# *Schistosoma mansoni* Worms Induce Anergy of T Cells via Selective Up-Regulation of Programmed Death Ligand 1 on Macrophages[1]

**Philip Smith,* Caitriona M. Walsh,* Niamh E. Mangan,* Rosie E. Fallon,* Jon R. Sayers,† Andrew N. J. McKenzie,‡ and Padraic G. Fallon[2]***

**Infectious pathogens can selectively stimulate activation or suppression of T cells to facilitate their survival within humans. In this study we demonstrate that the trematode parasite *Schistosoma mansoni* has evolved with two distinct mechanisms to suppress T cell activation. During the initial 4- to 12-wk acute stages of a worm infection both $CD4^+$ and $CD8^+$ T cells are anergized. In contrast, infection with male and female worms induced T cell anergy at 4 wk, which was replaced after egg laying by T cell suppression via a known NO-dependent mechanism, that was detected for up to 40 wk after infection. Worm-induced anergy was mediated by splenic $F4/80^+$ macrophages (M$\phi$) via an IL-4-, IL-13-, IL-10-, TGF-$\beta$-, and NO-independent, but cell contact-dependent, mechanism. $F4/80^+$ M$\phi$ isolated from worm-infected mice were shown to induce anergy of naive T cells in vitro. Furthermore, naive M$\phi$ exposed to live worms in vitro also induced anergy in naive T cells. Flow cytometry on in vivo and in vitro worm-modulated M$\phi$ revealed that of the family of B7 costimulatory molecules, only programmed death ligand 1 (PD-L1) was selectively up-regulated. The addition of inhibitory mAb against PD-L1, but not PD-L2, to worm-modulated M$\phi$ completely blocked the ability of these cells to anergize T cells. These data highlight a novel mechanism through which *S. mansoni* worms have usurped the natural function of PD-L1 to reduce T cell activation during early acute stages of infection before the subsequent emergence of egg-induced T cell suppression in the chronic stages of infection.** ***The Journal of Immunology*, 2004, 173: 1240–1248.**

Infectious pathogens have evolved a range of strategies that can positively or negatively modulate immune activation and thereby alter immune function to their own benefit. The trematode parasite *Schistosoma mansoni* is one such pathogen that preferentially stimulates Th2 cells (1). In addition to activating Th2 cell-biased responses, infection with schistosomes regulates lymphocyte function in vivo by suppressing T cell activation (2, 3). Conventional activation of naive T cells involves interaction of the surfaces of the T cell and APC, bringing together the TCR and peptide:MHC complexes along with ligation of the T cell costimulatory receptor CD28 by its ligands B7-1 and B7-2 (4). Alternatively, B7-1 and B7-2 can interact with CTLA-4 on the surface of activated T cells to stimulate an inhibitory signal that diminishes T cell activation. Thus, costimulatory molecules have an essential role in selectively inducing inhibitory or stimulatory T cell responses to respond to foreign pathogens as well as to regulate peripheral tolerance and thereby prevent autoimmune diseases. Although the interactions between CTLA-4 or CD28 and B7-1/B7-2 are the archetypal receptor ligands in T cell-APC activation, a number of additional functional receptors and B7 family members have been identified recently. These include programmed death ligand 1 (PD-L1),[3] also known as B7-H1, and PD-L2 (B7-DC) that interact with programmed death 1 (PD-1) on T cells, resulting in regulation of T cells (5, 6).

In this study we have found that distinct forms of in vivo suppression of T cells are induced dependent on the type/stage of schistosome infection. Conventional mixed sex schistosome infections, with male and female worms, suppress T cell activation after egg laying and throughout the chronic stages of infection via an NO-dependent mechanism. We also show in this study for the first time that schistosome worms can induce anergy of $CD4^+$ and $CD8^+$ T cells in vivo and in vitro. Worm-induced anergy occurred in the initial acute stages of infection. We have identified that worms induce anergy via selective up-regulation of PD-L1 on the surface of macrophages (M$\phi$), with blockade of this ligand restoring T cell activation.

## Materials and Methods

### *Mice*

BALB/c strain mice were obtained from Harlan Breeders (Bicester, U.K.). IL-4-, IL-13-, and IL-4/IL-13-deficient (−/−) mice, all on a BALB/c background, were previously described (7). Female mice, 6–8 wk of age, were used in all experiments. Mice were housed in a specific pathogen-free facility.

### *Parasitology*

A Puerto Rican strain of *S. mansoni* was maintained by passage in TO strain mice and albino *Biomphalaria glabrata* snails. To prepare worm-only infected snails, individual snails were exposed to a single *S. mansoni* miracidium. Cercariae shed from individual snails were sexed according to a method described by Grevelding (8), with slight modifications. DNA

*Department of Biochemistry, Trinity College, Dublin, Ireland; †Division of Genomic Medicine, University of Sheffield Medical School, Sheffield, United Kingdom; and ‡Medical Research Council, Laboratory of Molecular Biology, Cambridge, United Kingdom

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[2] Address correspondence and reprint requests to Dr. Padraic Fallon, Department of Biochemistry, Trinity College, Dublin 2, Ireland. E-mail address: pfallon@tcd.ie

[3] Abbreviations used in this paper: PD-L1, programmed death ligand 1; AA, alternatively activated; AC, accessory cell; AWA, adult worm Ag; D-NMMA, $N^G$-monomethyl-D-arginine; L-NMMA, $N^G$-monomethyl-L-arginine; M$\phi$, macrophage; PD-1, programmed death 1; PI, propidium iodide.



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from cercariae was isolated using the Wizard SV Genomic DNA Purification System (Promega, Madison, WI). Female-specific W1 and W2 sequences were amplified by PCR. Schistosome protein disulphide isomerase, present in both males and females, was used as a control gene. Snails shedding cercariae negative for both W1 and W2 were used to establish male-only infections. Mice were infected percutaneously with 50 (an acute infection) or 25 (chronic infection) male (worm infection) or male and female (worm plus egg infection) cercariae. Schistosome adult worm Ags (AWA) were prepared as previously described (9).

