IL-4Rα-Independent Expression of Mannose Receptor and Ym1 by Macrophages Depends on their IL-10 Responsiveness

Benjamin G. Dewals1,2*, Reece G. Marillier1,2, Jennifer C. Hoving1,2, Mosiuoa Leeto1,2, Anita Schwegmann1,2, Frank Brombacher1,2*

1 International Centre for Genetic Engineering and Biotechnology (ICGEB), University of Cape Town, Cape Town, South Africa, 2 Division of Immunology, Institute of Infectious Disease and Molecular Medicine (IIDMM), University of Cape Town, Cape Town, South Africa

Abstract

IL-4Rα-dependent responses are essential for granuloma formation and host survival during acute schistosomiasis. Previously, we demonstrated that mice deficient for macrophage-specific IL-4Rα (LysMcreIl4ra−/lox) developed increased hepatotoxicity and gut inflammation; whereas inflammation was restricted to the liver of mice lacking T cell-specific IL-4Rα expression (iLckcreIl4ra−/lox). In the study presented here we further investigated their role in liver granulomatous inflammation. Frequencies and numbers of macrophage, lymphocyte or granulocyte populations, as well as Th1/Th2 cytokine responses were similar in Schistosoma mansoni-infected LysMcreIl4ra−/lox liver granulomas, when compared to iLckcreIl4ra−/lox control mice. In contrast, a shift to Th1 responses with high IFN-γ and low IL-4, IL-10 and IL-13 was observed in the severely disrupted granulomas of iLckcreIl4ra−/lox and LysMcreIl4ra−/lox mice. As expected, alternative macrophage activation was reduced in both LysMcreIl4ra−/lox and iLckcreIl4ra−/lox granulomas with low arginase 1 and heightened nitric oxide synthase RNA expression in granuloma macrophages of both parasite strains. Interestingly, a discrete subpopulation of SSChighCD11b1-+I-A/I-Ehi+CD204+ macrophages retained expression of mannose receptor (MMR) and Ym1 in LysMcreIl4ra−/lox but not in iLckcreIl4ra−/lox granulomas. While aaMφ were in close proximity to the parasite eggs in Il4ra−/lox control mice, MMR/Ym1+ macrophages in LysMcreIl4ra−/lox mice were restricted to the periphery of the granuloma, indicating that they might have different functions. In vivo IL-10 neutralisation resulted in the disappearance of MMR/Ym1+ macrophages in LysMcreIl4ra−/lox mice. Together, these results show that IL-4Rα-responsive T cells are essential to drive alternative macrophage activation and to control granulomatous inflammation in the liver. The data further suggest that in the absence of macrophage-specific IL-4Rα signalling, IL-10 is able to drive mannose receptor- and Ym1-positive macrophages, associated with control of hepatic granulomatous inflammation.

Introduction

Schistosomiasis is a severe parasitic disease with more than 200 million people infected worldwide with an estimated 250,000 deaths per annum in sub-Saharan Africa alone [1,2]. In the murine model, mice infected with Schistosoma mansoni develop a severe liver pathology with granulomatous inflammatory responses directed towards the parasite eggs. During chronic infections, Th2-type inflammation in the liver results in fibrosis, which leads to portal hypertension, bleeding from collateral vessels and ultimately death [3,4]. At peak egg excretion, Th2-mediated granuloma formation appears to be indispensable for host protection and control of egg-induced inflammation.

Previous studies have demonstrated signalling via interleukin 4 receptor α-chain (IL-4Rα) to be essential for granuloma formation and host survival [5,6,7,8,9]. The cellular contributions of IL-4Rα to the mechanisms conferring protection to the host can be dissected using mice with IL-4Rα expression disrupted on specific cell types. Infection of macrophage/neutrophil-specific (LysMcreIl4ra−/lox) and T cell-specific (iLckcreIl4ra−/lox) IL-4Rα-deficient mice showed these mice to have high mortality during acute schistosomiasis irrespective of granuloma formation [6,10]. Eight weeks after infection, the absence of IL-4Rα-dependent T cell responses resulted in severe hepatotoxicity while an absence of IL-4Rα-activated macrophages was responsible for the development of severe inflammation and damage in both intestine and liver, with a subsequent increase in serum lipopolysaccharide (LPS) levels and aspartate transaminase levels in the serum, respectively [6]. Though the mechanism(s) explaining the high mortality rates observed in LysMcreIl4ra−/lox mice is(are) not fully understood, the observed increased Th1/type 1 in presence of normal Th2/type 2 cytokine and antibody systemic responses could be detrimental for the host survival [6].

Tissue macrophages were recently classified into three major categories based on their functions [11]. “Classical” macrophage...
Author Summary

Schistosomiasis is a tropical disease caused by one of the species of the parasitic worm *Schistosoma* which infects over 200 million people worldwide. Signalling via the IL-4 receptor alpha (IL-4Rα), which is the common receptor chain for the ligands IL-4 and IL-13, is essential for inducing protective Type 2 immune response and granuloma formation in response to the parasite eggs. In experimental *Schistosoma mansoni* infection and egg-induced inflammation studies with cell type-specific IL-4Rα-deficient mice, the role of IL-4Rα-activated alternative macrophages (aaMφ) and IL-4Rα-responsive T cells was investigated with focus on the control of hepatic inflammation and granuloma formation. Interestingly, aaMφ were not essential for the cellular composition or the Th1/Th2 cytokine profile in liver granulomas. In contrast, IL-4Rα-dependent T cell responses were important for predominant Th2 and IL-10 responses, as well as the presence of aaMφ in the granulomas, avoiding major disruption in the granuloma cell composition. Moreover, a macrophage subpopulation was identified and those cells expressed the two aaMφ markers, mannose receptor- and Ym1 in an IL-4Rα-independent but IL-10-dependent manner. These cells might be involved in the control of inflammation.

