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METHODS

In vitro modulation of spleen cells by live schistosome worms.

A Puerto Rican strain of *S. mansoni* was maintained by passage in albino *Biomphalaria glabrata* snails. BALB/c mice were infected percutaneously with 200 male and female cercariae to produce worms. Worms were isolated by portal perfusion of infected mice using conditions optimized to reduce damage or stress to worms. Spleen cells from IL-10-eGFP mice ($1x10^7$ per well) were seeded into 12 well transwell culture plates (Costar). 5 male worms were then placed in the transwell insert. The cells/worms were incubated at 37°C, 5 % CO_2 for 48 hours. As a control, cells were cultured in transwell plates with media alone. The worms were removed and the cells harvested and washed three times with fresh media and checked for viability[1, 2]. B cell populations were isolated by cell sorting (see Methods in the article).

Lung immunology

Processing of lung tissue and immunological analysis was as described[1, 3, 4]. In brief, the following was carried out:

BAL

Bronchoalveolar lavage (BAL) fluids were collected by cannulating the trachea and lavaging the lungs twice with 0.8 ml ice-cold PBS. BAL cells were pelleted, washed, and counted. BAL fluid was stored at –80°C for cytokine/chemokine ELISAs. BAL cells were used for cytospins or flow cytometry, see below. For cytospins, the numbers of eosinophils, neutrophils, macrophages and lymphocytes was determined by performing a differential count, with at least 400 cells/slide, on Giemsa-stained cytocentrifuge preparations.

Lung homogenates and digests

Whole lungs were removed from mice and either snap-frozen, placed in 10% formaldehyde-saline for histology, or digested with collagenase-D (Roche) for flow cytometry analysis, see below.

Lung Histology

Lungs were fixed in 10% formaldehyde-saline for histology. Paraffin-embedded tissue sections were stained with hematoxylin and eosin, and eosinophil infiltration was counted on Giemsa-stained sections. Airway mucus occlusion was analyzed on PAS-stained lung sections. A semi-quantitative score was used for airway occlusion as follows: 0, 0-10% occlusion; 1, 10-30% occlusion; 2, 30-60% occlusion; 3, 60-90% occlusion; and 4, 90-100% occlusion, as described[5]. For each mouse the mean score was determined from at least 10 individual airways.

Flow cytometry on BAL cells

BAL cells were prepared for surface staining as described previously[4]. Cell surface marker expression was assessed by flow cytometry using a CyAn (Beckman Coulter). Data were analyzed using FlowJo (Tree Star) software. Cells were stained with BD Biosciences mAbs; PerCP anti-CD4 (RM4-5), PerCP-Cy5.5 anti-CD19 (1D3), PerCP anti-CD8a (53-6.7) and PE anti-Siglec-F (E50-2440). Caltag mAbs; APC anti-CD25 (PC61 5.3). R&D Systems PE anti-CCR3 (83101). Flow cytometry buffer (PBS, 2% FCS, 0.05% sodium azide and 0.5µM EDTA) contained EDTA to exclude doublets. In brief, BAL cells were first gated on CD19, CD4 and CD8 vs forward side scatter (FSC). Lymphocytes were identified as FSC^{lo}, SSC^{lo} $CD19^{+}$, $CD4^{+}$ and $CD8^{+}$. Eosinophils distinguished as SSC^{hi}, $CD19^{-}$, $CD4^{-}$, $CD8^{-}$, ~~$CCR3^{+}$,~~ non-autofluorescent granulocytes that stained positive for CCR3 and/or SiglecF. Mononuclear cells were characterised as large (FSC^{hi}), granular (SSC^{hi}), autofluorescent cells that were CD4, CD8 and CD19 negative i.e. not in the lymphocyte gate. Macrophages were characterized as large highly autofluorescent mononuclear cells that were confirmed to be CD11b (Mac-1)$^{+}$ or F4/80^{hi}.

Cell preparation and Flow cytometry for spleen, lung, lung draining mediastinal lymph nodes and mesenteric lymph nodes

Spleen, lungs, lung draining mediastinal lymph nodes and mesenteric lymph nodes were collected and cells isolated for culture or flow cytometry analyses. Single cell suspensions were prepared from all the organs and erythrocytes lysed with 0.78% ammonium chloride solution where needed.

