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The C-Type Lectin SIGNR1 Binds *Schistosoma mansoni* Antigens In Vitro, but SIGNR1-Deficient Mice Have Normal Responses during Schistosome Infection▽

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The de novo immune response to infectious organisms arises from the innate recognition of pathogen-associated molecular patterns (PAMPs) by the host's pattern recognition receptors (PRRs). As the generation of type 2 cytokine responses by the human trematode parasite *Schistosoma mansoni* is glycan mediated, there is a particular potential role for a C-type lectin receptor (CLR) to mediate the innate recognition of schistosome PAMPs. One such CLR, dendritic cell-specific intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN; CD209), has been shown to recognize glycans expressed by *S. mansoni* eggs. We show that SIGNR1 (SIGN-related 1; CD209b), a murine homologue of DC-SIGN that is expressed on macrophages, also binds both schistosome-soluble egg antigens and worm antigens in vitro. The generation of schistosome egg-induced pulmonary egg granulomas was not altered in SIGNR1-deficient mice. Following *S. mansoni* infection, the SIGNR1-deficient mice had an unaltered phenotype with an intact immunological response and no difference in pathology. In this study we demonstrate that although SIGNR1 recognizes *S. mansoni* antigens in vitro, this CLR is redundant during infection. This study highlights the finding that although there was binding of SIGNR1 to immunogenic factors produced in the *S. mansoni* life cycle, this recognition does not translate to a functional in vivo role for the PRR during infection.

Schistosoma is a genus of parasitic digenetic trematodes that chronically infect more than 200 million people in developing countries, causing the tropical disease schistosomiasis. A major aspect of pathology during *Schistosoma mansoni* infection is immune mediated, with the infected hosts evoking inflammatory responses against the parasite eggs, which become trapped in host organs (25, 39). In *S. mansoni*-infected individuals there is a preferential deposition of parasite eggs in the liver and intestines. Consequently, these organs develop the granulomatous inflammation and fibrosis that are clinical features of *S. mansoni* infections. The characteristic immune response following *S. mansoni* infection is a polarized type 2 cytokine response, with elevated interleukin-4 (IL-4), IL-5, and IL-13 production and consequent eosinophilia, fibrosis, and production of immunoglobulin E (IgE). The genesis of the type 2 response following infection is primarily attributed to the potent type 2 cytokine-stimulating capacity of *S. mansoni* eggs and soluble egg antigens (SEAs) (36). SEA is highly glycosylated, and the antigen's capacity to induce type 2 responses is glycan dependent, with SEA deglycoslated by periodate treatment having diminished type 2 responses when administered to mice (22, 31). As these schistosome egg glycoconjugates evoke a marked skewing of the immune response toward a type 2-biased phenotype, there is considerable interest in the mechanisms of antigen-presenting cell (APC) recognition of glycans in SEAs. Schistosome glycoconjugates function as pathogen-associated molecular patterns (PAMPs) that modulate the innate immune response via interaction with pattern recognition receptors expressed on antigen-presenting cells.

While the Toll-like receptors represent the most extensively characterized family of pattern recognition receptors, there is an increasing understanding of the role of C-type lectin receptors (CLRs) in innate immune activation by schistosome glycans. One CLR expressed on human dendritic cells (DCs) is DC-specific intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN; CD209), which is implicated in the recognition of glycans from various pathogens including schistosomes (6, 33). DC-SIGN has been shown to bind glycans in *S. mansoni* SEAs, including the Lewis^X trisaccharide containing carbohydrate lacto-*N*-fucopentaose III (LNFPIII) and GalNAcb1-4(fuca1-3)GlcNAc (1, 32, 34). In addition, a DC-SIGN homologue, L-SIGN, that is expressed on liver sinusoidal endothelial cells also binds SEA (34) but recognizes different SEA glycans than DC-SIGN (20). In terms of the innate induction of type 2 responses by schistosomes, modulation of DCs to a DC2 phenotype following SEA treatment has been well described (10, 18), with the egg glycan LNFPIII driving the generation of DC2s (30).