### *mAbs and reagents*

mAbs against mouse CD3$\epsilon$ (145-2C11), CD28 (37.51), FITC-anti-CD80 (16-10A1), FITC-anti-CD86 (GL2), FITC-anti-CD40 (HM40-3), FITC-anti-CD54 (3E2), FITC-anti-H-2K$^d$ (SF1-1.1), FITC-anti-AI/EI (2G9), biotin-anti-OX40 ligand (RM134L), and anti-CTLA-4 (UC10-4F10-11) were obtained from BD Pharmingen (San Diego, CA). Tricolor-anti-CD4 (CT-CD4), Tricolor-anti-CD8 (5H10), Tricolor-anti-CD19 (6D5), Tricolor-anti-F4/80 (F4/80), and FITC-anti-Gr1 (RB6-8C5) mAbs were obtained from Caltag Medisystems (San Francisco, CA). PE-labeled anti-ICOSL (HK5.3), anti-ICOS (C3948.4A), anti-PD-L1 (MIH5), and anti-PD-L2 (TY25) were purchased from eBiosciences (San Diego, CA). For in vitro studies the following neutralizing/blocking mAbs were used: from R&D Systems (Minneapolis, MN): anti-IL-4 (30340), anti-IFN-$\gamma$ (37895), and anti-TGF-$\beta$ (1D11); from BD Pharmingen: anti-IL-10 (JES5-2A5) and an isotype control mAb (B39-4; rat IgG2a, $\lambda$); and from eBiosciences: anti-PD-L1 (MIH5; rat IgG2a, $\lambda$), anti-PD-L2 (TY25; rat IgG2a, $\kappa$), and an isotype control mAb (eBR2a; rat IgG2a, $\kappa$). $N^G$-monomethyl-L-arginine (L-NMMA) and $N^G$-monomethyl-D-arginine (D-NMMA) were purchased from Calbiochem (La Jolla, CA).

### *Cell preparation*

Spleens were removed from schistosome-infected mice or from age-matched naive mice, and single-cell suspensions were prepared. Splenic CD4$^+$ or CD8$^+$ cells were isolated using CD4 or CD8 enrichment columns (R&D Systems) resulting in >95% purity, as confirmed by flow cytometry. Spleen accessory cells (AC) were prepared by depletion of T and B cells from spleen cells with two rounds of complement-mediated (Lo-Tox rabbit complement; Cedarlane Laboratories, Hornby, Canada) lysis using anti-Thy-1 mAb and anti-B220 Ab. AC preparations were shown to contain <1% T or B cells by subsequent FACS analysis.

F4/80$^+$ M$\phi$ were purified from splenocytes by magnetic bead separation. Spleen cells ($4 \times 10^7$) were incubated on ice for 30 min with rat anti-mouse F4/80 mAb. Cells were then washed twice before addition of $2 \times 10^6$ sheep anti-rat IgG Dynabeads (Dynal Biotech, Great Neck, NY) and multiple rounds of magnetic separation. Purity was assessed by flow cytometry, with <1% contaminating CD11c$^+$, CD19$^+$, CD4$^+$, or CD8$^+$ cells. If the F4/80$^+$ M$\phi$ were to be used as accessory cells, they were irradiated.

### *Proliferation assays*

Spleen cells ($2 \times 10^5$/well) were cultured in 96-well, U-bottom plates for 72 h at 37°C. Spleen cells were activated with 0.5 $\mu$g/ml anti-CD3 mAb and, where indicated in the figure legends, rIL-2 (20 ng/ml) or anti-CD28 mAb (4 $\mu$g/ml) was added. In coculture proliferation assays, AC or F4/80$^+$ M$\phi$ were added to purified CD4$^+$ or CD8$^+$ cells at a range of effector: responder cell ratios (1:1, 1:2, 1:5, 1:10, and 1:20); the data presented are for a ratio of 1:5. In Transwell cultures, $1 \times 10^6$ CD4$^+$ cells were mixed or separated from $1 \times 10^6$ AC in 24-well tissue culture plates. All samples were set up in triplicate wells. Cultures were pulsed with 1 $\mu$Ci/well [$^3$H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) for the last 14 h of culture. Cells were harvested, and [$^3$H]thymidine incorporation was determined.

T cell proliferation was also determined by labeling splenocytes with CFSE (Molecular Probes, Eugene, OR). Total spleen cells or purified cell populations were incubated with 2.5 $\mu$M CFSE in PBS and gently mixed for 10 min. Unbound CFSE was quenched by the addition of an equal volume of FCS. Labeled cells were then washed twice with PBS, followed by two washes with culture medium. Cells were suspended in culture medium to give the desired concentration for in vitro culture. At 24-h intervals, cells were harvested, washed with FACS buffer, and labeled with Tricolor-conjugated anti-CD4 or anti-CD8 mAb. Cells were also stained with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) and then analyzed by flow cytometry.

### *Surface phenotyping*

Cells were incubated with the appropriate fluorochrome-conjugated Abs in FACS buffer for 45 min on ice, then checked for viability by addition of PI to the cells. Data were acquired and analyzed using FACSCalibur and CellQuest software (BD Biosciences, Mountain View, CA). Intracellular cytokine staining was performed as previously described (9).

### *Cytokine ELISAs*

Cytokines in supernatants from anti-CD3 mAb or AWA (25 $\mu$g/ml) cell cultures were analyzed using conventional sandwich ELISAs. Coating Abs, standards, and detecting Abs for IL-2, IL-4, and IFN-$\gamma$ were obtained from BD Pharmingen. Reagents for IL-13 detection were obtained from R&D Systems. Cytokine detection kits were used for the detection of IL-10 (R&D Systems) and TGF-$\beta$ (Promega); both assays were conducted according to the manufacturer's protocol. Total TGF-$\beta$ was detected after acid treatment.

### *In vitro modulation of cells by live worms*

Live male worms were isolated by portal perfusion of infected mice. Cells (either whole splenocytes or M$\phi$) were prepared as described above. Spleen cells ($1 \times 10^7$/well) or M$\phi$ ($1 \times 10^6$/well) were seeded into 12-well Transwell culture plates (Costar, Cambridge, MA). Various numbers of male worms were then placed in the Transwell insert. The cells/worms were incubated at 37°C at 5% $CO_2$ for various times up to 72 h. The worms were then removed, and the cells were harvested and washed three times with fresh medium. Cells were checked for viability. The addition of 10 worms for 48 h was optimal to modulate cells. Cell proliferation of worm-modulated cells was determined as described above.