activation occurring during Th1 immune responses, essentially via IFN-γ signalling is involved in cellular immunity against intracellular pathogens via the production of pro-inflammatory molecules and nitric oxide (NO). Macrophages can also develop a “deactivated” phenotype in association with IL-10 and TGF-β signalling. “Alternative” macrophage activation occurs via IL-4 and IL-13 signalling through their heterodimeric IL-4R during Th2 immune responses [11,12,13]. Alternative activation of macrophages results in the downstream activation of various molecules/markers among which arginase 1 (Arg-1) has been considered to be decisive for their functional activities [11]. Earlier studies suggested that alternatively-activated macrophages (aaMφ) may be important regulators of wound healing via arginase-1 activation. Arg-1 catalyses L-arginine to produce polyamines and proline, an important factor for collagen deposition [14,15,16]. More recent studies in LysM<sup>Cre<sup>Il4ra</sup></sup> mice showed that aaMφ are involved in immuno-modulation, suppression or pathology in infectious diseases such as schistosomiasis, cutaneous leishmaniasis and cryptococcosis, as well as in non-infectious diseases such as insulin resistance, EAE, and tissue repair. In these studies, IL-4Rα-deficient aaMφ have been shown to influence innate and adaptive immune responses with both beneficial or detrimental outcomes, depending on the nature of the disease [6,17,18,19,20,21,22]. Arg-1 activation by IL-4Rα signalling is able to inhibit the activation of inducible nitric oxide synthase (iNOS) and therefore NO production, directly repressing classical macrophage activation. In a recent study, mice selectively lacking Arg1 expression in macrophages during schistosomiasis were less susceptible than LysM<sup>Cre<sup>Il4ra</sup></sup> mice and developed higher Th2 cytokine responses and antigen-specific Th2 cell proliferation [23].

Besides inducing Arg-1, IL-4Rα signalling induces a large number of markers in macrophages [11,24]. Of these, macrophage mannose receptor (MMR, *Mrc1*), “resistin-like” (Relm) (also referred as “found in inflammatory zone” [FIZZ]) molecules and chitinase-like molecules (also referred as “Ym”) have come to be considered as markers of the aaMφ phenotype [11,25,26]. MMR, a C-type lectin, is induced in granulomas during schistosomiasis and is involved in the uptake of soluble antigens by antigen presenting cells [25,27,20]. Resistin-like molecule alpha (Relma/Relmα/FIZZ1) upregulation has been recently shown to play essential roles in suppression of helminth-induced Th2 inflammation [29,30]. Finally, though the chitinase-3-like 3 molecule (Ym1, *Ch3L3*) is strongly upregulated in macrophages during Th2-type immunity, its role remains unclear during helminth infection [31,32]. It is also apparent that these markers can be upregulated independently of IL-4 and IL-13, with IL-10 in particular being able to induce the expression of MMR along with other factors including glucocorticoids [33,34]. In addition, innate alternative activation of macrophages in response to chillin has also been reported [35]. Expression of these markers might therefore not be exclusively dependent on IL-4/IL-13 responsiveness of macrophages.

Although the absence of IL-4Rα-dependent aaMφ and T cell responses during schistosomiasis has been mainly studied at the systemic level [6,10], little is known of their specific role(s) in the liver granuloma microenvironment. As the major alterations of the immune response depending on IL-4Rα might be restricted to the direct environment surrounding the parasite eggs, we focus in this study on the liver granuloma level to investigate how macrophage- or T cell-specific IL-4Rα responses affect the local responses directed against *Schistosoma* eggs. Our results demonstrate that the Th1/Th2 balance and the cellular composition of liver granulomas are not affected during infection of LysM<sup>Cre<sup>Il4ra</sup></sup> mice; but are both severely modified in *iLck<sup>Cre<sup>Il4ra</sup></sup>* mice developing granuloma cytokine and cellular responses very similar to the global *Il4ra<sup>-/-</sup>* mice. Although LysM<sup>Cre<sup>Il4ra</sup></sup> and *iLck<sup>Cre<sup>Il4ra</sup></sup>* granuloma macrophages show a global reduction of expression of aaMφ markers, we demonstrate that MMR and Ym1 protein expressions remain high in LysM<sup>Cre<sup>Il4ra</sup></sup> granuloma macrophages. This IL-4Rα-independent MMR*Ym1<sup>+</sup> subpopulation was restricted at the periphery of LysM<sup>Cre<sup>Il4ra</sup></sup> granulomas, in contrast to *Il4ra<sup>-/-</sup>* control mice where those cells were in close proximity to the parasite eggs. We finally used a parasite egg model with IL-10R blockade, and demonstrate that in absence of macrophage-specific IL-4Rα expression, IL-10 signalling can induce the expression of MMR and Ym1 in macrophages.

### Methods

#### Mice

Macrophage/neutrophil-specific IL-4Rα-deficient mice (LysM<sup>Cre<sup>Il4ra</sup></sup>) and T cell specific IL-4Rα-deficient mice (*iLck<sup>Cre<sup>Il4ra</sup></sup>) were generated with hemizygous *Il4ra<sup>-/-</sup>* mice and homozygous *Il4ra<sup>-/-</sup>* mice were used as controls, as previously described [6,10]. All mice used were on a BALB/c background. 8–12 week old sex-matched mice were obtained from the University of Cape Town specific-pathogen-free animal facility. All experiments were approved by the University of Cape Town Animal Ethics Committee.

#### Parasite infections and antigen preparation

Mice were infected percutaneously with 100 cercariae of a Puerto Rican strain of *S. mansoni* obtained from infected *Biomphalaria glabrata* snails as described [10]. Snails and *S. mansoni* eggs isolated from livers of infected mice were obtained from Dr A.P. Mountford (University of York, UK). In some experiments, mice were injected intraperitoneally (i.p.) with 3,000 liver eggs in PBS. Soluble egg antigen (SEA) was prepared from purified eggs (liver) as described [36] and used at 20μg/ml.
Liver granuloma-associated leukocyte purification

Granulomatous livers were finely cut in small pieces and digested in culture media containing 50 μg/ml collagenase type IV (Sigma) at 37°C for 1.5h. Single cell suspensions were further passed through a 100-μm nylon mesh before leukocytes were isolated through a 30% Percoll cushion at 600g during 15 min. Liver granuloma cells were further treated for 2 min with NH4Cl lysis buffer to lyse erythrocytes. Purified granuloma cells were used for ex vivo restimulation or flow cytometry analysis.

