-γ secretion in T cell lines from helminth-infected MS subjects compared with uninfected MS patients and healthy controls (Fig. 2, A and B). In addition, Pamp3Cys stimulation significantly increased the IL-10 secretion by T cell lines from helminth-infected MS patients compared with uninfected MS patients and healthy controls (Fig. 2C). The TLR9 ligand CpG 1668 was used as a control did not induce changes in T cell proliferation or cytokine secretion. These observations suggest that the functional responses measured correlated with TLR2 expression patterns in helminth-infected MS patients, uninfected MS individuals, and healthy controls.

SEA up-regulates TLR2 expression on DCs and B cells from helminth-infected MS patients

Having shown previously that both DCs and B cells from MS helminth-infected patients exhibit increased TLR2 expression, we set out to assess whether SEA had a direct effect on TLR expression. Thus, monocyte-derived DCs and B cells were cultured for 48 h in the presence or absence of SEA, or purified protein derivative (PPD) used as control, and TLR expression was evaluated using flow cytometry. As illustrated in Fig. 3, both DCs and B cells expressed measurable levels of TLR2, TLR4, TLR5, and TLR9. Exposure of both cell populations to SEA resulted in significant up-regulation of TLR2 compared with cells not exposed to SEA (p < 0.001; Fig. 3, A, B, G, and H). In contrast, no significant changes in TLR2 expression were observed after exposure to PPD (Fig. 3, B, C, H, and D). Exposure to SEA did not affect TLR4, TLR5, or TLR9 expression on B cells (Fig. 3, D–F) or DCs (Fig. 3, J–L).

TLR2-stimulated DCs and B cells suppress T cell activation

To assess the role of TLRs on T cell activation during helminth-infections, DCs and B cells from helminth-infected MS patients were stimulated for 24 h with specific TLR2, TLR4, TLR5, and TLR9 agonists and then cultured with MBP peptide- or MOG peptide-specific T cell lines stimulated with specific Ags. Proliferation was measured after 48 h by [3H]thymidine incorporation and by IL-4, IL-5, IL-13, IL-17, and IFN-γ secretion using ELISPOT assay. Both TLR2-activated DCs (Fig. 4, A–C) and B cells (Fig. 4, F–H) significantly inhibited CD4^+ T cells proliferation as well as IFN-γ and IL-17 production (p < 0.001). In contrast, TLR4-, TLR5-, and TLR9-activated DCs as well as B cells failed to inhibit T cell proliferation or the production of IFN-γ and IL-17. However, both TLR2-activated DCs (Fig. 4, D and E), and B cells (Fig. 4, I and J) did induce a significant increase in the number of IL-5- and IL-13-secreting cells. In contrast, TLR4, TLR5, and TLR9 ligands did not induce IL-5 or IL-13 production. Notably, IL-4-secreting cells could not be detected in any of the cultures, even after restimulation of T cell lines with bound anti-CD3 mAb or PMA plus ionomycin.

SEA modulates cytokine secretion by DCs and B cells in a MyD88-dependent manner

We next examined whether SEA could modulate cytokine secretion by DCs or B cells. As illustrated in Fig. 5, A and B, SEA significantly suppressed LPS-induced IL-12 and TNF-α production by DCs. Likewise, SEA was also able to suppress IL-12 and TNF-α production by DCs pulsed with TLR9 agonist CpG DNA (data not shown). In contrast, SEA-exposed DCs significantly enhanced secretion of IL-10 (Fig. 5C). Likewise, anti-CD40-activated B cells significantly increased production of IL-10 after exposure to SEA (Fig. 5D). Inhibition of IL-12 and TNF-α production was not caused by SEA toxicity, because production of IL-10 by DCs was enhanced; also, cellular death was excluded by trypsin blue exclusion test. Interestingly, the addition of anti-IL-10 blocking mAbs to culture medium abrogated SEA effects on IL-12 and TNF-α secretion only partially, indicating that other factors must also be necessary.