## Results

### *Two distinct mechanisms of suppression of T cell activation are induced by different* S. mansoni *infections*

When examined after 8 wk of infection, spleen cells from BALB/c mice infected with a mixed sex (worm plus egg infection) or a male-only worm infection have a marked defect in anti-CD3 mAb-stimulated proliferation (Fig. 1*A*). Cells from both groups of infected mice consistently had 40–60% lower cell proliferation than naive spleen cells. The impaired anti-CD3-stimulated proliferation of infected splenocytes was not due to activation-induced cell death of T cells from schistosome-infected mice during in vitro culture (9–11), as trypan blue, annexin V plus PI staining and cytotoxicity assays demonstrated comparable in vitro cell death in all groups of mice (data not shown). As certain forms of T cell suppression can be restored by IL-2 treatment (12), we added exogenous IL-2 to in vitro splenocyte cultures. Addition of IL-2 partially restored proliferation of cells from worm-infected mice after anti-CD3 mAb stimulation (Fig. 1*A*). The addition of a range of other cytokines, including IFN-$\gamma$, IL-4, IL-7, IL-9, IL-10, and IL-15, did not restore proliferation (data not shown). In addition, anti-CD28 mAb treatment, to stimulate endogenous IL-2 (13), also restored T cell proliferation in worm-infected mice (Fig. 1*A*). In marked contrast, exogenous IL-2 or anti-CD28 mAb treatment did not restore anti-CD3 mAb-stimulated proliferation of splenocytes from worm- plus egg-infected mice (Fig. 1*A*). These data indicate that T cells from the spleens of both worm-infected and worm-plus egg-infected mice are unresponsive to anti-CD3 activation. The ability of IL-2 treatment to restore activation of T cells from worm-infected mice, but not worm- plus egg-infected mice after an 8-wk infection suggested there was a difference in the mechanism of T cell unresponsiveness induced by the different types of infection.

To further examine the differences in T cell suppression between the two types of infections, mice were chronically infected for up to 40 wk, and T cell proliferation was periodically evaluated. After 4 wk of infection, i.e., before the production of eggs, the worm-plus egg-infected mice had the same T cell anergy, restored by IL-2, as that observed in worm-infected mice at 4, 8, and 12 wk

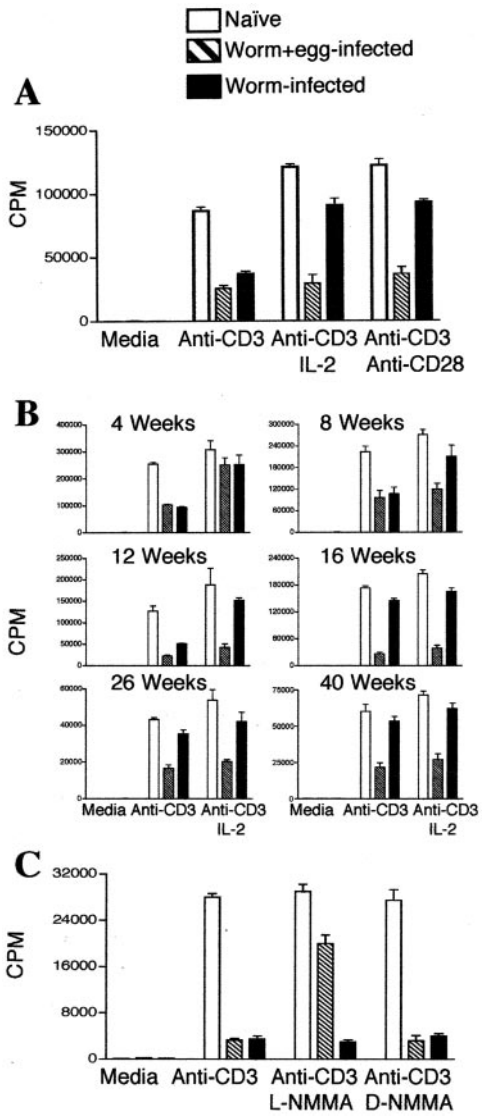


**FIGURE 1.** *S. mansoni* worm infection induces T cell anergy. *A*, Proliferation of spleen cells from naive mice or from mice infected for 8 wk with a worm plus egg or a worm-only schistosome infection. Cells were activated with soluble anti-CD3-alone (0.5 $\mu$g/ml), anti-CD3 with IL-2 (20 ng/ml), or anti-CD28 (4 $\mu$g/ml). *B*, Proliferation of spleen cells from mice infected with 25 ceracriae for a worm plus egg or worm infection that had been infected for 4, 8, 12, 16, 26, and 40 wk and from age-matched naive mice. *C*, Role of NO in defective proliferation of cells from infected mice was addressed by the addition of L-NMMA or D-NMMA (10 $\mu$M) to cell cultures. Proliferation was measured by $^3$H incorporation and expressed as cpm. Data shown are the mean $\pm$ SD of triplicate wells. In all experiments three to five spleens were pooled. Data presented in *A* and *C* are representative from at least four separate experiments. Data in *B* are from two separate experiments.

after infection (Fig. 1*B*). However, after 12 wk of infection the levels of anergy was reduced in worm-infected mice, whereas worm- plus egg-infected mice had marked suppression of T cell activation throughout the infection.

NO is a potent inhibitor of T cells and is implicated in suppression of T cells by filarial worms (14) and also by a schistosome egg glycan (15). The addition of the NO inhibitor L-NMMA did not restore proliferation of T cells from worm-infected mice (Fig. 1*C*). In contrast, the reduced anti-CD3 mAb stimulated proliferation of cells from worm- plus egg-infected mice was partially restored by inhibition of NO. An inert isomer of L-NMMA, D-NMMA, had no effect on proliferation in any group (Fig. 1*C*).

*Role of cytokines in worm infection-induced anergy*

A fundamental biological difference between worm and worm plus egg infections is the presence of Th2-inducing eggs in male and female worm-infected mice (1, 16). To examine differences in cytokine responses between the two types of infection, spleen cells were stimulated with anti-CD3 mAb or AWA. Cytokine production by splenocytes from both groups of infected mice were Th2 dominated, with anti-CD3 mAb-activated cells from infected animals having elevated IL-4 and IL-13 compared with cells from naive mice (Fig. 2). Cells from both groups of infected mice produced less IFN-$\gamma$ than naive mice. Consistent with previous studies (16, 17), stimulation of cells from infected mice with AWA induced Th2 cytokines, but not IFN-$\gamma$. Cells from infected mice had markedly limited IL-2 production after 24 h in cultures or when cultured longer (data not shown) compared with naive mice (Fig. 2). Consistent with the known regulatory role for IL-10 in acute murine schistosome infections (18, 19) the production of this cytokine was markedly elevated in worm- and egg-infected mice (Fig. 2). We also demonstrate that worm-infected mice had marked production of IL-10, with greater release of this cytokine from spleen cells from these mice compared with worm- plus egg-infected mice. The production of the regulatory cytokine TGF-$\beta$

