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SjHSP60 induces CD4⁺CD25⁺Foxp3⁺ Tregs via TLR4-Mal-driven production of TGF- β in macrophages

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Abstract

CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) play a pivotal role in limiting immunopathological damage to host organs after schistosome infection. Transforming growth factor- β (TGF- β) is an essential factor for the periphery conversion of CD4⁺CD25⁺ T cells into CD4⁺CD25⁺Foxp3⁺ Tregs by inducing the key transcription factor Foxp3. Antigen presenting cells (APCs), which highly express TGF- β , are involved in parasite antigen-induced Treg conversion in peripheral. However, the mechanisms underlying high TGF- β induction in APCs by parasite antigens remain to be clarified during schistosome infection. Here, we demonstrated that *Schistosoma japonicum* stress protein, heat shock protein 60 (SjHSP60), promoted TGF- β production in macrophages (M ϕ). Furthermore, we showed that activation of TLR4-Mal (MyD88 adaptor-like protein) signaling by SjHSP60 is necessary for induction of TGF- β expression in M ϕ , which subsequently promoted Treg induction. Our results not only demonstrate a novel mechanism of TGF- β production in M ϕ for inducing Tregs in mice with schistosomiasis, but also allude to the possibility of targeting parasite stress protein for potential therapeutics.

Keywords

Mal; M ϕ ; schistosomiasis; TGF- β production; TLR4; Treg induction

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

INTRODUCTION

After *Schistosoma japonicum* and *S. mansoni* infection, granuloma is triggered to form around eggs trapped in host liver. Stronger granulomatous response results in severer fibrosis in the liver and eventually causes extensive tissue scarring, which subsequently leads to irreversible impairment of the affected organs especially liver and even death of the host.^{1–3} However, the immunosuppression is typically established shortly in most of the patients after infection and mainly driven by regulatory T cells, transforming growth factor- β (TGF- β) and IL-10, which efficiently diminish the excessive Th2 response, thereby reducing granulomatous response and the severity of immune-mediated liver pathology.^{1,4–6} Immunosuppression in the host during the schistosome infection has been mainly attributed to regulatory cells.⁴ Although multiple regulatory cell types have been identified, CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) remain the most prominent population of immunoregulatory cells during helminth infections described and efficiently limit schistosome-induced immunopathology.^{7–9} Similarly, chronic infections with some other pathogens, such as *Leishmania* parasites and hepatitis B virus, have also been tightly associated with Tregs.^{10–12}

TGF- β plays an essential role in converting periphery CD4⁺CD25[–]T cells into CD4⁺CD25⁺Foxp3⁺ Tregs by targeting Runt-related class of transcription factors (RUNX), which binds to the Foxp3 promoter, to induce Foxp3 expression.^{13–15} Although numerous studies have shown that TGF- β is mass-produced upon infection,^{16–18} we still know very little about the exact mechanisms by which pathogens induce TGF- β expression.

By recognizing pathogen-associated molecular patterns, pattern recognition receptors such as Toll-like receptors (TLRs) sense pathogen invasion, trigger stress responses including innate immunity, and subsequently prime adaptive immunity.^{19–21} Although ligation of TLRs often enhances immunity and inflammation and TLRs are crucial for the host defense, it has become apparent that deregulation of TLR signaling is also strongly associated with immunopathology.^{22–24} However, recent data have indicated that TLR signaling can also lead to immunosuppression, presumably by induction of the immunosuppressive cytokines interleukin (IL)-10, TGF- β , or by enhancement of the expansion and immunosuppressive ability of existed Tregs.^{25–28} Our previous studies also have shown that the *S. japonicum* stress protein HSP60 (heat shock protein 60, SjHSP60) and a SjHSP60- derived peptide SJMHE1 significantly induce immunosuppressive Tregs both *in vitro* and *in vivo* through TLR on APCs (M ϕ or dendritic cells, DCs) by inducing higher levels of TGF- β .^{29,30} However, whether and how schistosome antigens manipulate TLR signaling to promote TGF- β production in APCs and thereby induce Tregs remains to be resolved.

In this study, we identified a novel consequence of TLR4-Mal signaling triggered by schistosome antigen that induces TGF- β production in M ϕ , which results in conversion of CD4⁺CD25[–] T cells into Tregs.