In view of the known role for glycans in immune modulation by schistosomes, we investigated the interactions of the mouse CLR SIGNR1 (SIGN-related 1; CD209b). SIGNR1 is one of 7 paralogues of DC-SIGN described in mice in a family which

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also contains a related pseudogene, SIGNR6 (24, 26). Whether SIGNR1 is expressed on DC subsets is not as yet clear, but it is expressed on spleen marginal zone (MZ), lymph node, and peritoneal macrophages (7, 12, 23, 24, 29). SIGNR1 is known to recognize glycans from different pathogens and has been shown to bind Lewis$^{X/Y}$- and Lewis$^{A/B}$-containing carbohydrates (6, 13). In this study we demonstrate that while SEAs and also schistosome worm antigens are bound by SIGNR1 in vitro, the use of SIGNR1-deficient (−/−) mice (16) reveals that in the absence of the receptor, no alterations in the immunological and pathological response occur following *S. mansoni* egg injection or during acute and chronic infection.

MATERIALS AND METHODS

Mice and parasite infection. SIGNR1$^{-/-}$ mice (16) were backcrossed for eight generations to the BALB/c strain. Mice were housed under specific-pathogen-free conditions. Animals were fed irradiated diet and housed on irradiated bedding in individually ventilated and filtered cages under positive pressure (Tecniplast, Northants, United Kingdom). A Puerto Rican strain of *S. mansoni* was maintained by passage in BALB/c mice and albino *Biomphalaria glabrata* snails. Six- to eight-week-old SIGNR1$^{-/-}$ and wild-type BALB/c mice were infected percutaneously with 25 cercariae. Mice were culled at 8 and 16 weeks postinfection. All animal experiments were performed in compliance with Irish Department of Health and Children regulations and approved by the Trinity College Bioresources ethical review board.

Parasitology and pathology. *S. mansoni* adult worm antigen (AWA) and SEAs were prepared as described previously (5). AWAs and SEAs were screened for endotoxin contamination, and only antigen batches containing <1 endotoxin unit/mg (Chromogenic LAL, Biowhittaker, MD) were used. Portal perfusion for worm recovery and liver, intestinal, and fecal egg counts were as described previously (4). Parasite fecundity was determined by dividing total tissue (liver and intestine) egg counts by the number of worm pairs present. Livers from mice were taken and fixed in 10% formaldehyde-saline for histology. Sections of liver were stained with hematoxylin and eosin for egg granuloma diameter measurements and Giemsa-stained for eosinophil quantification. Hepatic fibrosis was measured by quantification of collagen by differential staining of histology sections (4). Fibrosis is expressed as micrograms of collagen per milligram of protein. To quantify the extent of liver damage in mice, plasma was recovered for analysis of aspartate aminotransaminase (AST) levels (4).

Cell culture and immunological analysis. Spleens or mesenteric lymph nodes were removed and passed through 70-μm-pore-size sieves (Falcon) to prepare single-cell suspensions. Cells from liver granulomas were isolated as described previously (37). Splenocytes were depleted of erythrocytes by lysis with ammonium chloride solution. Cells were cultured in RPMI 1640 medium (Biowest, United Kingdom) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Labtech, United Kingdom), 2 mM L-glutamine (Gibco), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco). Cell suspensions were unstimulated (medium) or stimulated with 2.5 ng/ml phorbal 12-myristate 13-acetate (PMA; Sigma, United Kingdom) and 250 ng/ml ionomycin (Sigma, United Kingdom), SEA (20 μg/ml), or AWA (20 μg/ml) at 37°C. Supernatants were harvested after 72 h and stored frozen. Sandwich enzyme-linked immunosorbent assays (ELISAs) were performed to quantify levels of specific cytokines in the supernatants from cell cultures. Reagents for detection of IL-4 were from BD Pharmingen (United Kingdom), and gamma interferon (IFN-γ), IL-10, and tumor necrosis factor alpha (TNF-α) reagents were purchased from R&D Systems (United Kingdom). Serum antigen-specific antibody (IgG and IgE) responses to SEA were measured using ELISAs, as described previously (4).