Several studies have indicated a key role for TGF-β, IL-6, and IL-1β in Th17 induction. In contrast, IL-27 was recently shown to inhibit Th17 differentiation (19–22). Thus, we sought to investigate whether SEA-mediated inhibition of IL-17, as depicted in Fig. 4, was a consequence of this cytokine’s modulation by SEA. As illustrated in Fig. 5, E and F, SEA significantly suppressed LPS-induced IL-6 and IL-1β production by DCs. In contrast, SEA-exposed DCs significantly enhanced secretion of TGF-β (Fig. 5G). These observations support the notion that impaired production of IL-6 and IL-1β and enhanced secretion of TGF-β by DCs exposed to SEA could directly contribute to IL-17 production inhibition. Notably, stimulation of DCs with SEA did not result in either significant differences in IL-27 mRNA expression levels in DCs or in IL-27 secretion in culture supernatants when compared with

The Journal of Immunology

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unstimulated DCs (Fig. 5, H and I), suggesting that IL-27 does not alter IL-17 secretion levels during SEA stimulation.

It has been demonstrated that upon engagement with their ligands, TLRs recruit specific adaptor molecules that propagate downstream signaling. Thus, we investigated whether MyD88 was responsible for changes observed in cytokine secretion. All effects observed in relation to cytokine production were abrogated when MyD88 gene expression was silenced using the siRNA technique (Fig. 5, A–G), indicating that SEA-mediated signaling induced changes in cytokine secretion in a MyD88-dependent manner.

**FIGURE 1.** Baseline expression of TLRs on B cells and DCs. A–D, TLR2, TLR4, TLR5, and TLR9 expression was assessed in B cells (A and C) and monocyte-derived DCs (B and D) from helminth-infected MS patients, uninfected MS patients, and healthy controls using flow cytometry. MFI of TLR expression was corrected by subtracting the MFI of the isotype control. TLR2 expression in infected MS patients was found to be significantly higher ($p < 0.001$) than in that uninfected MS patients and healthy controls. For A and B, data represent mean values ± SEM from 12 subjects for each group. C and D show representative histograms of TLR2 expression on B cells and monocyte-derived DCs from helminth-infected MS patients, uninfected MS patients, and healthy controls. E, TLR2 expression on DCs isolated from peripheral blood and on monocyte-derived DCs. Data represent mean values ± SEM of MFI from 10 subjects for each group.

Suppression of T cell activation induced by SEA- and TLR2-activated B cells is IL-10 mediated

The increase of IL-10 production by SEA suggested this cytokine might be involved in CD4$^+$ T cell suppression. To examine this issue, B cells and DCs were pulsed with SEA and TLR2 agonist and then cocultured at a 1:4 ratio (for B cells), and a 1:1 ratio (for DCs) with CD4$^+$ MBP peptide- and MOG peptide-specific T cells. In another set of experiments, activated T cells received supernatants from SEA- and TLR2-activated B cells or DCs in the presence or absence of IL-10 blocking mAbs. When prestimulated B cells or B cell supernatants were added to cultures, a 68–82% reduction in proliferation...
was observed in response to myelin Ags (Fig. 6A). Likewise, secretion of IFN-γ was significantly lower in the presence of SEA or TLR2 agonist-prestimulated B cells or B cell supernatants (Fig. 6B). The presence of anti-IL-10 mAb, but not that of anti-TGF-β mAb, abrogated these inhibitory effects, which were then mimicked by the addition of rhIL-10 (Fig. 6, A and B). Together, these data demonstrated that IL-10 produced by B cells after SEA or TLR2 stimulation played a key role in suppressing T cell activation. Likewise, when SEA or TLR2 agonist-prestimulated DCs or DCs supernatants were added to cultures, a 65–85% reduction in proliferation and IFN-γ secretion was observed in response to specific Ag stimulation (Fig. 6, C and D). The presence of IL-10 blocking mAbs abrogated these inhibitory effects only partially, suggesting that other factors, i.e., costimulatory molecule modulation, may play a key role in suppressing T cell
activation. Using similar culture systems, IL-10 neutralization had no effect on IL-17 secretion following TLR2 and SEA activation (data not shown).

Suppression of costimulatory molecule expression mediated by SEA is MyD88 independent

Next, we investigated whether SEA exposure altered surface expression of costimulatory molecules on LPS-stimulated DCs. LPS significantly enhanced expression of CD40, CD54, CD80, and CD86 compared with unstimulated cells (Fig. 7). Flow cytometry data demonstrated that SEA significantly down-regulated the expression of LPS-induced costimulatory molecules \( (p < 0.001; \text{Fig. 7}) \). However, direct exposure of DCs to SEA resulted in marginal changes, if any, regarding costimulatory molecule expression compared with levels observed for unstimulated cells. To explore the role of MyD88 on the down-regulation of these costimulatory molecules, we used MyD88-null DCs and showed that the suppression was MyD88 independent (data not shown).

