The Mannose Receptor (CD206) is an important pattern recognition receptor (PRR) in the detection of the infective stage of the helminth *Schistosoma mansoni* and modulates IFNγ production

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ABSTRACT

In this study, infective larvae of the parasitic helminth *Schistosoma mansoni* were shown to contain a large number of glycosylated components specific for the Mannose Receptor (MR; CD206), which is an important pattern recognition receptor (PRR) of the innate immune system. MR ligands were particularly rich in excretory/secretory (E/S) material released during transformation of cercariae into schistosomula, a process critical for infection of the host. E/S material from carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)-labelled cercariae showed enhanced binding by cells lines that over-express the MR. Conversely, uptake was significantly lower by bone marrow-derived macrophages (MΦ) from MR$^{-/-}$ mice, although they were more active as judged by enhanced pro-inflammatory cytokine production and CD40 expression. After natural percutaneous infection of MR$^{-/-}$ mice with CFDA-SE-labelled parasites, there were fewer cells in the skin and draining lymph nodes that were CFDA-SE$^+$ compared with wild-type mice, implying reduced uptake and presentation of larval parasite antigen. However, antigen-specific proliferation of skin draining lymph node cells was significantly enhanced and they secreted markedly elevated levels of IFNγ but decreased levels of IL-4. In conclusion, we show that the MR on mononuclear phagocytic cells, which are plentiful in the skin, plays a significant role in internalising E/S material released by the invasive stages of the parasite which in turn modulates their production of pro-inflammatory cytokines. In the absence of the MR, antigen-specific CD4$^+$ cells are Th1 biased, suggesting that ligation of the MR by glycosylated E/S material released by schistosome larvae modulates the production of CD4$^+$ cell specific IFNγ.

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1. Introduction

Cercariae of the parasitic helminth *Schistosoma mansoni* gain entry to the mammalian host via the skin which provides the initial site for stimulation of the innate immune response. Schistosome larvae can take several days to migrate through the epidermis and dermis of the skin, facilitated by the secretion of proteases from the acetabular glands (Knudsen et al., 2005; Curwen et al., 2006), before exiting via vascular and lymphatic vessels (Wheater and Wilson, 1979). Using an amine-reactive tracer, we have labelled live cercariae to visualise the release of excretory/secretory (E/S) material after skin penetration and detect its uptake by host cells such as macrophages (MΦs) and dendritic cells (DCs) (Paveley et al., 2009). Both MΦs and DCs are activated after exposure to E/S products from schistosome larvae (Jenkins et al., 2005; Jenkins and

Mountford, 2005; Ferret-Bernard et al., 2008; Paveley et al., 2009), although they have a 'limited' maturation phenotype compared with cells matured with classical pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS) (Ferret-Bernard et al., 2008), and favour the induction of Th2 rather than Th1 lymphocytes (Jenkins and Mountford, 2005). However, although cytokine production by MΦs is MyD88-dependent (Jenkins et al., 2005), indicating an important role for non-phagocytic Toll-like receptors (TLRs), it is not known which pattern recognition receptors (PRRs) are involved in the uptake or phagocytosis of E/S material by host cells.

Phagocytic PRRs include scavenger receptors, complement receptors and C-type lectins (CLRs) such as the MΦ Mannose Receptor (MR; CD206) (Underhill and Ozinsky, 2002). CLRs are involved in the uptake of numerous pathogens through binding of glycans and have a diverse range of functions (Gazi and Martinez-Pomares, 2009; Kerrigan and Brown, 2009). The MR in particular can recognise sulphated sugars, collagen and sugars

terminating in D-mannose, L-fucose or N-acetylglucosamine (Taylor et al., 2005; Martinez-Pomares et al., 2006). It is expressed amongst others on tissue MΦs and DCs (Engering et al., 1997; Linehan et al., 1999; McKenzie et al., 2007) and is mainly intracellular with only 10–30% being expressed at the cell surface under steady state conditions (Schweizer et al., 2000). Furthermore, the MR can be up-regulated by cytokines such as IL-4, IL-13 and IL-10, or down-regulated with IFNγ (Harris et al., 1992; Doyle et al., 1994; Martinez-Pomares et al., 2003; Dewals et al., 2010).

Parasitic helminths express various carbohydrate containing glycoproteins on their surface and release glycan-rich E/S products that can potentially bind to various CLRs. The MR in particular binds to *Trichinella spiralis* muscle larvae (Gruden-Movsesijan and Milosavljevic Lj, 2006), *Trichuris muris* E/S (deSchoolmeester et al., 2009), and soluble schistosome egg antigen (SEA) from *S. mansoni* (Linehan et al., 2003; van Liempt et al., 2007). As MΦs are common in murine skin (Dupasquier et al., 2004; McKenzie et al., 2007), and the MR is highly expressed on dermal MΦs in murine and human skin (McKenzie et al., 2007; Ochoa et al., 2008), we suggest that they may play a major role in sensing invasive schistosome cercariae during infection of the skin and play a critical role in the development of the acquired immune response.

Here, we focus on the role of the MR in recognising and internalising E/S products released by infectious *S. mansoni* cercariae, and in the activation of the ensuing immune response. The presence of MR ligands in the E/S products released by transforming cercariae (termed 0–3hRP) was determined, while carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was used to label live cercariae in order to track uptake of E/S products by murine cells either in the presence or absence of the MR. We show that not only is the MR an important receptor in the uptake of E/S products by phagocytic cells, its absence leads to an increase in the ability of CD4^{+} lymphocytes in the skin draining lymph nodes (sdLN) to secrete antigen-specific IFNγ and reduce the production of IL-4.

2. Materials and methods

2.1. Animals

C57BL/6 mice were bred and maintained at the University of York, UK and housed alongside MR deficient (MR^{−/−}) mice on a C57BL/6 background (a kind gift from Prof. Michel C. Nussenzweig, Rockefeller University, New York, USA). All experimental procedures were undertaken within the guidelines of the United Kingdom Animals Scientific Procedures Act 1986 and approved by the University of York Ethics Committee.

2.2. Parasite material

Cercariae from a Puerto Rican strain of *S. mansoni* were shed from snails by exposure to light for 2 h. The live cercariae were then used immediately for labelling with CFDA-SE (see below) and/or infection. Alternatively, they were used for the production of a soluble cercarial antigen preparation (SCAP), or prepared for the collection of larval E/S products (0–3hRP) (Mountford et al., 1995; Jenkins et al., 2005; Jenkins and Mountford, 2005; Paveley et al., 2009) and soluble schistosomula antigen preparation (SSAP) (Mountford et al., 1995). Briefly, 1×10^6 cercariae were induced to be shed in 1,000 ml 'aged tap water' (ATW), then concentrated on ice and washed extensively (>5×) with sterile ATW prior to mechanical transformation in sterile RPMI medium. Parasite bodies were then incubated for 3 h in RPMI to induce the release of 0–3hRP (Paveley et al., 2009). All antigen preparations were prepared following sonication at 21 kHz at 6.5 μm amplitude for 90 s and centrifugation at 100,000g for 1 h (Mountford et al., 1995).