RESULTS

M ϕ from *S. japonicum*-infected mice are sufficient to induce Tregs and exhibit a tolerogenic phenotype characterized by expressing high levels of TGF- β

Studies have shown that tolerogenic APCs are involved in peripheral regulatory T-cell induction by helminth-derived antigens.⁴ M ϕ , one of most important APCs, plays a key role in schistosomiasis japonica.¹ As shown in Figure 1a, b, co-culturing of M ϕ from *S. japonicum*-infected mice with CD4⁺ T cells efficiently increased the proportion of CD4⁺CD25⁺Foxp3⁺ Tregs. M ϕ from spleens and livers of mice infected with *S. japonicum* expressed lower levels of MHC class II, CD40 and CD86 but much significantly higher levels of B7-H1 (Supplementary figure 1a, b), and expressed similar levels of TNF- α and IL-4, but much higher levels of TGF- β and IL-10 (Figure 1c, d) when compared to M ϕ from uninfected mice.

SjHSP60-treated M ϕ display a tolerogenic phenotype characterized by expressing high levels of TGF- β

Similarly, our previous study demonstrated that APCs, for example, M ϕ or DC, are necessary in SjHSP60, a major *S. japonicum* egg antigen, mediated conversion of CD4⁺CD25⁻ T cells into Tregs.³⁰ Here, our result further showed that in contrast to lipopolysaccharide-stimulated M ϕ (LPS-M ϕ), SjHSP60-treated M ϕ (SjHSP60-M ϕ) retained an immature morphology with a more rounded shape (Supplementary figure 1c). FCM (flow cytometry) analysis revealed that less profoundly increased expression of MHC class II and co-stimulatory molecules (CD40, CD80 and CD86), but more significant upregulation of co-inhibitory molecule (programmed death ligand 1, PD-1/B7-H1) on SjHSP60-M ϕ (Supplementary figure 1d). Moreover, SjHSP60-M ϕ expressed relatively low levels of proinflammatory mediators, tumor necrosis factor- α (TNF- α) and IL-12, but much higher immunosuppressive cytokines (TGF- β and IL-10) (Figure 2a, b). Moreover, both latent and active TGF- β showed similar significant increases in supernatants from SjHSP60-M ϕ (Supplementary figure 2).

Production of TGF- β by SjHSP60-M ϕ is necessary for conversion of CD4⁺CD25⁻ T cells into CD4⁺CD25⁺Foxp3⁺ Tregs

TGF- β is one of the most critical factors in the conversion of CD4⁺CD25⁻ T cells into Tregs by inducing the essential Treg transcription factor Foxp3.¹³ Paralleling the increase of Tregs *in vivo* and *in vitro*, serum levels of TGF- β were significantly elevated in both *S. japonicum*-infected mice (Figure 3a) and SjHSP60-injected mice (Figure 3b). In addition, supernatants from co-cultures of CD4⁺CD25⁻ T cells with SjHSP60-M ϕ also contained higher concentration of TGF- β than mock-treated T cells (Figure 3c). Blocking of TGF- β signaling by either a TGF- β RI inhibitor or an anti-TGF- β neutralizing antibody impaired SjHSP60-M ϕ -mediated conversion of Tregs (Figure 3d, e).

SjHSP60-triggered TGF- β production in M ϕ is TLR4 dependent

Studies have suggested that HSP60 is a natural ligand for TLR2 and TLR4,³¹ and we have demonstrated that SjHSP60 induces the conversion of CD4⁺CD25⁻ T cells into Tregs via

TLR4 pathway of M ϕ .³⁰ To further investigate the molecular mechanisms underlying TGF- β production in M ϕ upon S β HSP60 stimulation, TLR2 or TLR4 signaling pathway was blocked using TLR2- or TLR4-deficient M ϕ or using anti-TLR2 or anti-TLR4 blocking antibodies. As shown in Figure 4a, M ϕ from TLR2^{-/-} mice or M ϕ from wild-type (WT) mice treated *in vitro* with an anti-TLR2 blocking antibody produced high levels of TGF- β upon stimulation with S β HSP60. However, M ϕ from TLR4^{-/-} mice or M ϕ from WT mice treated *in vitro* with an anti-TLR4 blocking antibody showed significantly impaired TGF- β production but similar levels of TNF- α , IL-6 and IL-10 after S β HSP60 stimulation (Figure 4b and Supplementary figure 3). Taken together, these results suggested that TGF- β production by M ϕ in response to S β HSP60 stimulation was TLR4-dependent.