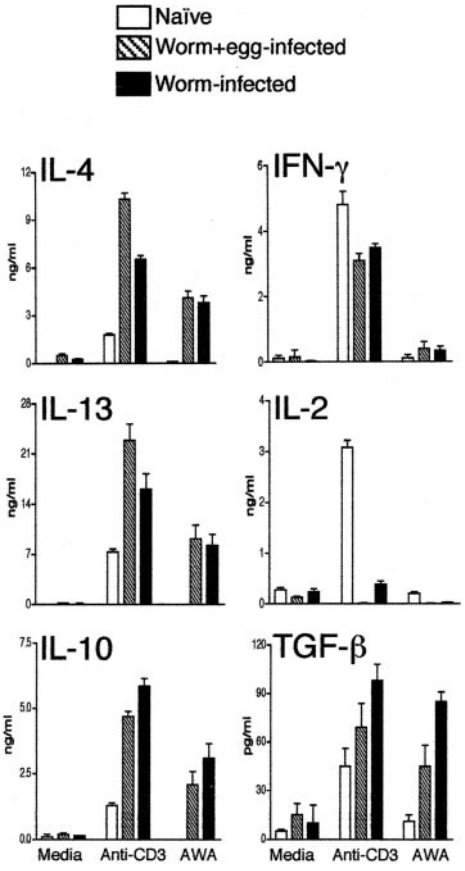


**FIGURE 2.** Th2-biased cytokine production by cells from worm- plus egg-infected and worm-infected mice. Spleen cells from naive mice or from mice infected for 8 wk with a worm plus egg or a worm-only schistosome infection were activated with anti-CD3 (0.5 $\mu$g/ml) or AWA (25 $\mu$g/ml). Supernatants were collected after 24 h (IL-2) and 72 h (IL-4, IL-10, IL-13, IFN-$\gamma$, and TGF-$\beta$) for cytokine ELISA. Data are from pools of spleens from two or three individual mice and are representative of three separate experiments.

by cells from infected mice was also elevated compared with naive cells, with greater release from cells from worm-infected mice than cells from worm- plus egg-infected mice (Fig. 2).

Worm-infected mice had a similar, but less marked, Th2-biased cytokine response as seen in worm- plus egg-infected animals, which is consistent with earlier studies (17). However, worm-infected had greater relative production of both IL-10 and TGF-$\beta$ compared with worm- plus egg-infected animals (Fig. 2). IL-10 and TGF-$\beta$ are regulatory cytokines that can suppress T cells (20). However, the addition of neutralizing anti-IL-10 mAb had no effect on the T cell activation defect (Fig. 3). Similarly, inhibition of TGF-$\beta$ did not restore T cell activation of cells from worm-infected mice.

### *Worm infection-induced anergy is mediated by accessory cells and is cell contact dependent*

The inability of T cells from the spleens of worm-infected mice to respond to anti-CD3 is analogous to functional characteristics of T cell suppression induced by regulatory $CD4^+CD25^+$ T cells (12, 20). Indeed, the frequency of $CD4^+$ cells that are $CD25^+$ and also coexpress CTLA-4 or GITR are elevated in the spleens of schistosome-infected mice (data not shown). To formally investigate whether anergy was mediated by $CD4^+$ cells, AC and $CD4^+$ cells were isolated from the spleens of naive or worm-infected mice and mixed in vitro, and anti-CD3 mAb proliferation examined. AC from worm-infected mice induced anergy of naive $CD4^+$ cells, whereas $CD4^+$ cells from worm-infected mice had normal anti-CD3 stimulated proliferation when cocultured with naive AC populations (Fig. 4*A*). Further confirmation that AC from worm-infected mice directly induced T cell anergy was demonstrated by the addition of exogenous IL-2 restoring $CD4^+$ cell proliferation (Fig. 4*A*). When separated by Transwell, ACs from worm-infected mice had no effect on proliferation of naive CD4 cells after anti-CD3 mAb treatment (Fig. 4*B*). These data demonstrate that worm infections induce anergy of naive $CD4^+$ cells via a splenic non-B non-T cell AC cell population involving a cell contact-dependent mechanism.

### *Worm infections mediate T cell anergy via splenic M$\phi$*

T cell suppression that occurs in worm- plus egg-infected mice has been shown to be induced by M$\phi$ (adherent cells) that are present within the granuloma surrounding eggs in the liver (21). Therefore, to investigate whether the splenic AC that induced T cell suppression during worm infections were also M$\phi$, we used the mouse

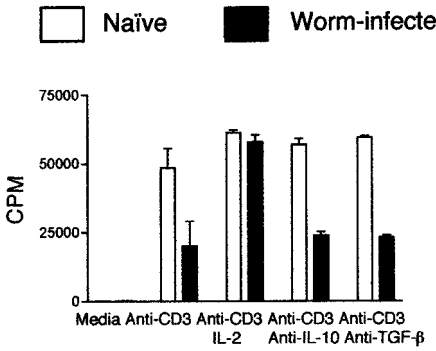


**FIGURE 3.** T cell anergy in worm-infected mice is independent of IL-10 and TGF-$\beta$. *A*, Cells from naive or worm-infected mice were processed as described in Fig. 1, with anti-IL-10 (10 $\mu$g/ml) or anti-TGF-$\beta$ (10 $\mu$g/ml) mAbs added to cultures. Proliferation was measured by $^3$H incorporation and expressed as cpm. Data shown are the mean $\pm$ SD of triplicate wells. Data are representative from four separate experiments.

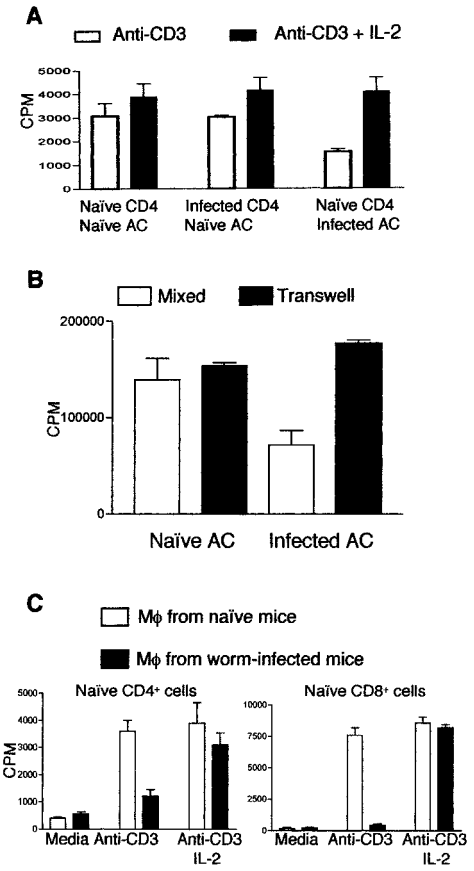


**FIGURE 4.** T cell anergy in worm-infected mice is mediated by M$\phi$. *A*, AC and $CD4^+$ cells from the spleens of naive or worm-infected mice were mixed. Cells were activated with anti-CD3 with or without IL-2. *B*, AC from worm-infected or naive mice were either mixed with naive $CD4^+$ T cells or separated by a Transwell. Cells were activated with anti-CD3. *C*, $F4/80^+$ M$\phi$ from the spleens of naive or worm-infected mice were added to naive $CD4^+$ and CD8 cells. Cells were activated with anti-CD3 with or without IL-2. For mixed cell cultures, a range of effector:responder cell ratios were evaluated; the data presented are at 1:5, which was the optimum inhibitory response. All data shown are the mean $\pm$ SD of triplicate wells. The results shown are representative of at least three independent experiments.