2.3. Parasite labelling

Live cercariae were cultured in the presence of 20 μM CFDA-SE (Invitrogen Ltd., Paisley, UK) at 28 °C for 1 h, washed, then incubated for a further 1 h to remove unconjugated tracer as previously described (Paveley et al., 2009). As reported previously, labelling of cercariae with CFDA-SE had no effect upon the viability of the cercariae to penetrate the skin or mature into egg-laying worms (Paveley et al., 2009). CFDA-SE-labelled cercariae were then used to infect mice or in the preparation of 0–3hRP. Alternatively, live parasites were labelled with 1 μg/ml of a recombinant fusion protein CTLD4-7-Fc (Linehan et al., 2001) which contains the MR CRD 4-7 fused to the Fc portion of human IgG$_1$, for 30 min at 28 °C, washed and incubated with anti-human IgG FITC. Labelled parasites were then fixed and imaged on a Zeiss LSM 510 meta confocal microscope (Carl-Zeiss Ltd., Welwyn Garden City, UK) at 488 nm/520 nm excitation/emission.

2.4. Detection of glycans within schistosome preparations

Components 0–3hRP, SCAP and SSAP were separated by 4–12% one-dimensional (1D) SDS-PAGE and stained with Coomassie® Blue. Gels were then oxidised with sodium meta-periodate (Perbo Science Ltd., Cheshire, UK) and stained with Pro-Q® Emerald before imaging on a Bio-Rad Molecular Imager FX Pro (Bio-Rad Laboratories, Hemel Hempstead, UK) at 280/530 excitation/emission. Glycoprotein content in the various preparations was determined with a glycoprotein estimation kit (Perbio Science Ltd.) against a D-mannose standard curve.

2.5. Analysis of carbohydrate ligands

Using CTLD4-7-Fc and CR-FNIII-CTLD1-3-Fc (Linehan et al., 2001), the specificity of mannose and galactose ligands present in 0–3hRP was determined using lectin-specific ELISAs according to the protocol of Zamze et al. (2002). Plates were coated with diluted schistosome antigen preparations, (10 μg/ml), mannan (1 μg/ml) and SO$_4$-3-gal (5 μg/ml) in PBS pH 7.6 and left overnight at 4 °C. Plates were washed and probed with CTLD4-7-Fc or CR-FNIII-CTLD1-3-Fc for 1.5 h at room temperature (RT) in the presence or absence of 10 mM mannose or galactose. After washing, plates were incubated with anti-human IgG alkaline phosphate conjugate (Sigma–Aldrich Ltd., Poole, UK) for 1 h and then developed with *p*-nitrophenyl phosphate substrate (Sigma–Aldrich Ltd.). Absorbance was measured at 405 nm.

To detect the presence of MR ligands by western blot, samples of 0–3hRP, SCAP and SSAP were separated by 1D SDS–PAGE, transferred to polyvinyl difluoride (PVDF) membranes (Invitrogen Ltd.) for 75 min at 30 V in Blot-cell (Bio-Rad Laboratories) and blocked with 5% non-fat milk. Membranes were probed with 1 μg/ml of CRD4-7Fc and then horseradish peroxidase (HRP)-conjugated anti-human IgG (Amersham Pharmacia Biotech Ltd., UK). Binding was revealed using ECL Plus™ reagent (Perbio Science Ltd.). Post-imaging, membranes were stripped of antibody, incubated with SYPRO® Ruby protein blot stain (Invitrogen Ltd.) and imaged at 488 nm/640 nm excitation/emission.

2.6. Production and stimulation of cell lines and bone marrow (BM) MΦs

Wild type CHO (WT; 85050302), MR-expressing CHO cells (Martinez-Pomares et al., 2003) and the MΦ cell lines J774A.1 and J774E, were a kind gift from Professor S. Gordon, University of Oxford, UK. MΦs derived from BMMΦs were generated from WT and MR^{−/−} mice as follows. BM cells (femurs) were resuspended in DMEM (Invitrogen Ltd.) supplemented with 10%

heat-inactivated low endotoxin FBS (Biosera, Ringmer, UK) and 20% L929 cell-conditioned medium, and cultured at 1×10^6/ml for 7 days at 37 °C, 5% CO_2.

Cell lines and BMMΦs were then stimulated with 1,000 unlabelled CFDA-SE-labelled cercariae or 40 µg/ml unlabelled/CFDA-SE-labelled 0–3hRP in the presence of 3 µg/ml polymyxin B (Jenkins et al., 2005). To determine non-specific uptake, cells were cultured with FITC-labelled IgG (Invitrogen Ltd.). After overnight culture, cell supernatants were recovered, while cells were analysed for increased fluorescence by flow cytometry.

2.7. Infection of mice and ex vivo cell recovery

Mice were infected via the pinnae with 1,000 unlabelled or CFDA-SE-labelled cercariae (Paveley et al., 2009) and the pinnae and sdLNs recovered at 24, 48 and 72 h p.i. Skin cells were obtained from the pinnae after first being separated along the central cartilage and then floated on RPMI 1640 containing 50 µg/ml of Liberase (Roche Products Ltd., Welwyn Garden City, UK) for 30 min before being macerated and incubated for a further 30 min. The sdLNs were then cut into small pieces and incubated with DNAse (0.02 mg/ml; Sigma–Aldrich) and collagenase D (0.5 mg/ml; Roche Products Ltd.) for 20 min after which cells were recovered and washed in culture medium.

2.8. In vitro culture of sdLN cells; proliferation and cytokine detection

On day 5 p.i., LNs draining the infection site were removed and cell suspensions (2×10^5) stimulated with SSAP (5 µg/ml) were cultured for 72 h; culture supernatants were then recovered for the detection of cytokines, and cell proliferation measured by the uptake of methyl-³H Thymidine (18.5 kBq) (Hewitson et al., 2006). IL-12/23p40 and TNF-α were measured from cellular supernatants by using DuoSet ELISA kits (Invitrogen Ltd.). IL-6 was captured using anti-IL-6 monoclonal antibody (mAb, clone # MP5-20F3), probed with biotinylated anti-IL-6 mAb (clone # MP5-32C110) and detected with streptavidin peroxidase conjugate (all BD Pharmingen, Oxford, UK). The lower sensitivity of the assays were 15 pg/ml (TNF-α), 20 pg/ml (IL-6) and 32 pg/ml (IL-12p40). Stimulation indices for the proliferation of sdLN cells were determined as c.p.m. in response to SSAP divided by c.p.m. of cells in the absence of antigen. For the detection of intracellular cytokines, sdLN were cultured with SSAP for 48 h, followed by 10 U/ml recombinant human IL-2 for 18 h, and then phorbol myristate acetate (PMA, 10 ng/ml) and ionomycin (1 µg/ml) for 1 h. Finally, 1 µl GolgiPlug was added for 2 h, and cells recovered for antibody labelling and flow cytometry.