S β HSP60-triggered TGF- β production for Treg conversion is MyD88-independent

It is well known that MyD88 is the most universal Toll/ IL-1 receptor (TIR) domain-containing adaptor molecule recruited by almost all TLRs except for TLR3 which mediates TLRs signaling. Consistently, our result in Supplementary figure 4 clearly showed that LPS stimulation induced significantly lower levels of cytokines including TGF- β in M ϕ from MyD88^{-/-} mice. However, surprisingly, similar to M ϕ from WT mice, M ϕ from MyD88^{-/-} mice retained the ability to produce TGF- β mRNA (Figure 5a) and protein (Figure 5b) in response to S β HSP60 treatment *in vitro*. In addition, FCM analysis showed that MyD88 deficiency in S β HSP60-M ϕ did not affect the conversion of CD4⁺CD25⁻ T cells into Tregs (Figure 5c, d). Altogether, these data suggested that MyD88 was not required for TGF- β production by S β HSP60-M ϕ and Treg conversion triggered by S β HSP60 via TLR4.

S β HSP60-induced TGF- β production for Treg conversion is Mal-dependent

Typically, TLR4 initially recruits Mal, also known as TIR domain-containing adaptor protein, and subsequently facilitates the recruitment of MyD88 to trigger the initial activation of NF- κ B.^{19,32} However, previous studies have suggested that Mal also can activate the NF- κ B pathway independently of MyD88.^{33,34} Our results in this study showed that S β HSP60 stimulation of both MyD88^{-/-} and WT M ϕ led to a significant upregulation of Mal mRNA expression (Figure 6a). In addition, TRIF, a TLR adaptor, also can mediate the MyD88-independent pathway from TLR4.^{19,35} However, S β HSP60 stimulation did not significantly change TRIF expression in M ϕ (Supplementary figure 5).

To further investigate whether Mal is the putative adaptor in the S β HSP60-TLR4 signaling pathway that is essential for triggering TGF- β production, specific small interfering (siRNA) duplexes were used to knock down Mal expression in M ϕ upon stimulation with S β HSP60. Mal siRNA duplex treatment efficiently reduced the expression of Mal, but did not change the expression of TLR4 or MyD88 (Figure 6b, c, and Supplementary figure 6). Furthermore, results showed that Mal knockdown significantly reduced TGF- β production but not other cytokines (TNF- α , IL-6 and IL-10) (Figure 6d and Supplementary figure 7) and impaired conversion of Tregs by S β HSP60-M ϕ (Figure 6e, f). Taken together, our results suggested that Mal, as the downstream adaptor of the TLR4 signaling pathway, played an essential role in TGF- β production and Treg conversion triggered by S β HSP60 via TLR4.

DISCUSSION

Compromised host immune responses in lieu of parasite eradication during the chronic infection are often needed for the host to circumvent tissue damage. Induction of immunosuppressive Tregs plays an indispensable role in maintaining immune homeostasis and limiting parasite-induced immunopathology in important host organs.^{8,36–39} TGF- β has been shown to cooperate with TCR stimulation and IL-2 to induce *de novo* Foxp3 expression in T cells and thereby promote Treg induction.^{40–42} However, little is known about the innate immune pathway that initiates or regulates TGF- β production in these cells. Here, we revealed that TLR4-Mal signaling in M ϕ triggered by schistosome antigen SjHSP60 leads to TGF- β expression, delineating a novel mechanism of TGF- β expression in M ϕ that eventually promotes Treg induction and the immunosuppression in mice with *S. japonicum* infection.