For surface staining, single cell suspensions were prepared in flow cytometry buffer (PBS, 2% FCS, 0.05% sodium azide and 0.5μM EDTA). Cells were blocked using Fc-block CD16/CD32 (2.4G2) (BD Biosciences). Biotin or directly conjugated antibodies with fluorescein isothiocyanate (FITC), r-phycoerythin (PE), peridinin-chlorophyll-protien complex (PerCP), allophycocyanin (APC) or Pacific Blue (PB) were used. Anti-CD1d (1B1), anti-CD5 (53-7.3), anti-CD62L (MEL-14), anti-IgM (11/41) and anti-IgD (11-26c.2a) were from BD Biosciences while, anti-CD4 (RM4-5), anti-CD19 (1D3), anti-CD21 (7G6), anti-CD23 (B3B4), anti-CD25 (PC61), anti-CD43 (R2/60) and anti-CD138 (281-2) were from eBioscience. All antibodies were used at optimal concentration after titration experiments. Cells were acquired using a Cyan (ADP Analyzer, Beckman Coulter). Gating of cells was based on the specific isotype control values as well as fluorochrome minus one (FMO) setting when needed[6]. All analyses were performed using FlowJo software (Tree Star Inc., Ashland, OR, USA). For intracellular FoxP3 staining, cells were first stained for surface CD4 and CD25 expression followed by fixation, permeablization and intracellular staining using Cytofix/Cytoperm Fixation/Permeablization Kit (BD Bioscience) according to the manufacturers protocol. Antibodies used were PE conjugated anti-FoxP3 (NRRF-30) and its matched isotype control rat IgG2a,κ (eBiosceinces). For intracellular IL-10, cells were cultured for 8h at $1x10^6$ cells/ml in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FCS (Labtech), 2mM L-glutamine (Invitrogen) and 50u/ml penicillin and 50 μg/ml streptomycin (Invitrogen), stimulated with PMA/Ionomycin 2.5 ng/ml and 250 ng/ml respectively or, cultured with worms or medium, in the presence of 10 μg/ml Brefeldin A (Sigma-Aldrich). In the cultures with worms the worms were taken out before Brefeldin A was added. Surface markers were stained first, followed by intracellular IL-10 (JES5-2A5) or matched isotype control IgG1 staining (Caltag).

Tetramer staining.

PE-conjugated CD1d-tetramers loaded with a-galactosylceramide (αGalCer)[7] were provided by Prof. Mitch Kronenberg. Streptavidin-PE labeled PBS-57-loaded tetramers specific for mouse CD1d[8] were obtained from Dr. May Stout at the NIH Tetramer Core Facility, Atlanta, US. Empty tetramers were used as an isotype control. In all experiments cells from $CD1d^{-/-}$ or $J\alpha18^{-/-}$ mice were included as additional controls for gating. First, cells were blocked with Fc-block and neutravidin (Molecular Probes, US; 0.4 $\mu g/10^6$ cells) for 15 min at RT to block non-specific binding. Cells were then incubated with FITC-conjugated anti-TCRβ and PE-loaded or unloaded tetramers for 30 min on ice. Stained cells with unloaded tetramers were used to set appropriate gates for CD1d-tetramer positive cells.

Active systemic anaphylaxis model.

For the induction of active systemic anaphylaxis, Penicillin V (Pen V)-OVA and Pen V-BSA conjugates were used, as described[1]. In brief, mice were sensitized by i.p. injection of 500 µg of Pen V-OVA with 2 x 10^9 *Bordetella pertussis* (Wako Pure Chemical) and 1.0 mg of Imject alum (Pierce). On day 14, anaphylaxis was elicited by i.v. challenge with 100 µg of Pen V-BSA. Temperature transponders (Bio Medic Data Systems) were implanted s.c. into recipient mice and temperatures recorded electronically (DAS-6007, Bio Medic Data Systems) just before challenge and subsequently every 10 min for the next 60 min.

Figure legends

Figure E1.

OVA sensitization protocol for acute and established airway inflammation.

A. Acute sensitization model: Mice were sensitized with 20 μg OVA in alum i.p. on day 0 and 14. Airway challenge with 1% OVA was performed on days 21-24, this concurrent with transfer of Breg cells. Lung analysis was performed on day 25. **B.** Established model: Mice were sensitized with 20 μg OVA in alum i.p. on day 0 and 12. Mice received airway challenge with 5% OVA on days 16-19 and days 25-28. On days 36-39 mice received Breg cells and 5% OVA aerosol. On day 44 mice received 5 % OVA aerosol challenge. Lung analysis was performed on day 45.

Figure E2.

Characterization of different B cell subpopulations in spleens of uninfected and worm-infected mice.