2.9. Flow cytometry

Flow cytometric analysis was performed using a Cyan ADP analyser (DakoCytomation, Ely, UK). Where required, cells were initially blocked with anti-CD16/32 mAb in PBS containing 1% FBS and 2 mM EDTA, and then labelled with directly conjugated antibodies; F4/80 Pacific Blue (clone # BM8), and CD40 allophycocyanin clone # 1C10, both from Insight Biotechnology Ltd., Wembley, UK. For intracellular cytokine staining, sdLN cells were first blocked as above, labelled with anti-CD4 allophycocyanin (#GK1.5; eBioscience), and then fixed and permeabilised with Cytofix (BD Pharmingen). Finally, cells were probed with anti IL-4-FITC (clone # BVD6-24G2) and anti-IFNγ-Pacific Blue (clone # XMG1.2; mAbs both from eBioscience). All antibody concentrations were optimised, and all analyses performed alongside irrelevant isotype controls. Data was analysed using Summit v4.3 (Dako, UK).

2.10. Co-localisation of intracellular CFDA-SE 0–3hRP and MR

J774E MΦs were cultured at a density of 0.2×10^6 on cover slips and stimulated with CFDA-SE-labelled 0–3hRP (40 µg/ml) for 1 h. Cells were then fixed in 2% paraformaldehyde and permeabilised using 0.2% saponin (Sigma–Aldrich Ltd.) for 30 min. The cover slips were then stained with DAPI (Sigma–Aldrich Ltd.) and anti-mouse MR/CD206 AlexaFluor 647 mAb (clone # MR5D3; AbD Serotec, Oxford, UK) or an irrelevant isotype control. The cell coated cover slips were washed three times and fixed to glass microscope slides with colourless nail varnish and Vectashield® (Vector laboratories, Peterborough, UK).

2.11. Statistics

Changes in cellular uptake of CFDA-SE-labelled antigen and differences in cytokine production were evaluated using a Student's t-test or one-way ANOVA (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

3. Results

3.1. Cercarial 0–3hRP contains a high concentration of glycans

As shown in Fig. 1A, the larval E/S preparation 0–3hRP contains a more limited range of proteins compared with SCAP and SSAP which comprise a number of cytosolic components from the different life cycle stages. Staining of 0–3hRP with Pro-Q® Emerald revealed the presence of several discreet glycosylated components ranging from 14 to >180 kDa, although there was a smear of very large molecules above 180 kDa (Fig. 1B). The strongest discreet band was evident at 50 kDa (Fig. 1B). The lack of fluorescence in an unoxidised gel confirms carbohydrate-specific staining (data not shown).

Using D-mannose as a standard for estimation of carbohydrate concentration, 0–3hRP had a high glycan content compared with SCAP and SSAP (Fig. 1C). Whilst SCAP should contain all of the molecular components of 0–3hRP, the high concentration of somatic proteins from the solubilised cercariae dilutes the carbohydrate content. SSAP had a lower quantity of carbohydrates, even compared with SCAP, confirming that glycans are mainly found in released E/S material which is largely absent in transformed larvae (Fig. 1C).

3.2. The CTLD4-7 region of the MR binds to parasite antigens

Various larval antigen preparations were probed with the recombinant Fc-fusion protein containing the MR domains CTLD4-7 (CRD4-7-Fc) (Linehan et al., 2001), to determine whether antigens from invasive larvae specifically bound to the MR. CRD4-7-Fc binding to mannan served as a positive control, whilst CR-FNII-CTLD1-3-Fc specific for SO_4-3-gal served as a negative control. The 0–3hRP product bound the greatest amount of CTLD4-7-Fc, almost twice as much as SCAP and SSAP (Fig. 2A). Moreover, the binding of CRD4-7Fc to all schistosome preparations was inhibited by the addition of 10 mM mannose but not galactose (Fig. 2B), confirming the glycan specificity of the fusion protein for MR ligands. Using western blotting, CTLD4-7-Fc recognised several glycosylated compounds between 37 and 100 kDa in 0–3hRP, SCAP and SSAP. The strongest bands in 0–3hRP at ∼37, 55, 66 and 75 kDa (Fig. 2C) co-localised with the presence of protein (Fig. 2D). Furthermore, as detected by binding of FITC-labelled CTLD4-7-Fc, MR ligands localised primarily to the acetabular glands of the freshly transformed schistosomula (Fig. 2E) and the E/S vesicles released after in vitro transformation (Fig. 2F) showing a high number of MR binding moieties in gland material which is secreted in the first few hours
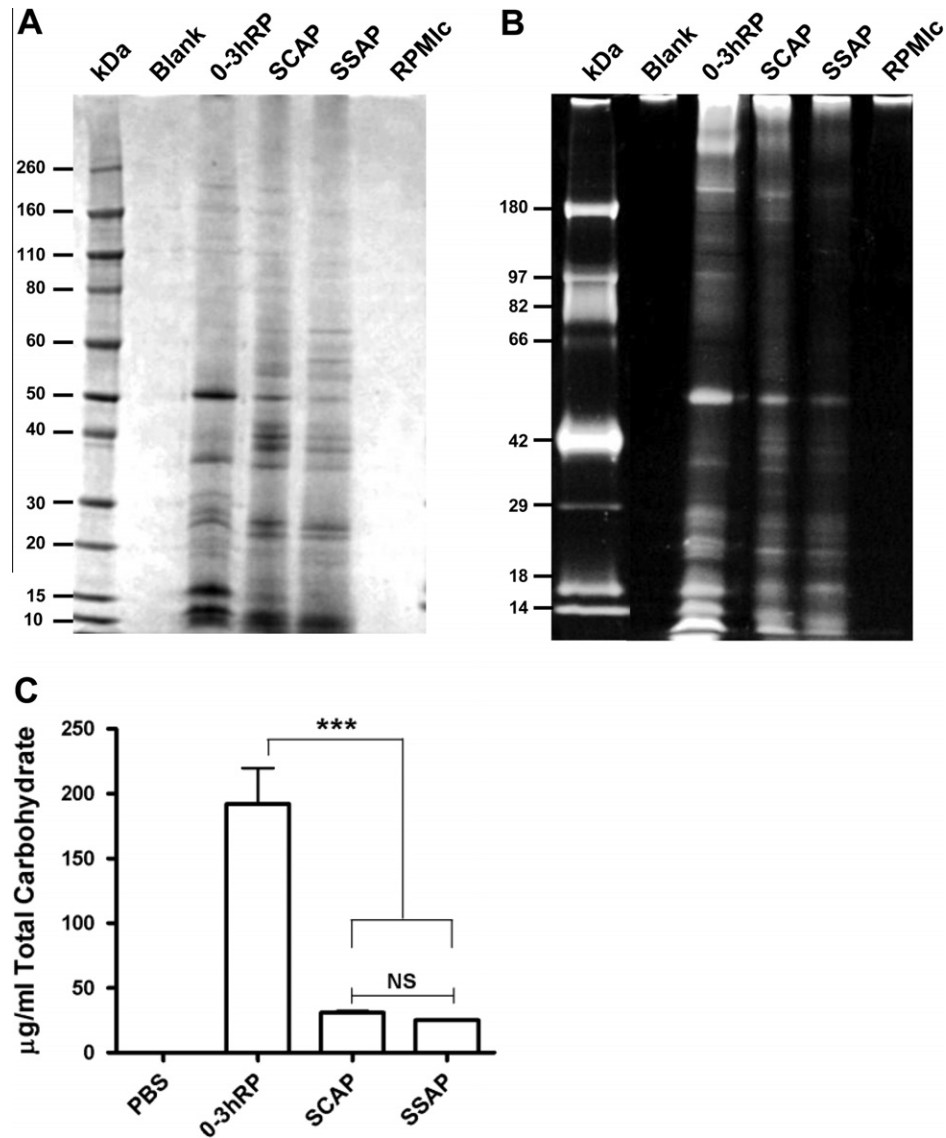
Fig. 1. Detection of glycans present within the cercarial 0–3 h released preparation (0–3hRP). (A) Protein detection by Coomassie Blue staining and (B) glycan detection by Pro-Q Emerald staining of 0–3hRP, soluble cercarial antigen preparation (SCAP), soluble schistosomula antigen preparation (SSAP) and RPMI concentrate (RPMIc). (C) Carbohydrate estimation of 0–3hRP, SCAP and SSAP (all 10 μg of protein) against a known carbohydrate standard curve. Gels (4–12% gradient) are representative of three independent experiments; bar graph is the mean of five different preparations ± S.E.M. Statistical significance is shown for 0–3hRP versus other antigens ($^{***}P < 0.001$). NS, not significant.