M$\phi$-specific marker $F4/80^+$ to isolate M$\phi$ from naive and worm-infected mice. Spleen $F4/80^+$ M$\phi$ from each group were cocultured with naive $CD4^+$ and $CD8^+$ T cells (Fig. 4*C*). $F4/80^+$ M$\phi$ from naive mice did not impair anti-CD3-stimulated cell proliferation of naive T cells. However, $F4/80^+$ M$\phi$ from worm-infected mice induced anergy of naive $CD4^+$ and $CD8^+$ cells (Fig. 4*C*). In support for a distinct role for M$\phi$ in this process after in vitro depletion of $F4/80^+$ cells from splenocytes from worm-infected mice, the remaining cells were unable to induce suppression of naive $CD4^+$ T cells (data not shown). The addition of IL-2 to $F4/80^+$ cells plus naive $CD4^+$ or $CD8^+$ cell cultures completely restored proliferation (Fig. 4*C*), which is consistent with anergy induced by whole spleen cells or ACs from worm-infected mice (Figs. 1, 3, and 4*A*).

In a model involving i.p. injection of *Brugia malayi* worms, a novel form of cell contact-dependent suppression of naive $CD4^+$ T cells is induced that involves the elicitation of alternatively activated (AA) M$\phi$, a process that is IL-4 dependent (22, 23). To determine whether the schistosome worm-induced T cell anergy was also due to the induction of AA M$\phi$, we infected mice deficient in IL-4, IL-13, or IL-4 plus IL-13 with schistosome male

worms. All worm-infected, cytokine-deficient mice developed comparable T cell anergy, as seen in infected wild-type mice (Fig. 5*A*). Therefore, the T cell suppression induced during schistosome worm infections is not IL-4 or IL-13 dependent and thus does not appear to be via alternative activation of M$\phi$. A schistosome egg glycan can induce T cell suppression via induction of novel F4/80$^+$Gr1$^+$ cells (15, 24, 25). However, the levels of Gr1 on F4/80$^+$ spleen cells from schistosome-worm infected mice is not up-regulated, with these mice having marginally lower Gr1 expression on F4/80$^+$ spleen cells compared with uninfected mice (Fig. 5*B*). In contrast, spleen cells from worm- plus egg-infected mice have elevated Gr1 expression on F4/80$^+$ cells relative to uninfected or worm-infected mice (Fig. 5*B*).

### *In vitro modulation of M$\phi$ by schistosome worms induces anergy of naive T cells*

To investigate whether schistosome worm infection induces T cell anergy via the secretion of worm Ags, we used live male worms ex vivo as a source of worm Ags. Exposure of naive spleen cells to 10 worms for 48 h in vitro induced T cell anergy, restorable by IL-2 (Fig. 6*A*). A concern with the use of this worm coculture method was worm-derived "cold" thymidine reducing [$^3$H]thymidine incorporation during the analysis of T cell proliferation. We therefore labeled worm-modulated spleen cells with CFSE and then examined the division of CD4$^+$ and CD8$^+$ by flow cytometry after anti-CD3 activation. There was a marked impairment in cell division of CD4$^+$ and CD8$^+$ T cells in worm-treated spleen cells compared with untreated cells (Fig. 6*B*). PI staining confirmed that cell death of anti-CD3 mAb-activated CD4$^+$ and CD8$^+$ cells was comparable between cells precultured in medium or worms. Finally, to confirm that M$\phi$ were the specific cells inducing T cell anergy, we isolated spleen M$\phi$ from naive mice and exposed them to live worm, as described above, added naive CD4$^+$ and CD8$^+$ cells, and assessed anti-CD3 proliferation. In vitro worm-modulated M$\phi$ induced anergy of both T cell populations (Fig. 6*C*).

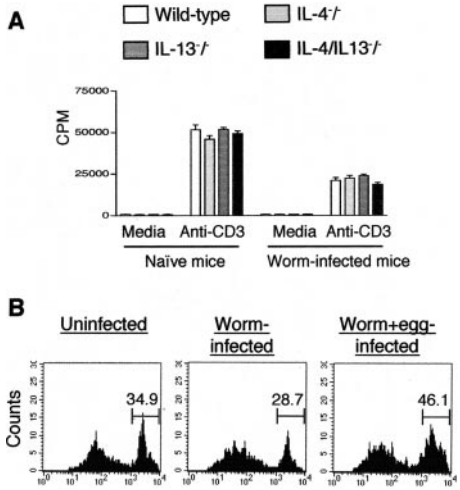


**FIGURE 5.** T cell anergy in worm-infected mice is independent of IL-4, IL-13, and Gr1. *A*, Anti-CD3-stimulated proliferation of spleen cells from naive or worm-infected wild-type or IL-4-deficient (−/−) mice, or IL-13$^{-/-}$ and IL-4/13$^{-/-}$ mice. Spleen cells from 8-wk infected mice were processed for T cell proliferation as described in Fig. 1. All proliferation data shown are the mean ± SD of triplicate wells. *B*, Gr1 expression on F4/80-gated spleen cells from uninfected, worm-infected, or worm- plus egg-infected mice. The mean percentage of Gr1$^+$ cells from three individual uninfected or infected mice is shown. The results shown are representative of at least three independent experiments.