Representative flow cytometry plots for uninfected and infected mice, and the absolute numbers for each subpopulation are shown; B cell ($CD19^+$), B1 B cells ($CD19^+IgM^+CD43^+IgD^-$), B2 B cells ($CD19^+IgM^{low}IgD^+CD43^-$), B1a B cells ($CD19^+CD5^+$), T1 B cells ($CD19^+IgD^-CD62L^-CD21^-CD23^-$), T2 B cells ($CD19^+IgD^+CD62L^+CD21^+CD23^+$), Marginal zone B cells (MZ B cell) ($CD19^+CD21^+CD23^-$), Follicular zone B cell (FO B cell) ($CD19^+CD21^-CD23^+$), $CD1d^{high}$ B cells ($CD19^+CD1d^{high}$) and Plasma cells ($CD19^-CD138^+$). Cell populations are gated on live cells first and then on $CD19^+$ B cells with 20,000 cells shown. Plasma cells are gated on live cells and then $CD19^-$ cells.

Figure E3.

Role for CD1d in *S. mansoni*-mediated protection against allergic airway inflammation and anaphylaxis.

A. Lung resistance (R_L) in response to methacholine of OVA-sensitized uninfected mice, and infected mice treated with an anti-CD1d mAb or a control mAb. Data represent the mean ± SEM change from baseline values for each group. **B.** Drop in temperature in Penicillin-V-sensitized uninfected mice, and infected mice treated with an anti-CD1d mAb or a control mAb. Anaphylaxis was induced by i.v. injection of Penicillin-V. Data is Mean ± SEM change in temperature.

Figure E4.

S. mansoni-infected mice are refractory to allergic airway inflammation independent of iNKT cells.

Flow cytometry plots and absolute numbers of iNKT cells, identified by staining with mCD1d-PBS056 loaded tetramer and anti-TCRβ mAb, in **A.** spleens and **B**. lungs of uninfected and infected wild type (WT) and $CD1d^{-/-}$ mice that were sensitized to OVA or PBS. **C**. Lung resistance (R_L) in response to methacholine of OVA-sensitized uninfected and infected wild type (WT) and $J\alpha18^{-/-}$ mice.

Figure E5.

Expansion of a $CD1d^-$ B cell population that exacerbates allergic airway inflammation, in the spleens of worm-infected mice.

A. Representative flow cytometry images of increased $CD1d^-$ B cells in spleen cells from infected mice compared to uninfected mice. **B.** Flow cytometry histograms showing gated $CD1d^-$ $CD19^+$ B cells from infected mice compared with whole $CD19^+$

population from uninfected mice and isotype control for expression of CD23, CD21, CD5, IgD and IgM. **C.** Lung resistance (R_L) in response to methacholine of OVA-sensitized uninfected mice transferred Breg cells or $CD1d^-$ B cells sorted from the spleens of infected mice. Recipients of $CD1d^-$ B cells died from 30-60 mg/ml methacholine exposure. **D.** Total cell and **E**, eosinophil counts in BAL from mice treated as described. Data are the mean ± SEM. Student's *t*-test was used to test for statistical difference between groups as indicated: $^{**}P<0.01$; $^{***}P<0.001$

Figure E6.

B regulatory cells generated *ex vivo* by worms mediate protection against allergic airway inflammation, and induce T regulatory cells *in vivo*.

Spleen cells from uninfected mice were cultured for 48h with 5 worms or in medium. **A.** Spleen cells from IL-10-eGFP mice cultured in media or with worms for flow cytometry. Following culture, the $IL\text{-}10^+$-eGFP spleen cells were gated and the numbers of $CD19^+$ B cells plotted. $CD19^+IL\text{-}10^+$ B cells from worm cultures (blue histogram) or media (red histogram) are shown. $CD19^+IL\text{-}10^+$ cells induced by worms express CD1d (blue histogram); isotype controls (black histogram). **B.** Representative hematoxylin and eosin-stained sections of lungs from OVA-sensitized mice that received *ex vivo* generated Breg cells. **C.** Lung resistance (R_L) in response to methacholine, of OVA-primed mice receiving *ex vivo* generated Breg cells. Untreated mice (PBS-Alone) or OVA sensitized mice (OVA-Alone) were used as controls. **D**. Total number of Treg cells ($CD4^+CD25^+Foxp3^+$) detected in BAL of mice sensitized with OVA and receiving no cells, Breg cells from medium cultures and *ex vivo* worm generated Breg cells. Student's *t*-test was used to tests for statistical difference between groups as indicated: $^{**}P<0.01$

Online Repository References

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