during infection of the skin (Paveley et al., 2009). Control schistosomula and vesicles labelled with IgG FITC demonstrate the specificity of CTLD4-7-Fc binding (Fig. 2Ga and b).

3.3. Cellular recognition and uptake of parasite antigen via MR

As reported previously (Paveley et al., 2009), labelling of cercariae or 0–3hRP with CFDA-SE has no effect upon the ability to stimulate antigen presenting cells such as MΦs and does not appear to change recognition via glycan moieties as the label attaches to protein components. Binding of E/S material released from CFDA-SE-labelled cercariae and CFDA-SE-labelled 0–3hRP was significantly elevated in CHO cells transduced to express MR ($15.3 \pm 0.6\%$ and $36.2 \pm 0.5\%$, respectively) compared with WT CHO cells ($P < 0.01$; Fig. 3A and B). There was no increase in the fluorescence of MR-expressing CHO cells cultured with control FITC-labelled IgG, confirming that this is not due to non-specific uptake (Fig. 3A). In addition, a MΦ cell line which over-expresses MR (J774E)

(Du et al., 2001) bound significantly greater quantities of E/S material from labelled cercariae and labelled 0–3hRP ($33.3 \pm 0.7\%$ and $49.6 \pm 2.4\%$, respectively) compared with control MΦs (J774A.1) (Fig. 3C and D). Moreover, CFDA-SE-labelled 0–3hRP was observed to co-localise with CD206$^+$ inside the phagosomes of J774E MΦs (Fig. 3E), providing further supporting evidence that MR is an important PRR aiding the internalisation of parasite E/S antigens. However, when stimulated with cercariae, J774E MΦs produced substantially lower levels of IL-12/23p40 and IL-6 compared with control cells (Fig. 3F), indicating that engagement of the MR with cercarial E/S antigens regulates cytokine production by MΦs.

3.4. Absence of MR leads to reduced antigen uptake but increased cell activation

A significantly greater proportion of WT BMMΦs phagocytosed E/S material released from CFDA-SE-labelled cercariae and labelled 0–3hRP compared with MR$^{-/-}$ BMMΦs ($P < 0.001$; Fig. 4A and B).
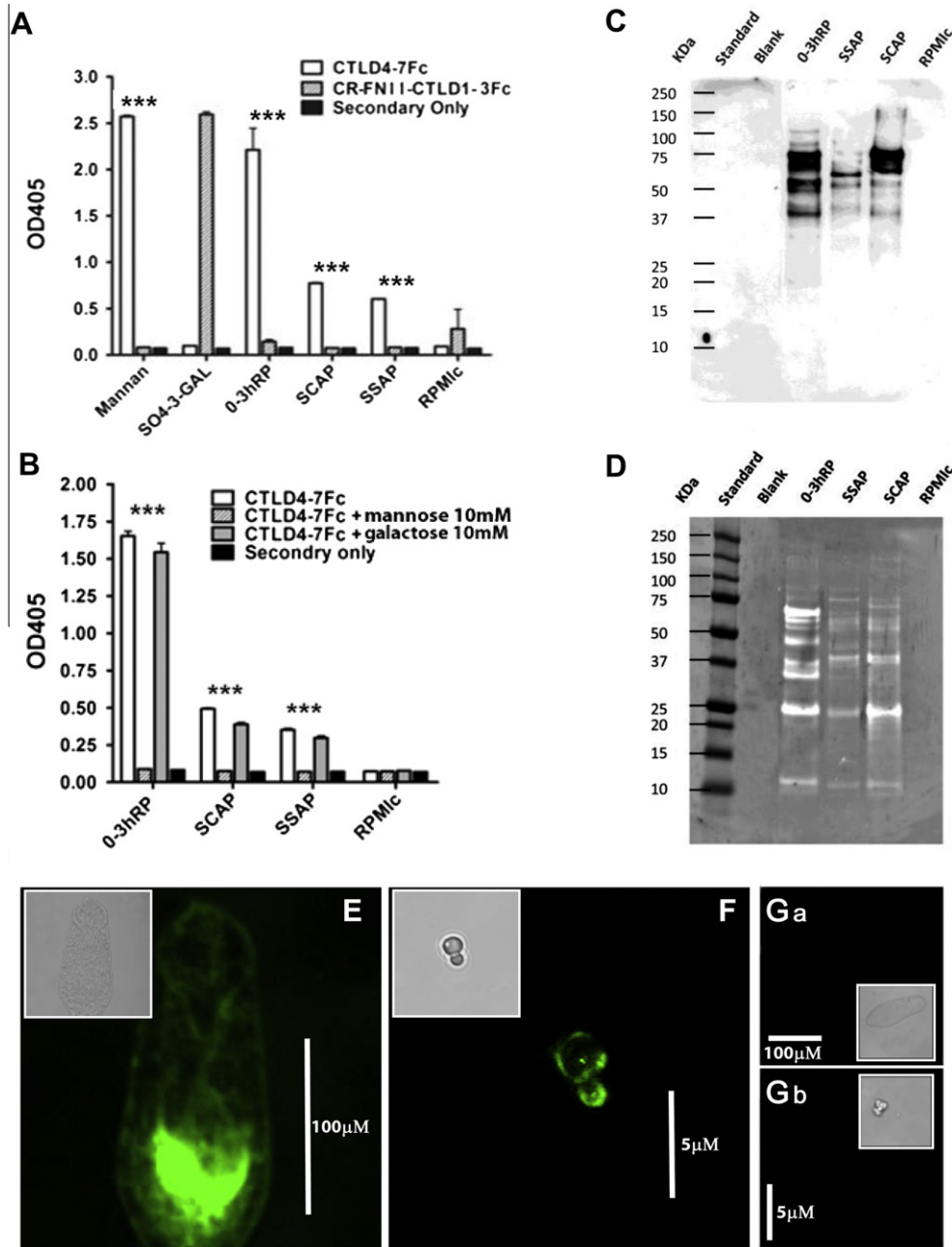
Fig. 2. Binding of the mannose receptor (MR) probe (CTLD4-7-Fc). (A) Binding of CTLD4-7-Fc (open bar), CR-FNII-CTLD1-3-Fc (hatched bar) and secondary antibody conjugate only (closed bar) to 0–3 h released preparation (0–3hRP), soluble cercarial antigen preparation (SCAP), soluble schistosomula antigen preparation (SSAP) (all 10 μg protein/ml), RPMI concentrate (RPMIc, equivalent volume to 0–3hRP), mannan (1 μg/ml) and SO$_4$-3-Gal (5 μg/ml). (B) Binding of CTLD4-7-Fc to schistosome antigen preparations (10 μg/ml) in the absence or presence of mannose (10 mM), and galactose (10 mM) as a mean of five different antigen preparations ± S.E.M. (C) Representative western blot probed with CTLD4-7-Fc and (D) subsequently stained with Sypro Ruby for protein detection. Localisation of CTLD4-7-Fc labelling in (E) whole cercarial head and (F) excretory/secretory (E/S) vesicle released from cercariae. Images were captured on a Zeiss LSM 510 meta confocal microscope at 488 nm/520 nm excitation/emission with CTLD4-7-Fc conjugated to FITC IgG. Control schistosomula and vesicles labelled with IgG FITC are shown in (G). Statistical significance is shown for binding of CTLD4-7-Fc versus CR-FN11-CTLD1-3Fc (a), or versus CTLD4-7-Fc plus mannose (b) (***$P < 0.001$).