**FIGURE 3.** SEA up-regulated TLR2 expression on B cells and DCs from helminth-infected MS patients. B cells and monocyte-derived DCs were cultured for 48 h in the presence or absence of SEA or PPD used as control. TLR expression was evaluated using flow cytometry as described above. Following exposure to SEA, TLR2 was markedly up-regulated in B cells and DCs from helminth-infected MS patients (A, B, G, and H). We did not observe significant changes in TLR2 expression after PPD exposure (B, C, H, and I). Likewise, exposure to SEA did not affect TLR4, TLR5, or TLR9 expression (D, E, F, J, K, and L). Data represent mean values \( \pm \) SEM of MFI from 12 subjects. B and H show representative histograms of TLR2 expression on B cells and DCs after 48 h of culture in the presence or absence of SEA or PPD.
of LPS-induced costimulatory molecules mediated by SEA, siRNA was used to inhibit MyD88 expression. As shown in Fig. 7, silencing of MyD88 had little effect on the levels of CD40, CD54, CD80, and CD86 expression. These results appear to indicate that SEA down-regulated LPS-induced costimulatory molecules on DCs in a MyD88-independent manner.

Pam3Cys- and SEA-induced ERK signaling

The MAPK family is composed of three major groups: p38, ERK1/2, and JNK1/2. To gain insight into potential signaling mechanisms mediating different DCs responses, we investigated the role of MAPK signaling pathways by assessing their phosphorylation in DCs after stimulation with the TLR2 agonist PamCys3 and SEA. As shown in Fig. 8, A and B, both Pam3Cys and SEA induced ERK phosphorylation in DCs to levels seven times above baseline. ERK phosphorylation was not detectable until after 20 min and persisted for 60–90 min, an activity abrogated when cells were preincubated with U0126, a specific inhibitor of upstream ERK1/2 activation. In contrast, neither Pam3Cys nor SEA had a measurable effect on p38 or JNK1/2 phosphorylation (Fig. 8, A and B). As positive control, LPS stimulation of DCs led to phosphorylation of all three MAPK species. Likewise, p38 phosphorylation was induced after treatment with anisomycin, a well-established activator of this pathway.

We next wanted to correlate ERK activation with IL-10 and IL-12 secretion by DCs. To determine this link, cells were treated with increasing concentrations of U0126 and IL-10 as well as IL-12 concentrations measured after PamCys3 and SEA stimulation. Addition of the ERK1/2 inhibitor U0126 was associated with dose-dependent IL-10 inhibition and a reciprocal enhancement in the production of IL-12, both strongly correlated with ERK1/2 inhibition (Fig. 8, C and D). To exclude the possibility that the positive effect of ERK inhibition on IL-12 could have been due to decreasing amounts of IL-10, neutralizing
Abs were added to the culture medium. IL-10 neutralization had no significant effect on increasing IL-12 production (Fig. 8, C and D). These results indicated that ERK phosphorylation was required for IL-10 production and IL-12 inhibition by DCs activated by SEA to occur.

Recent data have indicated that ERK signaling results in phosphorylation and stabilization of c-Fos. Expression of c-Fos on DCs after PamCys3 and SEA stimulation using flow cytometry was therefore also evaluated. As shown in Fig. 8, E and F, both stimuli induced enhanced levels of phosphorylated c-Fos expression, relative to unstimulated cells, as assessed by mean fluorescence intensity of staining. Likewise, the percentage of cells expressing phosphorylated c-Fos in PamCys3- and SEA-stimulated cells was significantly greater (p = 0.002) than that in unstimulated cells (data not shown). In contrast, no changes in phosphorylated c-Myc expression were observed.

SEA modulatory effects are TLR2 dependent

To assess the possible role of TLR2 in the recognition of SEA by DCs and B cells, we used the siRNA technique to inhibit its expression. Suppression of LPS-induced IL-12 and TNF-α production mediated by SEA was overcome when TLR2 expression was silenced (p < 0.001; Fig. 9, A and B). Similarly, silencing of TLR2 led to significant inhibition of IL-10 secretion induced by SEA in both DCs and B cells (p < 0.001; Fig. 9, C and D). These results indicated that TLR2 was required for the modulation of cytokine production by SEA. As was observed...
for cytokines, down-regulation of LPS-induced costimulatory molecules mediated by SEA was overcome by silencing TLR2 (p < 0.001 to p = 0.002; Fig. 9, E–H). These data therefore indicated that TLR2 was also required for DC phenotype modulation by SEA.