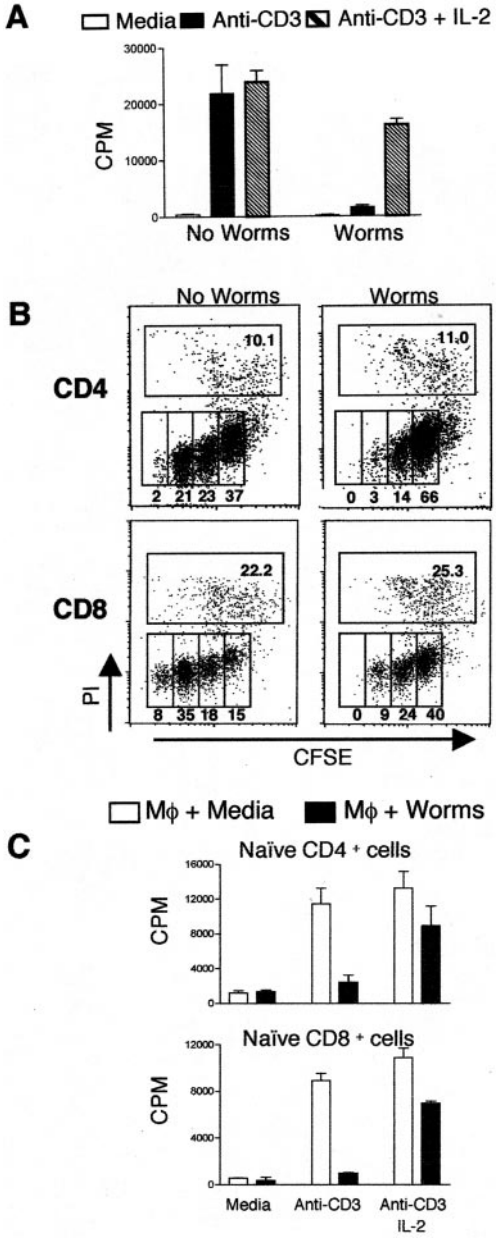


**FIGURE 6.** Schistosome worms induce anergy of T cells in vitro via M$\phi$. *A*, Ten live male schistosome worms were incubated, using Transwell inserts, over 5 × 10$^6$ naive spleen cells for 48 h. Cells were washed and stimulated with medium alone, anti-CD3, or anti-CD3 plus IL-2 for an additional 48 h. *B*, Spleen cells were treated with live worms and labeled with CFSE before anti-CD3 activation for 48 h. The division of CD4$^+$ and CD8$^+$ cells was analyzed by flow cytometry. Data shown are the percentage of CD4$^+$ or CD8$^+$ cells in different stages of division (small boxes). Cells were PI-stained to check the percentage of dead cells (large box). *C*, Naive F4/80$^+$ spleen M$\phi$ were exposed to worms in vitro before the addition of naive CD4$^+$ and CD8$^+$ cells, and anti-CD3, with or without IL-2, proliferation was analyzed. The results shown are representative of at least three independent experiments.

### *Schistosome worms induce T cell anergy via selective up-regulation of PD-L1 expression on M$\phi$*

As the T cell anergy induced by schistosome worms is cell contact dependent and restored by costimulating with anti-CD28 mAb, it is suggestive that the anergy of the T cells was induced by the absence, or up-regulation, of costimulatory molecules on the M$\phi$. Spleen cells were removed from worm-infected mice 8 wk after

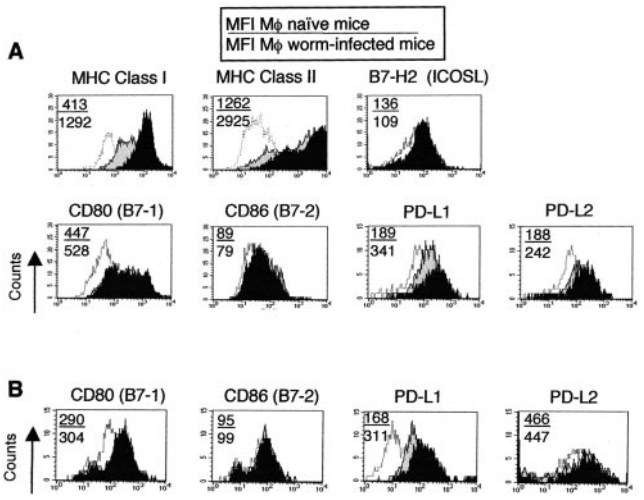


**FIGURE 7.** Selective alterations in expression of surface markers on in vivo or in vitro worm modulated F4/80$^+$ M$\phi$. *A*, Expression of markers on F4/80$^+$-gated cells from naive (light shading) or worm-infected (dark shading) mice. Control Ab is indicated (hatched line, no shading). *B*, Expression of B-7 family members on F4/80$^+$-gated cells in spleens cells exposed to worms in vitro. Medium-treated cells (gray shading), worm-treated cells (dark shading), and control Ab-treated cells (no shading) are shown. All data represented are the geometric mean of mean fluorescence intensities (MFI) from duplicate wells. Data are representative of at least three or four separate experiments.

infection and the level of B-7 costimulatory ligands on F4/80$^+$ M$\phi$ analyzed (Fig. 7*A*). Both MHC class I and II expression were both markedly up-regulated on splenic M$\phi$ from worm-infected mice compared with expression on naive cells (Fig. 7*A*). The costimulatory molecule CD80 was marginally elevated on M$\phi$ from infected mice, whereas CD86 and B7-H2 (ICOSL) were slightly reduced. Expression of PD-L1 (B7-H1) was up-regulated (MFI of 189 on M$\phi$ from naive mice compared MFI of 341 on M$\phi$ from infected mice), whereas the related molecule PD-L2 (B7-DC) was slightly elevated. M$\phi$ surface expression of OX40 ligand and CD40 were down-regulated, with a moderate increase in CD54 (ICAM-1) expression (data not shown). Collectively, with respect to altered secondary signal activation of T cells the most striking data on alterations in M$\phi$ surface expression by schistosome worm infection were the net decrease in costimulatory molecules with the exception of a moderate increase in CD80 and PD-L2 and a more pronounced up-regulation of PD-L1.

As we have shown that splenic M$\phi$ exposed to schistosome worms in vitro also induce anergy of naive T cells (Fig. 6*C*), we examined surface expression of molecules on these cells. The surface expression of PD-L1 on worm-treated M$\phi$ was consistently increased (MFI of 168 vs 311), whereas other B7 ligands, CD80, CD86, and PD-L2, were all unchanged or down-regulated on in vitro worm-treated M$\phi$ (Fig. 7*B*). Expression of a range of additional surface markers on worm-modulated M$\phi$ were also reduced or unchanged (data not shown). The specific up-regulation of PD-L1 on both in vivo and in vitro worm-modulated M$\phi$ prompted us to investigate the potential role of PD-L1 in worm-induced T cell anergy.

We isolated F4/80$^+$ M$\phi$ from the spleens of worm-infected mice, and before addition of naive CD4$^+$ or CD8$^+$ T cells, the

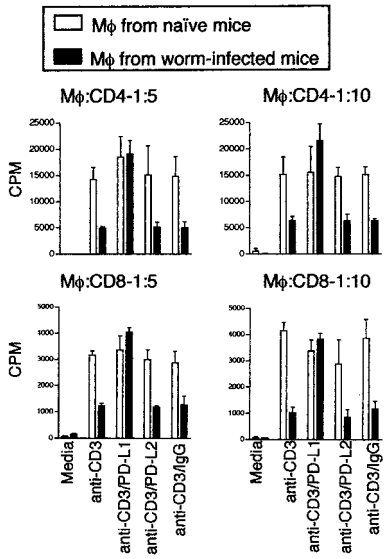


**FIGURE 8.** Blocking PD-L1 prevents F4/80$^+$ M$\phi$ from schistosome worm-infected mice from inducing T cell anergy. F4/80$^+$ M$\phi$ were isolated from the spleens of naive or worm-infected mice and incubated with blocking anti-PD-L1 (rat IgG2a, $\lambda$), anti-PD-L2 (rat IgG2a, $\kappa$), or control (rat IgG2a, $\kappa$) mAb (all at 500 ng/ml). A rat IgG2a, $\lambda$ L chain, Ab was all used as a negative control Ab. CD4$^+$ and CD8$^+$ cells (1:5 or 1:10) were added to F4/80$^+$ M$\phi$, and cells were treated with anti-CD3. Data shown are the mean ± SD of triplicate wells and are representative of three separate experiments.