SIGNR1 binding assay. NIH 3T3 fibroblasts stably expressing SIGNR1 were generated as described previously (29) and cultured in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, and 0.6 mg/ml G418 (Invitrogen). Ligands for SIGNR1 were assessed by competitive binding assays as previously described (29). In brief, NIH 3T3 cells expressing SIGNR1 were plated in 24-well plates the day before experiments. Cells were washed three times with ice-cold medium prior to addition of mannan as an inhibitor for 1 h at 4°C. After pretreatment, fluorescein isothiocyanate (FITC)-labeled zymosan (at a zymosan-to-cell ratio of 20:1) was added for 1 h at 4°C. After incubation, unbound zymosan was removed by four washes with medium, and cells were lysed with 3% Triton X-100. FITC in lysates was quantified using a Titer-Tek Fluoroskan II instrument (Labsystems Group, Basingstoke, United Kingdom) and expressed as relative fluorescence units.

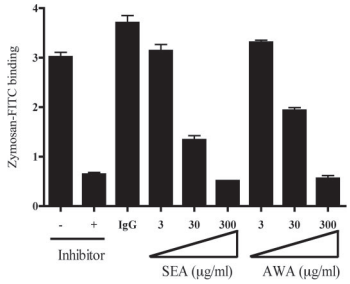

FIG. 1. Testing binding of *S. mansoni* antigens to SIGNR1 in vitro. SIGNR1-transfected NIH 3T3 cells were preincubated with no inhibitor (−), a known SIGNR1 binding sugar (mannan; 200 μg/ml), *S. mansoni* SEAs, or AWAs (3, 30, and 300 μg/ml), or rat IgG. FITC-conjugated zymosan was added, and bound FITC was quantified using a fluorimeter (as described in Materials and Methods) and is expressed as relative fluorescence units. Data are representative of two experiments and are expressed as means ± standard error of the means of duplicate samples.

Statistical analysis. All in vivo experiments were performed at least two separate times. The difference between groups was analyzed by a Student's *t* test. *P* values of <0.05 were considered significant.

RESULTS

SIGNR1 binds schistosome worm and egg antigens. As *S. mansoni* SEA glycans are known to bind to DC-SIGN (1, 32), we investigated whether soluble antigens from *S. mansoni* eggs and worms were recognized by SIGNR1. Schistosome SEAs or AWAs were tested in a FITC-zymosan binding assay using NIH 3T3 cells transfected with SIGNR1 (29). In this assay the binding of FITC-zymosan to SIGNR1-transfected cells was blocked by addition of mannan as an inhibitor, which is bound by SIGNR1 (21) but not by rat IgG (Fig. 1). Both SEA and AWA bound to SIGNR1 in a dose-dependent manner (Fig. 1). Therefore, using an in vitro cell-binding assay, we show that glycans within *S. mansoni* egg and worm antigens are recognized and bound by SIGNR1.

SIGNR1-deficient mice have normal egg-induced pulmonary granulomas. SIGNR1 has been shown to bind glycans from a range of pathogens in vitro, including *Streptococcus pneumoniae* (11). Following *S. pneumoniae* infection of SIGNR1$^{-/-}$ mice, a protective role for the in vivo interactions between *S. pneumoniae* and SIGNR1 has been shown, with altered pathogenesis and pulmonary response in SIGNR1$^{-/-}$ mice (15, 16). As egg antigens were bound by SIGNR1 in vitro (Fig. 1), we used the schistosome egg-induced pulmonary granuloma model to investigate whether SIGNR1$^{-/-}$ mice had an intact capacity to generate egg-specific pulmonary immune responses. In this model schistosome eggs are injected intravenously to induce Th2-mediated eosinophil-rich granuloma in the lungs, with the result that mice presensitized by intraperitoneal egg injection have greater secondary pulmonary responses (19). The innate response following intravenous injection of eggs into mice evoked granulomatous inflammation around eggs trapped in the lungs, with no difference between wild-type and SIGNR1$^{-/-}$ mice in the sizes of the cell infiltration surrounding the eggs (Fig. 2A). Similarly, the sizes of