Discussion

Several studies in both humans and in animal models have demonstrated that helminths are powerful modulators of host immune responses (23, 24). Extracellular parasites characteristically induce predominantly Th2 responses and repress Th1 cytokine production even after exposure to nonparasite Ags, illustrating a potentially beneficial effect derived from helminth infections on Th-1 mediated diseases (23). Supporting this concept, we recently demonstrated that in helminth-infected MS patients, parasite regulation of host immunity significantly alters the course of disease (10). Similarly, investigations in animal models have demonstrated that prior infection with S. mansoni or pretreatment of mice with S. mansoni OVA significantly reduced the incidence of EAE and delayed its onset (25, 26).

In this study, we provide evidence that the SEA modulation of DCs and B cells that biases the immune response to an anti-inflammatory profile requires TLR2 expression to be present on both cell populations. Additionally, our data indicate that SEA and TLR2 agonists mediate intracellular pathways, resulting in suppression of IL-12 production and activation of IL-10 production, and are dependent on ERK signaling.

TLR regulation may be altered by helminth parasites, affecting its function and level of expression. The results presented in this study showed that surface expression of TLR2 on both B cells and DCs was significantly higher in helminth-infected MS patients compared with uninfected subjects and that the exposure of both cell populations to SEA resulted in significant up-regulation of TLR2 in helminth-infected MS patients, but not in uninfected individuals. It is well known that a variety of pathogens can cause increased TLR expression (27). Some authors...
have found results similar to our observations, indicating higher expression of TLR2 on PBMCs and enhanced response to the Pam3Cys ligand in *Plasmodium falciparum*-infected children compared with their uninfected counterparts (28). Still other reports, however, show down-regulation of TLR expression to be an important immune evasion strategy induced by some protzoan parasites (29) and observed in filaria-infected individuals (30). Interestingly, cells from uninfected subjects did not exhibit any alteration of TLR expression, suggesting that the diminished expression of TLR is a consequence of chronic Ag stimulation. The fact that helminths can both activate and negatively regulate TLR expression indicates tight immune response control of helminth infections. Factors such as total body parasite burden, and infection duration may play an important role in determining TLR expression levels. It is feasible that TLRs are desensitized in uninfected individuals and, consequently, that SEA is not effective in inducing changes in expression levels. Supporting this notion, one study has shown that gut TLRs are constitutively nonresponsive to the TLR4 ligand LPS (31). Another possibility is that differences in polymorphisms within TLR genes or in signaling pathway proteins may alter responses to different infectious agent stimuli (32–34). Finally, lack of SEA effect on uninfected cells may be related to the need for the engagement of a second receptor, which is absent in uninfected cells. TLR-stimulated IL-10 levels have been shown to be enhanced by a second signal generated through FcγR on monocytes (35). Additional experiments are currently underway to investigate these hypotheses.

It is becoming clear that TLR2 recognizes a wide range of pathogen-associated molecular patterns with distinct chemical properties, ranging from proteins to heat shock proteins and peptidoglycans (36). TLR2 was implicated as a possible receptor for SEA immunomodulatory activity after observing that schistosome-derived lipids elicited tolerogenic DCs and IL-10-producing regulatory T cells via TLR2 recognition (37). Moreover, during experimental infections TLR2 absence was associated with enhanced Th1 response and reduced Th2 response, causing disease to be more severe (38). It is believed in general that schistosome-derived lipids signal via TLR2, whereas dsRNA from schistosomal eggs activate TLR3 (39). In a different experimental setting, lipophosphoglycans from *Leishmania major* bound to TLR2, up-regulating both mRNA as well as its membrane expression (40). Our findings are in agreement with these results. However, recent studies using DCs derived from TLR2−/− and TLR4−/− mice demonstrated that TLR2 and TLR4 were not required for SEA-pulsed DCs to induce anti-inflammatory responses in naive mice, suggesting that other receptors, such as C-type lectins, might be implicated in the ability of DCs to respond to SEA (41). There is also evidence that components of the Lewis glycan present at various schistosome life cycle stages (42) drive in vitro differentiation of naive DCs to a DC2 phenotype via a TLR4-dependent pathway (43). Discrepancies between these investigations may be due to the presence of different moieties in SEA preparations or, alternatively, differences in parasite Ag recognition between human and mouse TLR2 (44).
To establish chronic infections, many helminths have evolved immunomodulatory mechanisms that subvert a host’s immune system. Several experimental models have demonstrated that different parasite molecules promote Th2 polarization through interaction with APCs (45, 46). We have shown that SEA and TLR2 agonists can modulate intracellular pathways to result in the suppression of IL-12, IL-6, IL-1β, and TNF-α production by DCs and in the activation of TGF-β as well as IL-10 production by both DCs and B cells. Likewise, previous studies have demonstrated that both TLR4 and TLR5 induce uncommitted DCs to adopt a Th1 profile via IL-12 production and in the activation of TGF-β as well as IL-10 production by both DCs and B cells. Likewise, IL-10 production by B cells can be induced through stimulation with sugar molecules present in S. mansoni eggs interacting with TLR4 (43).