The median fluorescent intensity (MFI) of CFDA-SE$^+$ BMMΦs from MR$^{-/-}$ mice stimulated with 0–3hRP was also significantly lower than WT BMMΦs ($P < 0.01$; Fig. 4B). A significantly greater proportion of MR$^{-/-}$ BMMΦs were positive for the co-stimulatory marker CD40 (cercariae; MR$^{-/-}$ = 54.0 ± 2.5%, WT = 37.1 ± 2.4%, $P < 0.01$; 0–3hRP; MR$^{-/-}$ 77.1 ± 1.0, WT 62.1 ± 1.0, $P < 0.01$; Fig. 4C), although the percentage of BMMΦs from MR$^{-/-}$ compared with WT mice that were MHC-II$^+$ was not significantly different (data not shown). The production of IL-12/23p40 ($P < 0.05$) and IL-6 ($P < 0.01$) were both significantly elevated in culture supernatants of MR$^{-/-}$ BMMΦs (Fig. 4D). This was evident regardless of whether the cercariae or 0–3hRP had been labelled with CFDA-SE demonstrating that the presence of this dye did not disrupt immune recognition of cercarial E/S material. As demonstrated previously (Jenkins et al., 2005), neither SCAP or SSAP at equivalent concentrations induced IL-12p40 or IL-6.
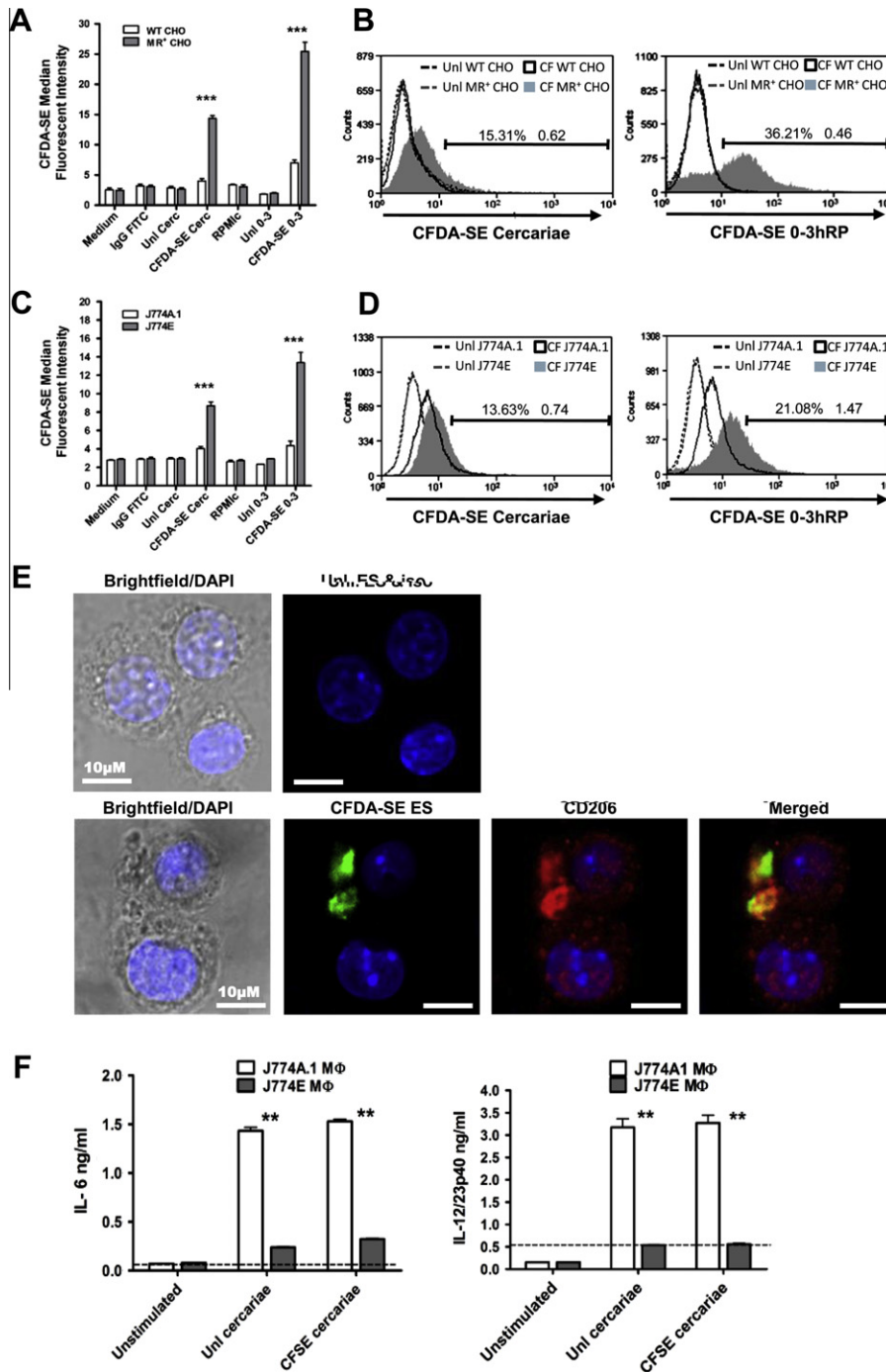
Fig. 3. Increased uptake of carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) labelled schistosome antigen by cell lines that over-express mannose receptors (MRs). CFDA-SE-labelled parasite antigen binding to (A) wild type (WT) and MR-expressing CHO cells, and (B) representative flow cytometry histograms illustrating the difference between WT and MR transgenic cells. (C) J744A.1 and J774E macrophage (MΦ) binding of CFDA-SE parasite material; (D) with representative flow cytometry histograms showing the fluorescence intensity of cells incubated with labelled (CF, solid) versus unlabelled (Unl, hatched) material. (E) Intracellular staining of J774E MΦs stimulated with unlabelled or CFDA-SE-labelled 0–3 h released preparation (0–3hRP), co-stained with DAPI and anti-MR/CD206 monoclonal antibody (mAb) AlexaFluor647 or an irrelevant isotype control; scale bar = 10 μm. (F) Production of IL-12/23p40 and IL-6 by J774A.1 and J774e MΦs after stimulation with unlabelled (Unl) and labelled (CFSE) cercariae, as a mean of five different preparations ± S.E.M. Cerc, cercariae; Unl 0–3, Unlabelled 0–3hRP; RPMIc, RPMI concentrate; iso, isotype ($^{***}P < 0.001$, $^{**}P < 0.01$).