Schistosomiasis is a typical chronic infection which well illustrates how parasites can induce the immunosuppressive responses including generation of Tregs.^{8,36–39} However, the mechanisms are never clearly defined. Our previous studies showed that schistosomal stress protein SjHSP60 highly expressed in parasites responding to stresses in the host, and a synthetic peptide SJMHE1 which is homologous to residues 437–460 of SjHSP60, significantly and specifically induce immunosuppressive Tregs both *in vitro* and *in vivo* through TLR.²⁹

TLRs play important roles in both responses to infection stress and innate and adaptive immunity.^{19–21} TLR2 and/or TLR4 are natural receptors and initiate the signal pathway on innate immune cells for HSP60.³¹ Activation of TLR2/4 signaling usually promotes proinflammatory cytokine production from innate immune cells such as M ϕ and DC and subsequently activates the adaptive immune system.¹⁹ In this study, however, our dataset suggest that TLR4 signaling upon SjHSP60 stimulation turned out to mediate the production of anti-inflammatory cytokine TGF- β in M ϕ and hence Treg induction. Thus, our current study further revealed a novel mechanism by which *S. japonicum* can use the host TLR4 signaling pathway to induce Tregs and promote immunosuppression during chronic infection.

The cytoplasmic tail of a TLR can recruit Toll/IL-1 receptor domain-containing adaptors. MyD88, the first such adaptor protein identified, is universally used by all TLRs except TLR3. Mal is another adaptor protein specifically used by TLR2/4. Previous studies have suggested that in the MyD88-dependent pathway, TLR2/4 initially recruits the Mal protein, which subsequently facilitates the recruitment of MyD88 to trigger the activation of NF- κ B, resulting in upregulated expression of proinflammatory cytokines.¹⁹ Mal has also been suggested to be sufficient in the MyD88-independent pathway for the activation of NF- κ B to induce inflammatory cytokines, for example, IFN- β and IFN- γ , although the response is delayed.⁴³ However, to the best of our best knowledge, our data represent heretofore the first demonstration that the adaptor protein Mal, rather than MyD88, mediates the induction of TGF- β from M ϕ to convert Tregs without inducing inflammatory cytokines. This finding underscores a novel mechanism of the induction of immunosuppression to contribute to the restriction of the severe immunopathology in vital host organs.

Together, our results illustrate that TLR-Mal-dependent activation drives TGF- β production in M ϕ in response to schistosomal antigen stimulation, indicating a novel mechanism underlying the Treg induction to regulate the immunopathology in host with schistosome infection. In addition, our study suggests a possible role for the pathogen stress protein in immune crosstalk between the pathogen and its host, which may have important therapeutic implications in exploitation of molecules derived from parasites for allergy, autoimmune diseases and graft-rejection during transplantation.

METHODS

Ethics statement

All animal studies were performed in strict accordance with the guidelines for the Administration of Affairs Concerning Experimental Animals (1988.11.1), and all efforts were made to minimize animal suffering. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University for the use of laboratory animals (Permit Number: IACUC-1703011).

Mice

Female 8-week-old C57BL/6, C57BL/10, TLR2^{-/-} (C57BL/6), TLR4^{-/-} (C57BL/10) and MyD88^{-/-} (C57BL/6) mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All mice were bred in a specific pathogen-free animal facility.

Cell line

The mouse M ϕ cell line RAW264.7 (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's minimal essential medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) and antibiotics (penicillin and streptomycin; Gibco).

Expression and purification of SjHSP60

SjHSP60 was prepared as previously described in Supplementary Methods.³⁰

Infection of mice with *S. japonicum*

C57BL/6 mice were infected percutaneously by abdominal skin exposure for 20 min to 12 *S. japonicum* cercariae of the Chinese mainland strain from infected snails (*Oncomelania hupensis*) purchased from the Jiangsu Institute of Parasitic Diseases (Wuxi, China).

Immunization

Twelve C57BL/6 mice were randomly divided into two immunized groups consisting of six mice in each group. Mice were subcutaneously (s.c., inguinal region) injected twice with 100 μ L of 1:1 (v/v) mixture of antigen (20 μ g SjHSP60 or PBS alone) and incomplete Freund's adjuvant (Sigma-Aldrich) on days 0 and 14. Serum samples were collected 10 days after the last injection.

Magnetic cell sorting

Single-cell suspensions of spleens and LNs (axillary, inguinal and mesenteric lymph nodes) were used to isolate CD4⁺ T or CD4⁺CD25⁻ T cells by negative selection, respectively, using CD4⁺ T cell or CD4⁺CD25⁺ regulatory T cell isolation kit for magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany), achieving >92% purity as determined by FCM analysis.

To purify CD11b⁺ M ϕ , splenic mononuclear cells were isolated using Percoll density-gradient centrifugation as described previously,⁴⁴ and then incubated with anti-CD11b (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the isolated CD11b⁺ M ϕ was >95%.