Recent analysis of signaling pathways induced in DCs by carbohydrates from schistosome eggs have indicated a preferential activation of the MAPK ERK1/2 and implicated both TLR2 and TLR4 in this pathway (43, 47). Interestingly, blocking ERK1/2 activity by using the specific inhibitor U0126 significantly enhanced IL-12 production and inhibited IL-10 production induced by TLR2 agonists (47, 54). Our results are consistent with these findings. In addition, we provide evidence that SEA stimulation of function of B cells, because mice with a B cell-specific deletion of TLR2 and TLR4 or the TLR adaptor MyD88 do not recover from EAE (51). Only particular TLR agonists trigger regulatory B cell function. TLR9 contributes to EAE severity (52) but has no influence on B cell-driven disease resolution (51). Infectious agents use TLR signaling in B cells to limit host immune response by inducing the production of IL-10. Thus, infection by S. mansoni stimulates IL-10 production by B cells and protects mice from fatal anaphylaxis (53). Likewise, IL-10 production by B cells can be induced through stimulation with sugar molecules present in S. mansoni eggs interacting with TLR4 (43).
DCs produces similar results. In contrast, TLR4 and TLR5 agonists have been shown to preferentially induce IL-12 via a mechanism involving p38 and JNK1/2 (47). Counter-regulation between p38 and ERK1/2 seems to be important in the decision of whether to follow a Th1 or Th2 type of effector response. Consistent with this view, treatment of Leishmania-infected mice with anisomycin, a p38 activator, along with CD40L as well as an ERK1/2 inhibitor resulted in rapid recovery from parasitemia associated with Th1 expansion and an increased production of IL-12 (55). Moreover, imidazole inhibitors of p38 block IFN-γ production by Th1 cells in a dose-dependent manner but have no effect on IL-4 production by Th2 cells (56). ERK1/2 signaling induced by TLR2 agonists and SEA resulted in c-Fos phosphorylation as previously described (47). Silencing of c-Fos caused marked enhancement of IL-12 production, behaving like a Th1 stimulus, thus showing that the bias in Th response toward the Th2 pathway observed after TLR2 or SEA stimulation is mediated by c-Fos phosphorylation (47).

Although SEA induced IL-10 production and IL-12 inhibition through a MyD88-dependent pathway, we also found that SEA reduced LPS-induced expression of costimulatory molecules on DC surface in a MyD88-independent manner. These patterns of DC response are similar to those observed after stimulation using Giardia extracts (57), suggesting that SEA is able to regulate multiple pathways downstream of TLRs and that schistosomes have multiple mechanisms that contribute to the maintenance of an anti-inflammatory milieu. Experiments with
mice have demonstrated that sequential activation of MyD88, IRAK, and TRIFβ is involved in MyD88-dependent pathways, whereas sequential activation does not seem to participate in MyD88-independent pathways, although both lead to NF-κB activation (58, 59). It is unclear at present whether SEA stimulation affects other signaling cascades, such as the PI3K/Akt pathway. Identification and characterization of signaling molecules involved in MyD88-independent pathways remain a challenge for the future.

Parasites inhabit immune-competent hosts for long periods and can therefore develop modulatory molecules generating strong anti-inflammatory responses that restrict host tissue damage on one hand and enhance parasite survival on the other (60). Further investigation is warranted to identify which molecules dampen inflammatory responses that restrict host tissue damage on one hand and enhance parasite survival on the other (60).

Disclosures

The authors have no financial conflict of interest.

References