3.5. Uptake of CFDA-SE parasite E/S by CD45⁺ cells in the skin is reduced in the absence of MR but the production of IFNγ in the sdLN is enhanced

Following percutaneous infection of WT and MR$^{-/-}$ mice with live CFDA-SE-labelled cercariae, significantly fewer CD45$^+$ cells that had taken up CFDA-SE-labelled antigen were recovered at 24 h from the skin of MR$^{-/-}$ mice compared with WT mice ($P < 0.05$; Fig. 5A). By 48 h, both strains of mice contained fewer CFDA-SE$^+$CD45$^+$ cells in the skin, which further declined by 72 h due to the movement of labelled schistosomula/cells out of the skin (Fig. 5A). In contrast, a peak in the number of CFDA-SE$^+$CD45$^+$ cells in the sdLN was reached at 48 h, presumably following the migration of antigen-laden cells from the skin (Fig. 5B).
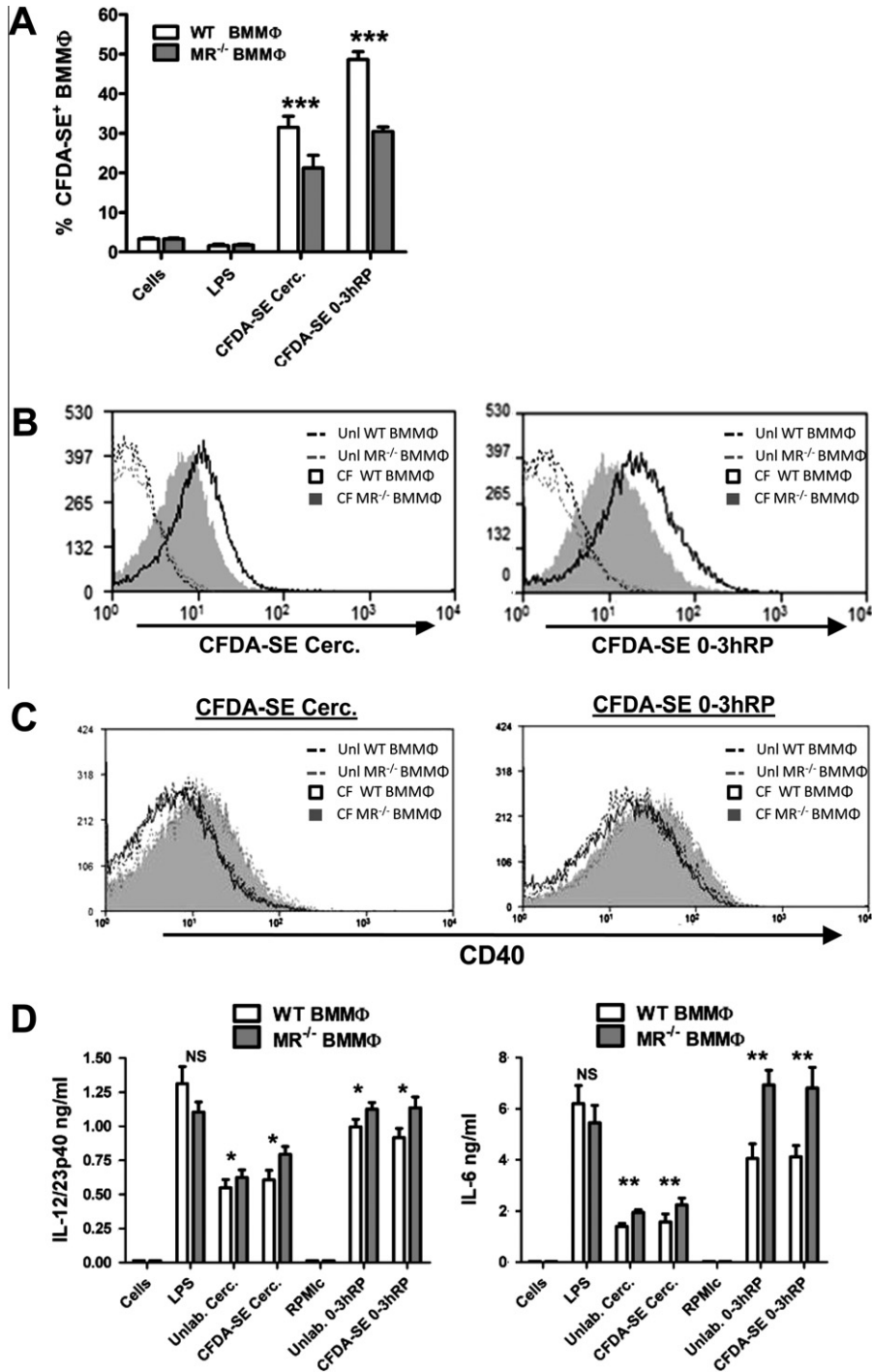
Fig. 4. Bone marrow derived macrophages (BMMΦs) from mannose receptor deficient (MR$^{-/-}$) mice are compromised in their ability to internalise carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)-labelled antigens from cercariae and 0–3 h released preparation (0–3hRP) but exhibit elevated levels of CD40 and increased cytokine production. (A) The percentage of CFDA-SE$^+$ BMMΦs after culture with labelled cercarial excretory/secretory (E/S) products and 0–3hRP, and (B) representative flow cytometry histograms showing uptake of CFDA-SE cercarial E/S and CFDA-SE 0–3hRP by wild type (WT) cells (mean fluorescence intensity, MFI for cercariae = unlabelled (Unl) 1.9 ± 0.3, CF 11.5 ± 0.4; and for 0–3hRP Unl 2.0 ± 0.4, CF 18.3 ± 1.3) and MR$^{-/-}$ cells (MFI for cercariae = Unl 1.9 ± 0.2, CF 7.2 ± 0.7 and for 0–3hRP = Unl 2.0 ± 0.1, CF 10.0 ± 0.4). (C) Representative histograms of CD40 expression on BMMΦs in response to CFDA-SE-labelled cercariae (Cerc.) (WT Unl 7.0 ± 1.1, CF 7.1 ± 0.6; MR$^{-/-}$ Unl 12.7 ± 1.3, CF 13.0 ± 2.2) and 0–3hRP (WT Unl 20.8 ± 0.7, CF 20.6 ± 0.6; MR$^{-/-}$ Unl 26.1 ± 2.0, CF 26.8 ± 1.1) (D) Production of IL-12p40/70 and IL-6 by WT and MR$^{-/-}$ BMMΦ in response to unlabelled and CFDA-SE labelled cercariae and 0–3hRP. Mean expression values ± S.E.M. (n = mean of nine mice). RPMIc, RPMI concentrate; CF, CFDA-SE; NS, not significant; ($^{***}P < 0.001$, $^{**}P < 0.01$, $^*P < 0.05$).