Anti-IL-10 receptor treatment

Mice were inoculated intraperitoneally with PBS or PBS containing 4 μg anti-mouse IL-10Rα (R&D Systems, goat IgG) at day 0, 4 and 6 after injection of eggs.

Antibodies and flow cytometry

mAbs targeting the following cell surface markers were used (the respective mAb clone names are given in itallic): CD11b (APC-, FITC- or PE-conjugated, M1/70), I-A/E-1 (MHC-II, FITC-conjugated or biotinylated, M5/114), F4/80 (PE-conjugated, A3-1, Caltag), Gr-1 (FITC- or PE-conjugated, RB6-8C5), Siglec-F (PE-conjugated, E50-2440), CD204 (Scavenger receptor-A, FITC-conjugated, 2F8, Serotec), CD206 (MMR, PE-conjugated or biotinylated, 5D3, Serotec), CD68 (macrophain, FITC-conjugated, F4-11, Serotec), Dectin-1 (biotinylated, 2411, gift from Dr. G. Brown), CD80 (B7-1, PE-conjugated, 16-1041), CD86 (B7-2, PE-conjugated GL1), CD4 (PE-, FITC-, PE-conjugated, GK1.5, PerCP-conjugated, RM4-5), CD3 (PE- or FITC-conjugated, 145-2C11), CD8 (FITC-conjugated or biotinylated, 33-67), TCRβ (FITC-conjugated, H57-597), TCRγδ (biotinylated, GL3), CD19b (pan-NK, biotinylated, DX3), CD19 (FITC-conjugated, 1D3), CD124 (IL-4Rα, PE-conjugated or biotinylated, M-1). Staining specificity was verified with the appropriate isotype-matched antibody controls and compensation performed with single-stained samples before acquiring the multi-coloured samples. Incubations with antibodies were performed in washing buffer (PBS containing 0.1% BSA, 5mM EDTA and 2mM NaN3) supplemented with heat-inactivated rat serum (2%) and rat anti-mouse FeRII/III mAb (2.4G2, 10μg/ml). PerCP or APC-conjugated-streptavidin was used to detect biotinylated mAbs. For detection of intracellular Ym1 liver cells were fixed for 20 min on ice in 2% (wt/vol) paraformaldehyde and permeabilized during 30 min with 0.5% saponin buffer and further stained with biotinylated goat anti-Ym1/ECF-L (R&D systems). mAbs were from BD Pharamingen except where noted otherwise. Acquisition was performed using a FACS-Calibur (BD Immunocytometry Systems), and data were analyzed by FlowJo software (TreeStar). In some experiments, liver granuloma cells were sorted to high purity (≥98%) using a FACSVantage (BD Immunocytometry Systems) cell sorter. Sorted cell populations (SSC<median>CD11b<median>I-A/E<median>F4/80<median>Gr-1<median>high granulocytes or CD11b<median>I-A/E<median>F4/80<median>Gr-1<median>high macrophages) were either used for RNA extraction or stained with Dill-Quick (Rapidiff set - Clinical Science Diagnostics) after cytosin for direct microscopic examination.

Ex vivo restimulation and cytokine detection

Single-cell suspensions of mesenteric lymph node (mLN) cells or liver granuloma-associated leukocytes were cultured overnight in Iscove’s modified Dulbecco medium (IMDM) containing 10% FCS, 2mM L-glutamine, 0.1 mM non essential amino acids, 1 mM Na Pyruvate (Invitrogen) and 50 μM 2-mercaptoethanol (Sigma) in presence or absence of 20 μg/ml SEA before cytokine detection by flow cytometry. IL-4, IL-10 and IFN-γ secreting cells were detected with the cytokine-specific mouse secretion assay detection kits (PE) (Milenyi) according to the manufacturer’s instructions. Dead cells were excluded from analysis by using 7-amino-actinomycin D (7-AAD, Sigma). For detection of intracellular IL-13, cells were further incubated 4h with monensin, fixed for 20 min on ice in 2% (wt/vol) paraformaldehyde and permeabilized during 30 min with 1× permeabilization buffer (eBioscience) before being stained with anti-IL-13-PE (eBioscience, eBio13a).

Immunohistology

Liver tissue was embedded in OCT (Tissue-Tek, Sakura) before cryopreservation at −80°C. Seven-μm cryosections were cut and mounted onto 3-aminopropyltriethoxysilane (APES) coated slides, dehydrated overnight at 4°C before being fixed in ice-cold acetone. Washes were performed in 0.05% Twem-20 in PBS and staining steps in PBS containing 0.1% BSA. We used the primary antibodies to the following: CD206-Biotin (MRR, 5D3, Serotec), Ym1/ECF-L-Biotin (goat IgG, R&D Systems), CD204-FITC (Scavenger receptor-A, 2F8, Serotec), or iNOS (rabbit IgG, provided by Dr J.Pheilschifter, Germany). PE-conjugated streptavidin (BD Biosciences) was used to detect biotinylated primary antibodies after an avidin/biotin blocking step (Vector). FITC-conjugated goat anti-rabbit IgG secondary antibody (Abcam) was used for the detection of iNOS staining. Sections were washed in PBS before coverslipped in anti-fade fluorescent mounting medium (Dako). Staining specificity was verified by using irrelevant antibody controls. Images were taken with a Nikon Eclipse E400 microscope (Nikon), control by a NIS-Elements Basic Research 3.0 imaging system. Exposure times and fluorescence intensities were verified to appropriate control images. Photomicrographs of liver granuloma focusing on autofluorescent parasite eggs were captured using a Nikon 5.0 mega pixels color digital camera (Digital SIGHT DS-SCm). Images were photographed separately for each fluorescent channels and were merged using Adobe Photoshop 7.0.