Cell culture

To investigate the impact of SjHSP60 on M ϕ , SjHSP60 (0.1 μ g mL⁻¹ in 24-well plates at 2.5×10^5 cells mL⁻¹ per well) was used to *in vitro* stimulate RAW264.7 cells, CD11b⁺ M ϕ purified from TLR2, TLR4, or MyD88 deficient mice and their control littermates, or RAW264.7 cells/purified M ϕ treated with 20 μ g mL⁻¹ anti-TLR2/TLR4 blocking antibody or isotype antibody (eBioscience, San Diego, CA). After 2 days, stimulated cells and culture supernatants were collected.

To investigate Treg conversion *in vitro*, M ϕ (1×10^5 cells well⁻¹) which had been cultured under the same conditions as described previously were extensively washed, suspended in fresh medium, or CD11b⁺ M ϕ (1×10^5 cells well⁻¹) were purified from normal or *S. japonicum*-infected mice at 13 weeks post-infection, and then co-cultured in triplicates with purified CD4⁺ T or CD4⁺CD25⁻ cells (2×10^5 cells well⁻¹) in 96-well round-bottom culture plates. After 3 days, supernatants were harvested for cytokine quantification by ELISA, and/or cells were collected for FCM analysis. In some co-cultures, a neutralizing antibody to TGF β 1 (5 μ g mL⁻¹; R&D Systems, Inc. Minneapolis, MN) or an inhibitor of TGF- β RI signaling (20 μ mol L⁻¹; SB-431542; Sigma-Aldrich) was added.

Immunofluorescence staining and FCM

To evaluate the induction of CD4⁺CD25⁺Foxp3⁺ Tregs, the cells from co-cultures were stained using the Mouse Regulatory T Cell Staining Kit (eBioscience).

For phenotypic analysis of M ϕ , single-cell suspensions of splenocytes or liver cells from each mouse or from *in vitro* cultures were prepared and surface stained with anti-CD11b-APC and anti-F4/80-FITC. The cells were washed and then stained with PE-conjugated antibodies against MHC II, CD40, CD80, CD86, B7-H1, or the isotype control antibodies (eBioscience).

To detect expression of TNF- α , IL-4, IL-6, TGF- β and IL-10 in M ϕ , single-cell suspensions of splenocytes or liver cells from each mouse or from *in vitro* cultures were prepared. Each sample (1×10^6 cells) was stimulated with 25 ng mL⁻¹ phorbol myristate acetate and 1 μ g mL⁻¹ ionomycin (Sigma-Aldrich, St Louis, MO) in complete RPMI 1640 medium in the presence of 1 μ L mL⁻¹ Golgistop (BD Biosciences, San Jose, CA) for 6 h at 37°C in 5% CO₂. After 6 h, the cells were collected and surface stained with anti-CD11b-APC

(eBioscience) and anti-F4/80-FITC (eBioscience). The cells were washed, fixed, permeabilized with Cytofix/Cytoperm buffer (BD Biosciences) and subsequently intracellularly stained with PE-conjugated antibodies against TNF- α , IL-4, IL-6, TGF- β 1, IL-10 or isotype antibodies (eBioscience) as control.

Following staining, cells were analyzed by FCM using a FACS Calibur (BD Biosciences) and CellQuest software (BD Biosciences).

siRNA duplexes and transfections

The siRNA duplexes (ON-TARGETplus SMARTpool and siGENOME SMARTpool, Dharmacon, Lafayette, CO) target murine Mal were purchased. The specific siRNA sequences for Mal are listed in Supplementary table 1.

M ϕ were plated in 6-well plates at 1.0×10^6 cells per well in 2 mL of DMEM supplemented with 10% FBS. After 24 h of incubation, M ϕ was transfected with Mal siRNA or control siRNA. The transfection procedures were performed following the manual of the FuGENE HD Transfection Reagent (Roche Applied Science, Meylan, France). The silencing effect was assayed by qPCR or western blot at 2 days post-transfection.