Significantly, the number of CFDA-SE$^+$CD45$^+$ cells in the sdLN of MR$^{-/-}$ mice at this time point was lower than in WT mice ($P < 0.05$; Fig. 5B).

Five days after exposure of MR$^{-/-}$ and WT mice to infective cercariae when the CD4$^+$ response in the sdLN reaches a peak (Hogg et al., 2003), the acquired immune response in the sdLN was examined in response to stimulation with parasite antigen in vitro. The ability of sdLN cells from MR$^{-/-}$ mice to proliferate was markedly greater (stimulation index 9.6 ± 1.7 versus 5.3 ± 0.85, $P < 0.05$; Fig. 5C). The enhanced ability of MR$^{-/-}$
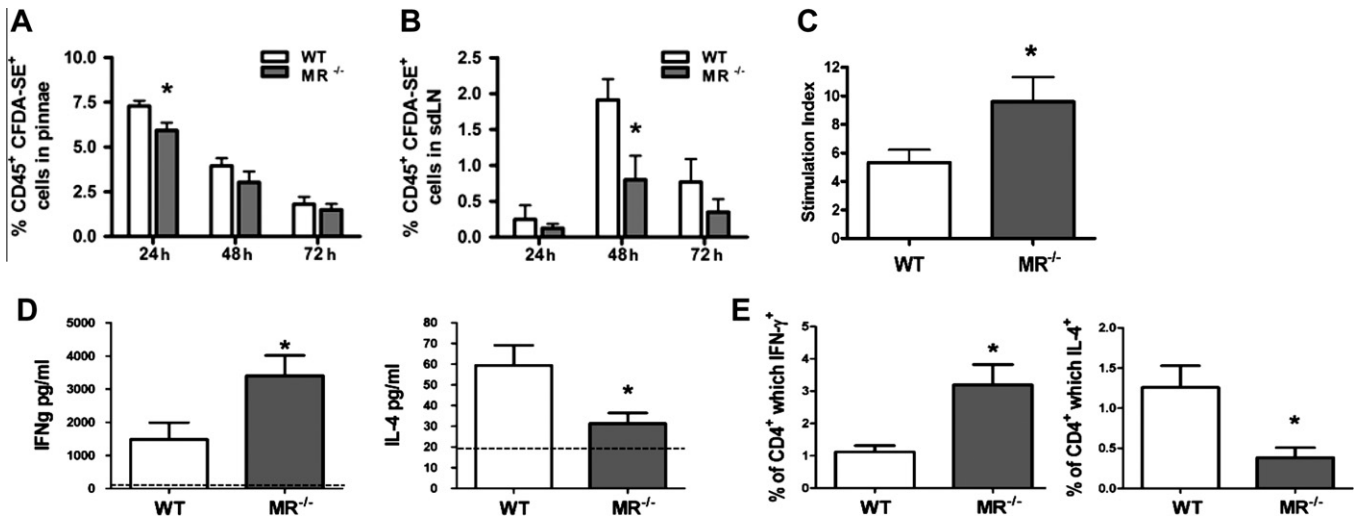
Fig. 5. Mannose receptor deficient (MR$^{-/-}$) mice infected with cercariae exhibit enhanced skin draining lymph node (sdLN) cell proliferation and CD4$^+$ IFNγ production. (A) The number of carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)$^+$ CD45$^+$ cells in the pinnae and (B) the sdLN of wild type (WT) and MR$^{-/-}$ mice after percutaneous infection with CFDA-SE labelled cercariae. (C) Proliferation of sdLN cells taken 5 days after infection cultured in vitro shown as the stimulation index of soluble schistosomula antigen preparation (SSAP) antigen-stimulated over unstimulated cells. (D) Production of IL-4, and IFNγ in pg/ml by sdLN cells cultured in the presence or absence of SSAP (mean ± S.E.M., n = 5–9 mice). (E) IL-4$^+$CD4$^+$ and IFNγ$^+$CD4$^+$ as percentage of total CD4$^+$ cells stimulated with SSAP (mean ± S.E.M., n = 3–5 mice). Significance is shown for MR$^{-/-}$ mice versus WT mice as $^*P < 0.05$.

lymphoid cells to proliferate was matched by the secretion of significantly greater levels of antigen-specific IFNγ ($P < 0.05$; Fig. 5D). On the other hand, the production of antigen-specific IL-4 was significantly reduced ($P < 0.05$; Fig. 5D). Combined, these data show that in the absence of MR, the immune response polarises towards an increased amount of IFNγ. Indeed, intracellular cytokine staining of antigen-stimulated sdLN cells revealed that the proportion of CD4$^+$ cells that are IFNγ$^+$ was nearly three-fold greater in MR$^{-/-}$ compared with WT mice (Fig. 5E). Moreover, although the overall numbers of IL-4$^+$CD4$^+$ cells in WT mice was low (Fig. 5E), as would be expected at this early time point after infection (Hogg et al., 2003), the proportion of IL-4$^+$CD4$^+$ cells in MR$^{-/-}$ mice was ~3-fold lower than in the WT mice.

4. Discussion

A number of glycan moieties have been identified in different pathogenic helminths that potentially bind various CLRs which in turn act as sensors of the innate immune system. Here, we have focussed on the role of MR/CD206, a type IV CLR, in the detection and subsequent uptake of E/S antigens released by schistosome cercariae. Our studies conclusively demonstrate that glycosylated antigens released by invasive larvae are recognised by MRs and, following natural parasite infection through the skin, are important in regulating the development of Th1 type immune responses.

E/S material released by transforming cercariae is rich in proteins (Curwen et al., 2006) and glycomic analysis of 0–3hRP reveals it to be rich in mannosylated and fucosylated residues (Jang-Lee et al., 2007). We too observed that 0–3hRP was rich in glycoproteins and they were more common than in SCAP and SSAP. The 0–3hRP antigen preparation is rich in ligands for CTLD4-7-Fc which specifically recognises MR ligands (Linehan et al., 2001) and had approximately three times the binding capacity of SCAP or SSAP. Binding of CTLD4-7-Fc to several bands on western blots of 0–3hRP demonstrates the presence of multiple glycosylated components that could bind to the MR.

Confocal imaging of CRD4-7Fc binding to schistosome larvae established that MR ligands were enriched within the acetabular glands. These glands contain numerous proteolytic enzymes and

other molecules that are secreted upon infection to aid the entry of larvae into the skin (Knudsen et al., 2005; Curwen et al., 2006). The gland contents are released as the larvae migrate through the epidermis, and in aqueous medium in vitro, they have the appearance of membrane-less vesicles with a micelle-like structure (Paveley et al., 2009). The release of cercarial E/S material into the skin highlights their potential role in stimulating cells of the innate immune response. We have previously shown that uptake of 0–3hRP by BMMΦs was inhibited by ethylene glycol tetra acetic acid (EGTA) and cytochalasin D (Paveley et al., 2009), indicating that CLRs, some of which are calcium-dependent and require actin polymerisation for phagocytosis (Kerrigan and Brown, 2009), are required for the uptake of E/S molecules. In accordance with cercarial E/S material and 0–3hRP being rich in ligands for the MRs, we observed enhanced binding of CFDA-SE-labelled antigens to CHO cells expressing MR. This receptor is thought by some not to be a typical professional phagocytic receptor (Le Cabec et al., 2005), whilst others show that the MR is essential for the phagocytosis of Pneumocystis carinii (Ezekowitz et al., 1991; Koziel et al., 1998) and Leishmania (Peters et al., 1995; Ueno et al., 2009). In this respect, we show that a greater proportion of MR$^+$ J774E MΦs were CFDA-SE$^+$ compared with MR-deficient J774A.1 MΦs, suggesting they had taken up labelled antigen in a MR-specific manner. Moreover, CFDA-SE-labelled 0–3hRP co-localised with the MR in the endosomes of J774E MΦs, supporting the view that MR is critical in the phagocytosis of cercarial E/S material.