Quantitative RT-PCR

Total RNA was extracted and purified with RNeasy Microprep kit (Qiagen) and cDNA synthesised with Transcriptor First Strand cDNA synthesis kit (Roche). Real-time PCR was performed using Lightcycler® FastStart DNA MasterPLUS SYBR Green I reaction mix (Roche) and the reactions run on a Lightcycler® carousel-based system (Roche). Primers used are listed in Table 1.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 4 software (www.graphpad.com). One-way ANOVA test was used to determine significant differences, with Bonferroni’s multiple comparison post test applied to calculate significance values between samples.

Results

Characterization of macrophagic/granulocytic cell populations within liver granulomas in schistosomiasis

The major leukocytic cell types associated with the liver granuloma in S. mansoni infected wild-type mice are T cells, eosinophils and macrophages [3]. IL-4/IL-13-activated macrophages have been shown to play a crucial role in host survival during acute schistosomiasis [6]. Studies to define tissue macrophages are however complex as these cells can express different markers depending on the organ or tissue, in particular eosinophils are known to express high levels of both CD11b and F4/80 [37].
Here, we used a 4-colour flow cytometry strategy to detect and isolate liver granuloma-associated macrophages and granulocytes. Following exclusion of dead cells, a first gate was placed on CD11b<sup>+</sup>-I-A<sup>+</sup>-E<sup>+</sup> cells for granulocytes. As opposed to inflammatory macrophages, Kupffer cells express low levels of CD11b [38,39].

Therefore, a second gate was placed on CD11b<sup>+</sup>-I-A<sup>+</sup>-E<sup>+</sup> and CD68 (Fig. 1B). Granuloma macrophages were therefore defined as CD11b<sup>+</sup>-I-A<sup>-</sup>-E<sup>-</sup> cells and CD204<sup>+</sup>, MMR, Dectin-1 (and Siglec-F<sup>+</sup>) neutrophils, confirmed by morphologic analysis of cell composition and cell-specific cytokine responses within liver granulomas. At 8 weeks post-infection (p.i.), corresponding to the peak of Th2 responses, leukocytes were isolated from granulomas of infected Il4ra<sup>−/−</sup>, LysM<sup>Cre</sup>/Il4ra<sup>−/−</sup>, Ilk<sup>Cre</sup>/Il4ra<sup>−/−</sup>, and global Il4ra<sup>−/−</sup> mice and analyzed by flow cytometry. Il4ra<sup>−/−</sup> littermates were used as controls. Results obtained in Table 2 show that the relative frequencies of macrophages, neutrophils, eosinophils and lymphocyte subpopulations in granulomas were not affected by the specific impairment of IL-4R<sub>a</sub> on macrophages, with eosinophils (CD11b<sup>+</sup>-F<sub>4/80</sub>-Gr<sup>1<sup>+</sup></sub>-Siglec-F<sup>+</sup>) dominating granulomatous cell composition in LysM<sup>Cre</sup>/Il4ra<sup>−/−</sup> and infected Il4ra<sup>−/−</sup> control mice. We observed however dramatically disrupted granuloma cell populations in Ilk<sup>Cre</sup>/Il4ra<sup>−/−</sup> mice with increased frequencies of macrophages (CD11b<sup>+</sup>-I-A<sup>-</sup>-E<sup>+</sup>-<sub>ab</sub>CD204<sup>+</sup>), neutrophils (CD11b<sup>+</sup>-F<sub>4/80</sub>-Gr<sup>1<sup>+</sup></sub>-), and γ-t cells. Though we previously showed that these mice developed bigger granulomas with reduced numbers of eosinophils per granuloma [10], Lek<sup>Cre</sup>/Il4ra<sup>−/−</sup> mice showed high numbers of eosinophils in their liver (143.1±10<sup>5</sup> cells in Lek<sup>Cre</sup>/Il4ra<sup>−/−</sup> mice vs. 110.5±10<sup>5</sup> cells in Il4ra<sup>−/−</sup> mice). This apparent discrepancy could be explained by the fact that these mice had globally increased cell numbers per liver (data not shown). The cellular changes in Lek<sup>Cre</sup>/Il4ra<sup>−/−</sup> mice were very similar to the disruption of the granuloma cell frequencies observed in Il4ra<sup>−/−</sup> mice, also showing increased CD4<sup>+</sup> T cells and B cells. The disruption of the granulomas in Il4ra<sup>−/−</sup> mice could be explained by the 4-fold-reduced numbers of eosinophils (25.4±10<sup>5</sup> cells in Il4ra<sup>−/−</sup> mice vs. 115.9±10<sup>5</sup> and 116.0±10<sup>5</sup> cells in LysM<sup>Cre</sup>/Il4ra<sup>−/−</sup> and Il4ra<sup>−/−</sup> mice, respectively) (Table 2). CD4<sup>+</sup>CD62L<sup>+</sup> naive T cell numbers however changed with a 4-fold increase in infected Ilk<sup>Cre</sup>/Il4ra<sup>−/−</sup> mice and a 10-fold increase in infected Il4ra<sup>−/−</sup> mice compared to Il4ra<sup>−/−</sup> control mouse over-proportionally (28.3±10<sup>5</sup> cells in Il4ra<sup>−/−</sup> mice and 11.9±10<sup>5</sup> cells in Lek<sup>Cre</sup>/Il4ra<sup>−/−</sup> mice vs. 4.3±10<sup>5</sup> and 2.9±10<sup>4</sup> cells in LysM<sup>Cre</sup>/Il4ra<sup>−/−</sup> and Il4ra<sup>−/−</sup> mice, respectively). In contrast, NK (CD11b<sup>+</sup>-SSC<sup>high</sup>TCR<sub>B</sub> CD49b<sup>+</sup>) and NT (CD11b<sup>+</sup>-SSC<sup>high</sup>TCR<sub>B</sub> CD49b<sup>+</sup>) cells showed significant lower frequencies in the liver of infected Il4ra<sup>−/−</sup> mice compared to Il4ra<sup>−/−</sup> control mice (10.3% vs. 17.3% and 8.2% vs. 25.7%, respectively), resulting in a 2-fold cell number reduction of NK-T cells compared to control mice (2.9±10<sup>5</sup> cells in Il4ra<sup>−/−</sup> mice vs. 5.8±10<sup>5</sup> cells in Il4ra<sup>−/−</sup> mice) (Table 2). Following ex vivo SEA restimulation of mesenteric lymph node (mLN) cells or liver granuloma-associated leukocytes, cytokine productions of CD4<sup>+</sup> T cells and non-CD4<sup>+</sup> cells were analyzed by multicolour flow cytometry, either by catch secretion assay (IFN-γ, IL-4, IL-10) or intracellular staining (IL-13) (Fig. 2). Cytokine catch secretion assays give information on cytokines secreted by a specific cell-type, gathering therefore advantages of both ELISA and intracellular staining. In both tissues, lymphocytes from LysM<sup>Cre</sup>/Il4ra<sup>−/−</sup> mice produced similar levels of IL-13, IL-10 and IFN-γ cytokines compared to Il4ra<sup>−/−</sup> control mouse with slightly but significant higher levels of IL-4 in liver CD4<sup>+</sup> cells compared to Il4ra<sup>−/−</sup> control mice (Fig. 2B). In contrast, lymphocytes from both Ilk<sup>Cre</sup>/Il4ra<sup>−/−</sup> and Il4ra<sup>−/−</sup> mice produced less Th2 cytokines but more IFN-γ revealing a shift towards Th1-type responses within the granulomas, explaining the observed differences in cellular composition.