Quantitative real-time PCR (qPCR)

Total RNA from cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed with random hexamers using the SuperScript III First-Strand cDNA Synthesis System (Invitrogen). The synthesized cDNA samples were then used as templates for qPCR performed on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the FastStart SYBR Green Master Mix (Roche Applied Science). Gene expression levels were normalized and shown as fold increases over controls. The previously described primers used for qPCR are listed in Supplementary table 2.^{45–54}

Cytokine assays

Levels of TNF- α , IL-12, IL-10 and TGF- β in supernatants or serum samples were determined by specific sandwich ELISAs using commercial kits (Bender MedSystems, Biovision, CA) according to the manufacturer's instructions. The amounts of active and latent TGF- β in M ϕ supernatants were also determined with ELISA kits (Biolegend, San Diego, CA). The cytokine concentration in each supernatant was extrapolated from a standard curve.

Western blot analysis

M ϕ transfected with either Mal siRNA or control siRNA were washed with PBS and disrupted with lysis buffer (50 mmol L⁻¹ Tris-HCl, 250 mmol L⁻¹ NaCl, 5 mmol L⁻¹ EDTA, 1 mmol L⁻¹ dithiothreitol, 5% glycerol, 0.2% Nonidet P-40 at pH 8) containing a protease inhibitor cocktail (Roche Applied Science). Protein concentrations were determined using the DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of whole cell lysates (50–100 μ g) were loaded in each lane for separation by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking in Tris-buffered saline containing Tween 20 (0.1%) (T-TBS) and milk (5%) at room temperature for 2 h, the membrane was then incubated at

4°C overnight with primary antibodies. After washing with T-TBS, the membrane was incubated at room temperature for 1 h with HRP-conjugated anti-rabbit IgG secondary antibody (Cell Signaling Technology, Danvers, MA). The primary anti-Mal (Abcam, Cambridge, UK), anti-MyD88 (Cell Signaling Technology), anti-TRIF (Novus Biologicals, Littleton, CO), anti-TLR-4 (Novus Biologicals) and anti- β -actin (Sigma- Aldrich) antibodies were diluted and used as described in the manufacturer's instructions. The density of each band was quantified by densitometric analysis with Image J software (Image Processing and Analysis in Java) from the NIH (<https://imagej.nih.gov/ij/>).

Statistical analysis

Statistical analysis was performed using the SPSS program (version 11.0 for Windows; SPSS, Inc., Chicago, IL). The differences between two groups were analyzed by the Student's *t*-test. The differences between more than two groups were analyzed by one-way analysis of variance (ANOVA) with an LSD post hoc test. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Mφ from *S. japonicum*-infected mice are sufficient to induce Tregs and exhibit a tolerogenic phenotype characterized by expressing high levels of TGF-β and IL-10. **(a, b)** Purified CD4⁺ T cells from normal mice were co-cultured with Mφ from normal or *S. japonicum*-infected mice for 3 days. CD4⁺CD25⁺Foxp3⁺ Tregs were analyzed by FCM and cells were gated on CD4⁺ T cells. **(c, d)** At 13 weeks post-infection, single-cell suspensions of splenocytes or liver cells from *S. japonicum*-infected mice were stimulated with phorbol myristate acetate and ionomycin in the presence of Golgistop for 6 h. Cells were surface stained with anti-CD11b and anti-F4/80 then intracellularly stained with antibodies against TNF-α, IL-4, IL-6, IL-10, TGF-β1, or isotype antibody and analyzed by FCM. Cells were gated on CD11b⁺F4/80⁺ Mφ. FCM plots are representative of three independent experiments. Data are means ± s.d. of triplicate cultures or 5 mice and representative of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Figure 2.

SjHSP60-treated M ϕ display a phenotype resembling that of tolerogenic M ϕ characterized by expressing high levels of TGF- β and IL-10. **(a)** Purified M ϕ from normal mice were cultured for 2 days in medium alone or in the presence of $0.1 \mu\text{g mL}^{-1}$ SjHSP60 or $1 \mu\text{g mL}^{-1}$ LPS. The mRNA expression of cytokines in M ϕ were determined by qPCR and expressed as fold increases over control. **(b)** ELISA quantification of cytokines in supernatants of M ϕ cultures. Data are means $\pm$ s.d. of triplicate cultures and representative of three independent experiments. ** $P < 0.01$.

Figure 3.