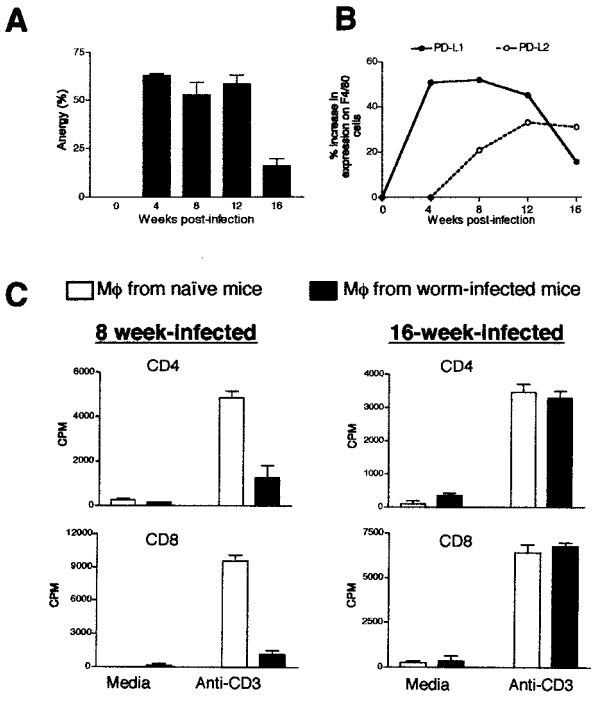


**FIGURE 9.** Temporal changes in anergy and PD-L1 and PD-L2 expression during acute and chronic schistosome worm infection. *A*, Levels of anergy in worm-infected mice 4, 8, 12, and 16 wk after infection. Data are presented as the mean percent increase in anergy in infected mice (at least three experiments per time point) relative to that in uninfected mice. *B*, Increase in PD-L1 or PD-L2 expression on gated F4/80$^+$ spleen cells from worm-infected mice relative to their expression on cells from age-matched and uninfected mice. At each time point flow cytometry was performed on pools of spleen cells from two to four uninfected or infected mice. Data are representative of four (wk 4 and 8) or two (wk 12 and 16) separate experiments. *C*, In vitro suppression of anti-CD3 mAb activation of naive CD4$^+$ or CD8$^+$ cells by F4/80$^+$ M$\phi$ isolated from the spleens of uninfected or 8- or 16-wk worm-infected mice. The assay was performed as described in Fig. 4. Data shown are the mean ± SD of triplicate wells. The results shown are representative of at least two independent experiments.

cells were treated with blocking mAbs against PD-L1, PD-L2, or a control IgG2a isotype control mAb. We first confirmed that after the addition of blocking anti-PD-L1 mAb (500 ng/ml) to M$\phi$, the ligand was fully blocked, with a fluorochrome-conjugated anti-PD-L1 mAb being unable to bind to the cells, whereas an anti-PD-L2 mAb stained the same cells (data not shown). Blockage of PD-L1 completely ablated the capacity of M$\phi$ from infected mice to induce anergy of T cells (Fig. 8). In contrast, blocking of PD-L2 had no affect on the ability of M$\phi$ from infected mice to induce anergy, with comparable cell activation as the control Ab-treated groups. Furthermore, blocking of PD-L1 on in vitro worm-modulated spleen cells also blocked their ability to induce anergy of naive $CD4^+$ and $CD8^+$ T cells (data not shown).

*PD-L1 expression on F4/80$^+$ spleen M$\phi$ is reduced during chronic stages of schistosome worm infection*

In worm-infected mice the levels of anergy peak at wk 4–12, but decline by wk 16 of infection (Figs. 1*B* and 9*A*). We analyzed PD-L1 and PD-L2 expression on F4/80$^+$ spleen M$\phi$ during the first 16 wk of worm infection to address whether the loss of anergy after 12 wk of worm infection was associated with changes in PD-L1 levels on M$\phi$. The levels of PD-L1 on spleen M$\phi$ rose in the initial acute stages of infection, peaking at wk 8–12, followed by a decline in expression at wk 16 (Fig. 9*B*). In contrast, PD-L2 M$\phi$ expression peaked at wk 12, but did not decline by wk 16 (Fig. 9*B*). We isolated F4/80$^+$ M$\phi$ from 8- or 16-wk infected mice to formally address whether the absence of anergy in vivo in chronic worm infection was also manifested in an inability of isolated splenic M$\phi$ to induce anergy of naive T cells in vitro. As shown earlier (Fig. 4*C*), F4/80$^+$ M$\phi$ from the spleens of mice infected for 8 wk with schistosome worms induce anergy of both naive CD4 and CD8 cells (Fig. 9*A*). In contrast, F4/80$^+$ M$\phi$ from the spleens of 16-wk worm-infected mice that had reduced PD-L1 expression were unable to induce anergy of naive T cells (Fig. 9).

## Discussion

The propensity of schistosome infections to modulate T lymphocyte responses by evoking a type 2 cytokine-biased response is extensively documented (1). In contrast, there are relatively limited studies on the mechanism of the less characterized phenomenon of T cell hyporesponsiveness that is induced during schistosome infection. An objective of this study was to address the underlying mechanisms of T cell hyporesponsiveness during murine *S. mansoni* infection. We have demonstrated that schistosome infection stimulates two forms of $CD4^+$ cell hyporesponsiveness that are induced by different stages of the parasite life cycle and are active at different stages of infection. The T cell suppression observed in this study during a conventional egg-producing male and female worm infection is similar to observations in earlier studies (26–28). In contrast, relatively little is known about the T cell suppression induced by worm infection, although mitogen unresponsiveness has been previously reported in schistosome worm-infected mice (29, 30). In this study we describe a new mechanism by which schistosome worms induce anergy of T cells during the early stages of infection.