Mannose receptor and Ym1 expression in liver granuloma-associated macrophages of LysM<sup>Cre</sup>/Il4ra<sup>−/−</sup> mice

We further compared gene expression levels of markers used to distinguish classical from alternative macrophage activation within liver granulomas during acute S. mansoni infection. Liver granuloma macrophages (SSC<sup>high</sup>CD11b<sup>+</sup>-I-A<sup>-</sup>-E<sup>-</sup>CD204<sup>+</sup>)
Figure 1. Flow cytometry analysis of liver granuloma during acute schistosomiasis. BALB/c mice were infected with 100 S. mansoni cercariae, killed 8 weeks later and liver granuloma-associated leukocytes isolated before 4-colour flow cytometry analysis. (A) Gating strategy for analysis of surface expression of CD11b and I-A/I-E on SSC<sup>high</sup> and live (7-AAD<sup>−</sup>) liver granuloma-associated leukocytes. Outlined regions define CD11b<sup>+</sup>I-A/I-E<sup>−</sup> and CD11b<sup>+</sup>I-A/I-E<sup>high</sup>, respectively. (B) CD11b<sup>+</sup>I-A/I-E<sup>−</sup> and CD11b<sup>+</sup>I-A/I-E<sup>high</sup> gated populations in A were analyzed for F4/80, Gr-1, Siglec-F, CD204, MMR, Dectin-1, CD68, CD80, CD86 and IL-4R<sup>a</sup> expression, respectively. Greyscale histograms show relevant isotype control. (C) 4-colour flow cytometry analysis of liver granuloma-associated leukocytes gated on CD11b<sup>+</sup>I-A/I-E<sup>−</sup> cells as described in A. F4/80 and Gr-1 double staining contour plot is shown. Outlined regions were sorted for cytospin and morphological analysis. (D) 4-colour flow cytometry analysis of liver granuloma-associated leukocytes gated on CD11b<sup>+</sup>F4/80<sup>−</sup> cells as described in A. MHC-II (I-A/I-E) and scavenger receptor-A (CD204) double staining contour plot is shown. Outlined region was sorted for cytospin and morphological analysis. Data are representative of three independent experiments with similar results.

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were FACS-sorted to high-purity at 8 weeks p.i. (≥98%); RNA extraction performed, and gene expression profile analyzed (Fig. 3A). Il4ra (IL-4Rα) mRNA expression, observed in Iba1/lox control macrophages, was impaired in macrophages from LysMcreIl4ra/lox mice and global Iba1+/lox mice, confirming the efficiency of Cre-loxP mediated DNA deletion in granuloma macrophages. Interestingly, Il4ra expression was also reduced in LckcreIl4ra/lox macrophages, suggesting a predominant classical activation in the granulomas of this mouse strain. Consistent with the IL-4/I-13/IL-4Rα- and IFN-γ/IFN-γR-mediated macrophage activation by direct control of expression of the enzymes catalysing L-arginine, LysMcreIl4ra/lox, LckcreIl4ra/lox, and global Il4ra+/lox mice presented classically activated macrophages with heightened Nos2 but reduced Arg1 mRNA expression levels, compared to Iba1+/lox control mice. This was in line with the expression pattern of the known alternative activation marker gene Retnla (FIZZ1), significantly reduced in macrophages from LysMcreIl4ra/lox, LckcreIl4ra/lox and global Il4ra+/lox mice, compared to Iba1+/lox control mice. Of importance, the aMφ marker Chi3l3 and M1c1 gene expression levels were present although reduced for M1c1 in LysMcreIl4ra/lox macrophages, resulting in Ym1 and MMR surface expression in about 30% of SSChighCD11b1/1-A/I-EhighCD204+ macrophages from LysMcreIl4ra/lox mice. The expression of these two markers was however strongly reduced in both LckcreIl4ra/lox and global Il4ra+/lox mice, compared to control Il4ra+/lox mice (Fig. 3B). Mean fluorescent intensities (MFI) values of MMR and Ym1 staining were also significantly higher in macrophages from LysMcreIl4ra/lox compared to both LckcreIl4ra/lox and global Il4ra+/lox mice (Fig. 3C). The majority (76.3%) of MMR-expressing CD11b1/1-A/I-Ehigh granuloma macrophages also expressed Ym1 in the Il4ra+/lox control mice (Fig. 3D). Absence of surface IL-4Rα expression was further confirmed by 4-colour staining on granuloma macrophages (Fig. 3E). MFI of IL-4Rα staining on gated CD11b1/MMR- cells revealed similar expression levels of the receptor between LysMcreIl4ra/lox mice and isotype control (MFI = 5.54 and 5.52, respectively), whereas granuloma CD11b1/MMR- cells from Il4ra+/lox mice showed 2-fold increased expression of the IL-4Rα compared to LysMcreIl4ra/lox mice and isotype control (MFI = 12.69) (Fig. 3E). Together, these data suggest that MMR and Ym1 can be expressed independently of macrophage-specific IL-4Rα signalling, whereas IL-4Rα-dependent T-cell responses are necessary for driving expression of aAαMφ markers, including MMR and Ym1.