Production of TGF-β by SjHSP60-Mφ is necessary for conversion of CD4⁺CD25⁻ T cells into Tregs. **(a)** At 0, 3, 5, 8 and 13 weeks post-infection, serum TGF-β levels were measured by ELISA. **(b)** ELISA measurements of TGF-β in serum samples of mice injected with SjHSP60 or PBS. **(c)** ELISA measurements of cytokines in supernatants of purified CD4⁺CD25⁻ T cells cultured for 3 days in medium alone or with Mφ that had been unstimulated or exposed to SjHSP60 or LPS for 2 days. **(d)** Mφ were prestimulated with 0.1 μg mL⁻¹ SjHSP60 or medium for 2 days. Purified CD4⁺CD25⁻ T cells were cultured for 3 days with medium, SjHSP60, or prestimulated Mφ above. Co-cultures were set up with or without SB-431542 (20 μmol L⁻¹) or neutralizing TGF-β1 antibody (5 μg mL⁻¹). Frequency of CD4⁺CD25⁺Foxp3⁺ Tregs was determined by FCM. FCM plots are representative of three independent experiments with similar results and cells were gated on CD4⁺ T cells. **(e)** The bar graph indicates average percentages ± s.d. of Tregs. Data are expressed as means ± s.d. of 6 mice or triplicate cultures and representative of two or three independent experiments. **P* < 0.05, ***P* < 0.01.

Figure 4.

SjHSP60-triggered TGF- β production in M ϕ is TLR4-dependent. **(a, b)** Purified M ϕ from TLR2^{-/-}, TLR4^{-/-} mice or control WT littermates were stimulated with or without 0.1 μ g mL⁻¹ SjHSP60 for 2 days. In some cultures, purified M ϕ from control littermates were pretreated with medium alone, or with 20 μ g mL⁻¹ of anti-TLR2, anti-TLR4 blocking or isotype-matched antibodies for 1 h at 4°C. After stimulation, TGF- β production was detected by ELISA. Data are means $\pm$ s.d. of triplicate cultures and representative of three independent experiments. ** $P < 0.01$.

Figure 5.

SjHSP60-triggered TGF- β production and Treg conversion are MyD88-independent. **(a, b)** MyD88^{+/+} or MyD88^{-/-} Mφ were stimulated with 0.1 $\mu\text{g mL}^{-1}$ SjHSP60 for 2 days. TGF- β mRNA expression was detected by qPCR and expressed as fold increases over unstimulated MyD88^{+/+} Mφ **(a)**. Culture supernatants were collected for determining TGF- β production by ELISA **(b)**. **(c, d)** Purified CD4⁺CD25⁻ T cells were co-cultured with MyD88^{+/+} or MyD88^{-/-} Mφ prestimulated with 0.1 $\mu\text{g mL}^{-1}$ SjHSP60 or medium. After 3 days, percentages of CD4⁺CD25⁺Foxp3⁺ Tregs were analyzed by FCM. FCM plots are representative of three independent experiments with consistent results and cells were gated on CD4⁺ T cells. The bar graph indicates average percentages $\pm$ s.d. of Tregs. Data are means $\pm$ s.d. of triplicate cultures and representative of two or three independent experiments. $^{**}P < 0.01$.

Figure 6.

SjHSP60-triggered TGF- β production and Treg conversion are Mal-dependent. **(a)** Purified M ϕ from MyD88^{-/-} mice or control WT littermates were cultured with or without 0.1 μ g mL⁻¹ SjHSP60 for 2 days. Mal mRNA levels were determined by qPCR and expressed as fold increases over unstimulated MyD88^{+/+} M ϕ . **(b, c)** Effect of specific siRNA on endogenous Mal expression in RAW264.7 cells was analyzed by qPCR **(b)** and Western blot **(c)**. **(d, e)** RAW264.7 cells transfected with Mal siRNA or control siRNA were stimulated with 0.1 μ g mL⁻¹ of SjHSP60. After 2 days, TGF- β levels in supernatants were detected by ELISA **(d)**, while cells were washed and co-cultured with purified CD4⁺CD25⁻ T cells for three additional days. Percentages of CD4⁺CD25⁺Foxp3⁺ Tregs in co-cultures were measured by FCM and cells were gated on CD4⁺ T cells **(e)**. **(f)** The bar graph indicates average percentages $\pm$ s.d. of Tregs. Western blots and FCM plots are representative of three independent experiments with consistent results. Data are means $\pm$ s.d. of triplicate cultures and representative of three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001.