As cell lines often lack the full array of naturally occurring CLRs, MΦs were obtained from mice selectively deficient in the MR. This revealed that uptake of CFDA-SE-labelled E/S material was not completely abolished and indicates that other receptors bind and internalise material in MR-deficient cells. This is not unexpected, as there is considerable redundancy in the CLRs that bind mannosylated structures (McGreal et al., 2006). For example, SIGNR1 has a high specificity for mannose and fucose oligosaccharides (Galustian et al., 2004; Koppel et al., 2004), as well as Lewis^x, Lewis^Y and sialylated Le^x structures, all of which are present on various life cycle stages of schistosome parasites (Hokke and Yazdanbakhsh, 2005). The human form of this receptor (DC-SIGN) binds to SEA (van Die et al., 2003) and is thought to play a role in regulating signalling pathways through TLRs (van Liempt et al.,

2007; van Stijn et al., 2010). However, although SIGNR1 can bind SEA, there is no alteration in the pathological response in vivo to *S. mansoni* eggs in SIGNR1$^{-/-}$ mice (Saunders et al., 2009). Other CLRs expressed on MΦs that recognise mannose include Endo-180 and Dectin-2 (East et al., 2002; McGreal et al., 2006). However, Dectin-2 has a 50-fold lower affinity for mannose and fucose than MRs (McGreal et al., 2006), and Endo-180 has a distinct ligand repertoire compared with the MR and is not involved in phagocytosis (Howard and Isacke, 2002). We therefore conclude that MR is an important PRR in the detection and uptake of cercarial E/S material and 0–3hRP but that other, as yet un-defined, PRRs play a supporting role.

A major finding of our studies is that ligation of the MRs has profound effects upon the production of various cytokines and priming of the acquired immune response in response to antigens release by schistosome larvae. First, MR$^+$ J774E MΦs stimulated with cercariae had a markedly reduced ability to secrete IL-6 and IL-12/23p40. Second, stimulation of MR$^{-/-}$ BMMΦs with 0–3hRP enhanced the secretion of these cytokines and the expression of CD40. Third, sdLN cells from infected MR$^{-/-}$ mice had a greater capacity to proliferate and secrete IFNγ. Combined, these findings suggest that ligation of the MR has a major role in down-regulating the innate response to E/S products by limiting the production of pro-inflammatory cytokines, although in other infection models it can lead to increased nitric oxide (NO) (Gruden-Movsesijan and Milosavljevic Lj, 2006), cytokine production (Netea et al., 2006; Heinsbroek et al., 2008) and TH17 responses (van de Veerdonk et al., 2009).

In support of a regulatory function for MRs, binding of the MR-specific mAb (PAM-1) stimulates the production of regulatory DCs (Chieppa et al., 2003), engagement by mannose in alveolar MΦs protects against LPS-induced lung injury (Xu et al., 2010) and Man-Lam induces significantly reduced levels IL-12 through the ligation of MRs on LPS matured DCs via the induction of IRAK-M (Pathak et al., 2005). Restricting IL-12/23p40 would restrain the expression of CD40 and consequently the activation of antigen-specific T cells, both of which in the absence of MRs were increased in our studies following schistosome infection. The mechanism causing down-regulation is not known but MR ligands such as ManLam from *Mycobacterium tuberculosis* (Kang et al., 2005) and *Mycobacterium avium* (Sweet et al., 2010) delay phagosome lysosome fusion. Therefore, ligation of the MR by schistosome E/S material might operate in a similar way to cause the observed slow rate of lysosome fusion with 0–3hRP containing phagosomes (Paveley et al., 2009). This would lead to down-regulation in the efficiency of antigen processing and presentation of larval antigens, thereby dampening T cell activity. This hypothesis is supported by our results demonstrating elevated antigen-specific cell proliferation in the absence of MRs. Significantly, the absence of MRs leads to enhanced production of antigen-specific IFNγ but diminished IL-4 which indicates that ligation of MRs favours the development of Th2 responses. This observation supports the recent findings that the binding of MRs to the dust mite allergen Der p 1 was essential for IL-4 and partially for IL-13 and IL-5 production but its absence lead to enhanced IFNγ release (Royer et al., 2010). This is of particular interest as certain molecules released by cercariae (i.e. Sm21.7, TAL-2 a fructose 1,6-bisphosphate aldolase) (Harrop et al., 1999), which may have the potential to bind MRs, have very similar sequence homologies to dynein light chains from non-parasite allergens (Fitzsimmons et al., 2007).

MR expression in MΦs is up-regulated by IL-4, IL-13 and IL-10 (Doyle et al., 1994; Martinez-Pomares et al., 2003; Dewals et al., 2010) and significantly, these three cytokines are markedly up-regulated in the skin of mice exposed to multiple exposures to *S. mansoni* larvae (Cook et al., 2011). Enhanced MR expression in this situation should lead to increased uptake of parasite E/S material.

However, as ligation of MRs on BMMΦs by parasite E/S products down-regulates expression of IL-12p40 and CD40, priming of the immune response in vivo may be compromised, as was observed following multiple infection (Cook et al., 2011). In addition, where there is an increased amount of IL-4- and IL-3-dependent 'alternatively-activated' MΦs (Martinez et al., 2009), such as in the skin following damage caused by schistosome infection, the MR is also likely to facilitate wound healing and tissue re-modelling through the binding of types I and II collagens (Martinez-Pomares et al., 2006; Napper et al., 2006).

In conclusion, there is large amount of glycosylated molecules in cercarial released material and many of those have specificity for the MR. As cercarial E/S products stimulate cytokine production by MΦs in a MyD88-dependent, and partially TLR-4-dependent mechanism (Jenkins et al., 2005), it is possible that ligation of the MR regulates the signalling through other PRRs such as TLRs. In fact ligation of TLR-4 with schistosome glycan LNFPIII from SEA can modulate DC function (Thomas et al., 2003). However, we show that ligation of the MR regulates the activation state of MΦs both in vitro and in vivo, which in turn modulates the development of antigen-specific proliferation and the production of IFNγ. On the other hand, the MR is required for the induction of IL-4 in response to cercarial E/S material and illustrates the potential of the MR in polarising the development of T helper responses to the schistosome parasite. Indeed, as the MR recognises other glycosylated allergens, such as Der p 1, which drive Th2 cell polarisation (Royer et al., 2010), it would seem likely that the MR might prove significant in the recognition and internalisation of glycosylated antigens from the schistosome egg (e.g. Omega-1) (Everts et al., 2009), which are strong polarisers of Th2-type immune responses.

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