Peripheral localisation of IL-4Rα-independent MMR- and Ym1-positive macrophages within egg-induced liver granulomas of LysMcreIl4ra/lox mice

The results detailed in Figure 3 demonstrated that LysMcreIl4ra/lox granuloma macrophages have retained expression of MMR and Ym1. This was surprising as we previously showed that liver granuloma from LysMcreIl4ra/lox mice had low levels of MMR expression in close proximity to the eggs compared to Il4ra+/lox control mice [6]. To further determine the localisation of the MMR- and Ym1-positive macrophage subpopulation within the liver granuloma, immunofluorescent stainings were performed on liver cryosections at 8 weeks p.i. (Fig. 4). As expected from the expression data, MMR and Ym1 were highly expressed in granulomas from Il4ra+/lox control mice; and these cells co-expressing scavenger receptor A (CD204) were in close proximity to the parasite eggs in the centre of the granuloma (Fig. 4A and B). MMR- and Ym1-positive macrophages were restricted to the periphery of the granuloma in LysMcreIl4ra/lox mice and nearly undetectable in both LckcreIl4ra/lox and Il4ra+/lox mice. Instead, iNos-positive, MMR- and Ym1-negative macrophages were in close proximity around the egg in the granuloma of LysMcreIl4ra/lox, LckcreIl4ra/lox and Il4ra+/lox mice (Fig. 4B), confirming previous observations [6,10,41]. These results show that a peripheral IL-4Rα-independent MMR- and Ym1-positive macrophage subpopulation surrounds the iNos-producing classically activated macrophages present in close proximity to the egg within the liver granuloma of LysMcreIl4ra/lox mice.

IL-4Rα-independent MMR- and Ym1- positive macrophages depend on IL-10 signalling

IL-10 signalling can induce MMR expression in macrophages [34]. We therefore investigated whether IL-10 could drive the MMR and Ym1 expression in macrophages of LysMcreIl4ra/lox mice. To test this hypothesis we used a local egg model by injecting purified S. mansoni eggs intraperitoneally to induce Th2 responses and elicit macrophage activation in Il4ra+/lox, LysMcreIl4ra/lox and Il4ra+/lox mice. At 7 days p.i., corresponding
### A

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to the peak of Th2 responses [42]. MMR and Ym1 were highly expressed in peritoneal macrophages of Il4ra−/lox control mice (Fig. 5A). To investigate peritoneal macrophages, we placed a live gate on FSC<high>SSC<low>CD11b+F4/80+ cells. Here the association of the CD11b, F4/80 markers and side/forward scatter allows discrimination between eosinophils and macrophages, as previously described [37]. Similar to that observed in macrophages from liver granuloma, a subpopulation of cells expressing MMR and Ym1 was present in peritoneal macrophages of LysMcreIl4ra<lox/lox> mice but absent in peritoneal macrophages from Il4ra−/− mice (Fig. 5A). In vivo blockade of IL-10 signalling via anti-IL-10 receptor antibody treatment was sufficient to significantly reduce the protein expression of both MMR and Ym1 in peritoneal macrophages of LysMcreIl4ra<lox/lox> mice (Fig. 5B and C). These results demonstrate that IL-10 drives expression of MMR and Ym1 in macrophages independently of macrophage IL-4Rα.

Discussion

Recent S. mansoni infection studies demonstrated that LysMcre<lox/lox> mice, deficient for IL-4Rα-responsive aaMΦ succumb to acute S. mansoni infection, due to the development of egg-induced gut inflammation, severe liver damage, and despite the formation of egg-induced granulomas and unaffected fibrosis in the liver [6]. These observations were very similar to the pathology developed by infected global Il4ra−/− mice [6], or Il4−/− and Il4−/− Il13−/− mice which developed hepatotoxicity and endotoxaemia due to the degradation of the intestinal barrier [5,9]. Antibiotic treatment in infected LysMcre<lox/lox> mice resulted only in partial protection [6], suggesting that in addition to gut inflammation and systemic leakage of the intestinal content, hepatotoxic lesions could also explain the increased mortality. In addition to these findings, we also recently demonstrated, using iLckcreIl4ra<lox/lox> mice, that IL-4Rα responsiveness by T cells was required for host survival mainly for controlling liver damage caused by the parasites eggs [10]. As iLckcre<lox/lox> mice did not develop gut injury or endotoxaemia, we concluded that IL-4Rα responsiveness by T cells is not essential for the control of intestine-derived sepsis. In this study we focused on the liver granuloma microenvironment and the consequences of impaired IL-4Rα signalling in macrophages or T cells on the local cellular responses directed against the parasite eggs. Granulomas induced by S. mansoni contain both myeloid cells (mainly eosinophils and macrophages) and lymphoid cells (mainly T cells) [3]. Several markers are readily available for staining murine tissue macrophages but their expression can be highly tissue-dependent [43]. Most of the studies using such markers have been performed with blood, spleen or bone-marrow but little is known on granuloma macrophage markers during schistosomiasis. Liver granulomas are tightly organized, which renders their disruption difficult without affecting cell viability, especially macrophages. Though collagenase can in some conditions cleave surface proteins, comparison between collagenase-treated or untreated organs gave similar results concerning the expression levels of standard surface markers (including CD11b, F4/80, CD11c, Gr-1, and I-A/I-E), suggesting that collagenase treatment had no major influence on surface marker detection (data not shown). The combination of CD11b and/or F4/80 and MHC-II (I-A/I-E) was sufficient to specifically detect and isolate macrophages from granulomas. These macrophages were defined as SSC<high>CD11b+F4/80<−/−>I-A/I-E<+> cells, which showed a macrophage-like morphology and expressed high levels of macrophage-specific markers such as scavenger receptor-A (CD204), mannose receptor (MMR), β-glucan receptor (Dectin-1) and macrosidin (CD68). In parallel, we defined granuloma eosinophils as SSC<high>CD11b+F4/80<−/−>I-A/I-E<−>Gr-1<+>Siglec-F<+> and neutrophils as SSC<high>CD11b+F4/80<−/−>I-A/I-E<−>Gr-1<−>Siglec-F−.