Humans with chronic infectious diseases, including schistosomiasis, experience sustained immune activation that is often accompanied by T cell hyporesponsivess (3). In schistosomiasis patients, various studies have shown impaired parasite Ag-specific and nonspecific proliferation (2, 31, 32). As mechanisms by which helminth parasites have evolved to down-modulate T cell responses are investigated, it has become apparent that there may be multiple mechanisms involved, especially as both APC and T cells are capable of playing a regulatory or suppressive role (3). We describe in this study a new mechanism by which schistosomes regulate T cell activation during the early acute stages of infection via selective up-regulation of a costimulatory molecule on M$\phi$. The role for regulation of T cell activation during schistosome infection via costimulatory molecules has previously been demonstrated by the exacerbated pathology during *S. mansoni* infection of mice deficient in B7-1/2 (33), and in a more recent study of mice that had disrupted ICOS-LICOS interactions by mAb treatment (34). Earlier studies have shown a role for M$\phi$ during T cell suppression in schistosome worm- plus egg-infected mice (27, 35). More recently, a schistosome egg glycan can also elicit F4/80$^+$Gr1$^+$ suppressor cells when injected into the peritoneum of mice (15, 24). In contrast, the F4/80$^+$ M$\phi$ from the spleens of worm-infected mice we describe do not have elevated Gr1 expression. Similarly, a novel T cell-suppressive F4/80$^{dull}$ and nonadherent cell population isolated from the spleens of worm- plus egg-infected mice by Marshall et al. (35, 36) is distinct from the F4/80$^{bright}$, adherent, and contact-dependent suppressor M$\phi$ described in this report.

In mice, PD-L1 (B7-H1) is constitutively expressed on a range of cells, including T cells, B cells, M$\phi$, and dendritic cells (37). The expression of PD-L1 and also PD-L2 on APCs have been implicated as inducers of negative signals in T cells (6, 38–40). Thus, it has been shown that blocking PD-L1 impairs the ligand's ability to suppress T cell activation (40, 41). However, there is also conflicting evidence that PD-L1-PD-1 interactions may activate T cells (42–44). The data presented in this study indicate that schistosome worms up-regulate PD-L1 expression on M$\phi$ to induce T cell anergy. This observation is validated by the use of inhibitory mAb clearly showing that blocking PD-L1, but not PD-L2, on schistosome worm-modulated M$\phi$ prevents these cells from inducing anergy of both naive $CD4^+$ and $CD8^+$ T cells. PD-L1 expression during schistosome infection of mice has not, to our knowledge, being examined previously. However, it has been shown that PD-L2 is up-regulated on splenic dendritic cells in worm- plus egg-infected mice (45). As worm infections are Th2 biased (Fig. 2), the up-regulated expression of PD-L1, and not PD-L2, on M$\phi$ from worm-infected mice or in vitro modulated cells is surprising in view of the recent demonstration that IL-4-producing Th2 cells stimulate up-regulated PD-L2 expression on M$\phi$, whereas IFN-$\gamma$-producing Th1 cells preferentially induce PD-L1 expression on murine M$\phi$ (46). We are currently further investigating the role of selective up-regulation of PD-L1 or PD-L2 expression on other cells and tissue in schistosome-infected mice.

It is interesting that previous in vitro studies on PD-L1- and PD-1-mediated T cell inhibition (47) complement what we observe during a pathogen infection in vivo, in particular the impaired IL-2 production by T cells. We have also observed that worm-infected mice have approximately half the frequencies of IL-2-producing spleen $CD4^+$ and 3-fold fewer IL-2-producing $CD8^+$ compared with responses of T cells from naive mice (data not shown). With respect to a role for PD-1 on T cells, worm-infected mice had a 2- to 3-fold elevation in PD-1 expression on splenic $CD4^+$ and $CD8^+$ T cells compared with levels on naive spleen cells (data not shown). However, we have been unable to directly associate PD-1 expression on $CD4^+$ or $CD8^+$ cells with greater susceptibility of T cells to anergy induced by worm-modulated M$\phi$. This discrepancy may be due to the recent demonstrations of PD-1-independent activities for PD-L1/2 (48–50) and potential roles for additional T cell inhibitory receptors (51).

Morbidity during *S. mansoni* infection is primarily attributed to activated $CD4^+$ T cell-mediated granulomatous inflammation to the egg stage of infection, with limited worm-related pathology (1). Previously, worm infections of mice have been shown to drive

liver lymphocytes in the prepatent period toward a Th2 phenotype (52), thereby priming the liver for the onset of egg deposition and the generation of a Th2-mediated granuloma (53, 54). The data presented in this study demonstrate that schistosome worms induce anergy of T cells in the acute stages of infection, before the arrival of the eggs, with a decline in anergy during chronic stages of infection. This loss of anergy in worm-infected mice after 12 wk was coincident with a reduction in PD-L1 expression on M$\phi$, with M$\phi$ from chronically worm-infected mice being unable to suppress naive T cells in vitro. Our data support a hypothesis by which the worm has evolved to modulate M$\phi$ to suppress the potent initial T cell activation that is induced by the egg and thereby limit inflammation before the emergence of the egg-induced T cell suppression. In the context of proposing a role for PD-L1 in pathogen immune evasion strategies, it is relevant that tumors have also usurped the natural function of PD-L1 to escape from immune attrition (41, 55).

In agreement with a role for secretions from an active worm infection inducing cell modulation within 2 wk of drug treatment, with praziquantel and oxamniquine to kill male worms (56), the spleen cells from drug-treated mice had fully recovered their ability to proliferate in response to anti-CD3 mAb (data not shown). Interestingly, earlier studies have also shown that parasite Ag-specific hyporesponsiveness in schistosome-infected humans is lost after chemotherapeutic treatment (31, 32, 57). Exposure of bone-marrow M$\phi$ or J774 monocytic-M$\phi$ cells to live worms or worm excretions-secretions also modulates the cells to induce anergy of $CD4^+$ T cells (data not shown). It would be of interest to address the mechanism, for example, interactions via TLRs or C-type lectins, whereby worms influence M$\phi$ to express PD-L1 and induce anergy. Indeed, it has been shown that certain schistosome worm lipids can modulate APCs (human DCs), via TLR2 activation, to induce regulatory T cells (58). More recently, it has been shown that a schistosome carbohydrate (lacto-*N*-fucopentaose III) matures murine dendritic cells toward a DC2 phenotype via a TLR4-dependent mechanism (59). At least one worm molecule that is released by live worms in vitro, and also in vivo, has been shown to modulate M$\phi$, and we are currently further characterizing this molecule and delineating its mechanism of cell modulation.

In conclusion, this study is the first demonstration that a pathogen, *S. mansoni*, has evolved with the ability to selectively upregulate PD-L1 on M$\phi$ to subvert the immune system. Further investigation of the role of PD-L1 in inhibiting T cell activation and reducing immunopathology in schistosome infection may have implications for the control of other T cell-mediated diseases.

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