Despite increased liver granuloma sizes, previously reported in infected LysMcreIl4ra<lox/lox> mice [6], neither the cellular composition nor the Th1/Th2 cytokine profiles were affected. In contrast, we observed severe changes in granuloma cell composition and morphology of infected LysMcre<lox/lox> mice. Interestingly, a recent report showed that infected LysMcreArg1<lox/lox> or Tie2creArg1lox/lox mice (deficient for Arg1-expressing macrophages) did not develop lethal endotoxaemia or hepatotoxicity but showed increased Th2 responses and increased chronic disease [23]. Further, Arg1-expressing aMΦ were able to suppress schistosome-specific T cell proliferation, suggesting a possible mechanism for downmodulating granulomatous inflammation and slow the progression of Th2-driven fibrosis during chronic infection [23]. Similarly, studies in Retnla-deficient mice showed that absence of resistin-like molecule alpha (Retnla/VIZZ1) exacerbated Th2 cytokine responses in S. mansoni-egg-induced lung inflammation, suggesting that Retnla/VIZZ1 is able to control effector Th2 responses in S. mansoni egg-induced inflammation and infection [29,30]. As IL-4Rα-signalling is upstream from the induction of Arg1 and Retnla in aMΦ, it might be not surprising that LysMcre<lox/lox> mice have a more severe disease phenotype already detrimental in the acute phase. The relative normal granuloma morphology, cellular composition and Th1/Th2 cytokine responses in the liver of LysMcre<lox/lox> mice but more severe liver pathology in iLckcreIl4ra<lox/lox> mice, suggest that abrogation of IL-4–promoted Th2 responses in combination with impairment of aMΦ negatively affect the control of liver pathology in mice. Together with the chronic phenotype and aMΦ-mediated T cell suppression observed in LysMcreArg1<lox/lox> mice, aMΦ seem to control T helper cell responses in an optimal balance. Future studies need to further investigate the molecular mechanisms of how and which T cell subpopulations are controlled by aMΦ.

Interestingly, LysMcre<lox/lox> liver granulomas retained expression of MMR and Ym1 in a subpopulation of macrophages, confirmed in mRNA and protein (Chil3) or protein (Mrc1) expression analyses. Interestingly, MMR/Ym1+ macrophages were not observed in iLckcreIl4ra<lox/lox> mice, suggesting that IL-
4R-α-dependent Th2 responses drive MMR and Ym1 expression independently of IL-4Rα-responsive macrophages in Ly4M<sup>−/−</sup>Il4ra<sup>−/lox</sup> mice. After staining of scavenger receptor-A (CD204) in situ to specifically detect macrophages [44,45], we showed that macrophages were in close proximity to the parasitic eggs within the granuloma in all mouse strains. However, macrophages expressing MMR or Ym1 were only found at the periphery of the Ly4M<sup>−/−</sup>Il4ra<sup>−/lox</sup> granulomas, while present in close proximity to the eggs in the Il4ra<sup>−/−</sup> control granulomas. As previously demonstrated, MMR- or Ym1-positive cells were absent of global Il4ra<sup>−/−</sup> granulomas [25] and we observed nearly undetectable MMR or Ym1<sup>+</sup> cells in Lck<sup>−/−</sup>Il4ra<sup>−/lox</sup> granulomas. Instead classically activated macrophages, defined by their iNOS expression, were centred in the eggs of Ly4M<sup>−/−</sup>Il4ra<sup>−/lox</sup>, Lck<sup>−/−</sup>Il4ra<sup>−/lox</sup> and global Il4ra<sup>−/−</sup> granulomas (Fig. 4B). Increased iNOS activity in these mice strains could explain the heightened hepatic cellular damage observed after S. mansoni infection [6,10]. Together, these results suggest that IL-4Rα-responsiveness in aaMφ regulates their ability to migrate in close proximity and/or interact with the parasite eggs. The factors that could be responsible for the interaction with the eggs are not known, but a possible candidate could be MMR. This receptor encodes a C-type lectin, which has been involved in antigen uptake by antigen-presenting cells and components of S. mansoni eggs as well as a fraction of their egg-secreted molecules are ligands of MMR [25]. Furthermore, a study demonstrated that SEA can be internalized by DCs through the C-type lectin dendritic cell specific ICAM-3 grasping non integrin (DC-SIGN), macrophage galactose-type lectin (MGL) and MMR [28]. We did not look at the expression of other C-type lectins, but the presence of macrophages expressing MMR in the proximity of the parasite eggs could allow IL-4Rα-responsive aaMφ to interact with antigen-specific T cells and also play regulatory functions on effector T cells as previously suggested [6,23,29,30,46]. Although Ym1 was described as a chitinase-like secreted protein, it lacks any chitinase activity and S. mansoni eggs do not contain chitin [47,48]. A recent study described Ym1 to digest glycosaminoglycans and might therefore play a role in egg killing and/or antigen processing [49]. It has previously been suggested that Ym1 could encapsulate pathogens [31] and our observation of Ym1-expressing macrophages in the proximity of the parasitic eggs supports this hypothesis. Encapsulation of the parasite eggs may serve to protect the host from pro-inflammatory molecules and harmful factors released by the eggs. Macrophages isolated from granulomas or macrophages elicited with S. mansoni egg components have previously been proposed to act as `myeloid suppressive cells (MSCs) by inducing clonal anergy in egg-antigen-specific Th1 cells by unknown mechanisms [46,50,51,52,53,54]. Although we did not detect changes in cellular composition or cytokine production in the liver granulomas of Ly4M<sup>−/−</sup>Il4ra<sup>−/lox</sup> mice, these mice quickly die from increased systemic Th1-type responses and acute infection due to uncontrolled gut inflammation [6]. Recently established MMR-deficient mice [53], generation of Ym1-deficient mice should allow further studies to address the role of such aaMφ biomarkers.

Previous studies proposed that MMR and Ym1 expression by macrophages could directly be driven by bacterial endotoxin or chitin [35], and independently of IL-4Rα signalling [56]. Linehan and colleagues (2003) previously described a complete absence of MMR expression in the liver granulomas of global Il4ra<sup>−/−</sup> mice [25], demonstrating that IL-4Rα responsiveness is essential for MMR expression in macrophages recruited to the granulomas. These authors however described resident liver macrophages to retain MMR expression and we also observed MMR expression in resident Kupffer cells (Fig. 4A). As Kupffer cells express no/low levels of CD11b in contrast to inflammatory macrophages [38,39], we can exclude any contamination with these cells in our gating strategy targeting SSC<sup>high</sup>CD11b<sup>+/hi</sup>I-A/I-E<sup>high</sup>CD204<sup>−</sup> granuloma macrophages.

Our findings demonstrate that direct IL-4Rα signalling in a subpopulation of granuloma macrophages is not essential for MMR and Ym1 expression and these cells are restricted to the periphery of Ly4M<sup>−/−</sup>Il4ra<sup>−/lox</sup> granulomas. IL-10 signalling in macrophages can induce MMR expression [34] and Ym1 expression is downregulated in Il10<sup>−/−</sup> and Il10<sup>−/−</sup>Il4<sup>−/−</sup> deficient mice [32]. These observations, together with reduced IL-10 production and impaired aaMφ markers (including MMR and Ym1) in Lck<sup>−/−</sup>Il4ra<sup>−/lox</sup> and Il4ra<sup>−/−</sup> liver granulomas, prompted us to consider IL-10 as a good candidate to explain the IL-4Rα-independent expression of MMR and/or Ym1 in macrophages. In order to focus on the egg-induced inflammation in the Ly4M<sup>−/−</sup>Il4ra<sup>−/lox</sup> mice, we chose to use an egg model to induce acute Th2 responses following challenge in the peritoneum. Here, we showed evidence by in vivo IL-10R signalling blockade that IL-10 signalling was responsible for the retained expression of MMR and Ym1 expression in Ly4M<sup>−/−</sup>Il4ra<sup>−/lox</sup> peritoneal macrophages following S. mansoni egg exposure and induction of Th2 cytokine responses, while no significant effect could be observed in macrophages of Il4ra<sup>−/lox</sup> control mice. Although elicitation of macrophages in the peritoneal cavity by S. mansoni eggs might not exactly reflect what occurs in the liver granuloma during live infection, data generated with peritoneal macrophages gave similar results as those obtained with granuloma macrophages. Control Il4ra<sup>−/lox</sup> mice over-expressed both MMR and Ym1, whereas expression of those markers in
peritoneal macrophages from Il4ra<sup>-/-</sup> mice was strongly impaired. Moreover, LysM<sup>cre</sup>Il4ra<sup>-/-</sup> and iLck<sup>cre</sup>Il4ra<sup>-/-</sup> peritoneal macrophages also retained expression of MMR and Ym1 after injection of eggs and anti-IL-10R treatment blocked the expression of both markers. These results indicate that IL-10 plays a key role in the retained expression of MMR and Ym1 in the liver granulomas of LysM<sup>cre</sup>Il4ra<sup>-/-</sup> mice. The macrophages retaining expression of MMR and Ym1 in infected LysM<sup>cre</sup>Il4ra<sup>-/-</sup> mice could therefore represent a cellular subpopulation with IL-4R<sub>a</sub>-independent aM<sub>1</sub>-related regulatory activities, driven by IL-10. The presence of such macrophage population could explain why LysM<sup>cre</sup>Il4ra<sup>-/-</sup> liver granulomas do not have disrupted cellular or cytokine responses, in contrast to mice having impaired Th2 responses and showing strongly reduced IL-10 production such as iLck<sup>cre</sup>Il4ra<sup>-/-</sup> and Il4ra<sup>-/-</sup> mice. Supporting these results, Herbert et al. recently showed that in vivo IL-10R neutralization results in increased hepatocellular damage without affecting gut inflammation in schistosomiasis [57]. Hepatotoxicity was however observed in infected LysM<sup>cre</sup>Il4ra<sup>-/-</sup> mice [6], suggesting that IL-10 without IL-4/IL-13 signalling in macrophages might not be sufficient to control liver tissue damage. Future work on IL-4R<sub>a</sub>-independent MMR<sup>+</sup>Ym1<sup>+</sup> macrophages from liver granulomas may define whether those cells have an “alternative” or “deactivated” phenotype, which was previously described to be mediated by IL-10 signalling [11].

In conclusion, we demonstrated that IL-4R<sub>a</sub>-responsive macrophages are dispensable for the control of cellular and cytokine responses in liver granulomas during acute schistosomiasis, whereas IL-4R<sub>2</sub>-responsive T cells are necessary to sustain cellular composition and morphology of the liver granuloma. Though our results suggest that macrophage-specific IL-4R<sub>2</sub> expression is necessary for an adequate interaction between...
aaMΦ and the parasite eggs, we showed that MMR and Ym1 expression may be induced via IL-10R signalling in absence of IL-4Rα signalling in macrophages. Taken together, our results suggest that IL-4Rα-derived Th2 cytokine responses are essential to drive aaMΦ in the liver and demonstrate that in absence of macrophage-specific IL-4Rα, IL-10 signalling induces MMR+/Ym1+ macrophages. Future investigation may clarify whether IL-10-driven IL-4Rα-independent MMR+/Ym1+ macrophages have important functions in the control of liver granulomatous inflammation.

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References

Author Contributions
Conceived and designed the experiments: BGD FB. Performed the experiments: BGD RGM JCH. Analyzed the data: BGD RGM MI. AS. Wrote the paper: BGD FB.

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Gr1(+) macrophages that suppress naive CD4(+) T cell proliferation via an IFN-
adherent mononuclear cells and lymphocytes from granulomas of mice with
Gr1+IL-4-producing innate cells are induced in response to Th2 stimuli and
Schistosoma mansoni worms induce anergy of T cells via selective up-regulation
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