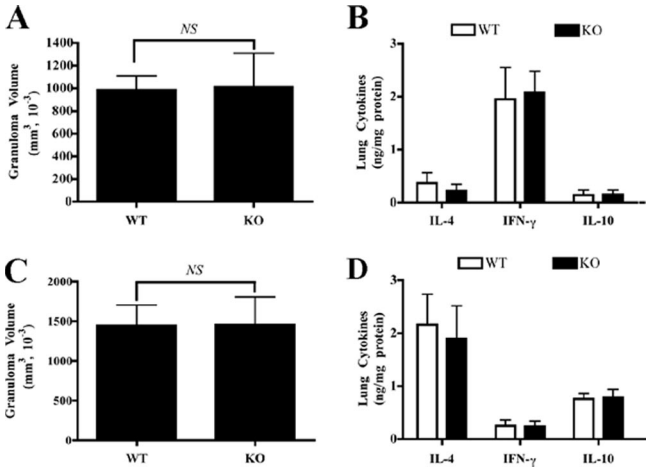

FIG. 2. Evaluation of primary and secondary *S. mansoni* egg pulmonary granulomas in wild-type (WT) and SIGNR1$^{-/-}$ (KO) mice. For study of the primary granulomas, mice were injected intravenously with 5,000 eggs. After 14 days the lungs were removed and fixed for histology sections for measurement of the volume of pulmonary egg granulomas (A), or lungs were isolated and homogenized for ELISA detection of Th1 (IFN-γ), Th2 (IL-4), and regulatory (IL-10) cytokine protein, adjusted to nanograms of cytokine per milligram of protein (B). To evoke secondary granulomas, mice were presensitized intraperitoneally with 5,000 eggs and injected 14 days later with 5,000 eggs intravenously. Similar to primary granulomas, 14 days later lungs were removed, and granuloma volume (C) or lung cytokine protein production (D) was evaluated. Data are means ± standard error of the means from 14 to 17 mice per group. A Student's *t* test was used to test for statistical differences between groups. NS, nonsignificant.

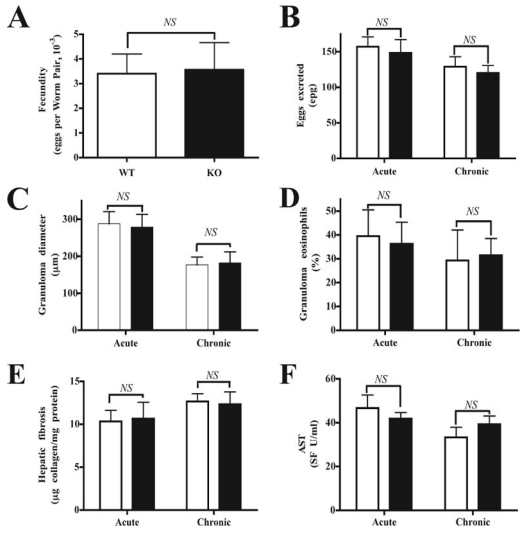

FIG. 3. Parasitology and pathology following *S. mansoni* infection of wild-type (WT) and SIGNR1$^{-/-}$ (KO) mice. Mice were infected with 25 cercariae and culled in acute (8 weeks) and chronic (16 weeks) stages of infection. Worm fecundity (eggs per worm pair) (A) and egg excretion (eggs per gram of feces; [epg]) in the feces (B) were determined. The diameter (C) and eosinophil composition (percentage of granuloma cells) (D) of granulomas surrounding eggs in the liver were quantified. Hepatic fibrosis, expressed as micrograms of collagen per milligram liver protein (E), and plasma levels of AST, a marker for hepatocyte damage in *S. mansoni* infection of mice (F), were analyzed. Data are means ± standard error of the means from 13 to 17 mice per group at 8 weeks and from 9 to 12 mice per group at 16 weeks. A Student's *t* test was used to test for statistical differences between groups at acute and chronic stages of infection. NS, nonsignificant.

pulmonary egg granulomas were comparable in both groups of mice that had been presensitized to schistosome eggs (Fig. 2C), with no differences in the cell composition of the granuloma between wild-type and SIGNR1$^{-/-}$ mice (data not shown). The levels of Th1 (IFN-γ), Th2 (IL-4), and regulatory (IL-10) cytokine proteins in the lungs of both groups were no different following primary (Fig. 2B) and secondary (Fig. 2D) egg challenge. These data show that there is no defect in pulmonary response in SIGNR1$^{-/-}$ mice after challenge with *Schistosoma* eggs.

SIGNR1-deficient mice have normal acute and chronic *S. mansoni* infections. SIGNR1 is expressed primarily on peritoneal and spleen MZ macrophages (12), with very little or no SIGNR1 expression being found on DCs, alveolar macrophages, B cells, or T cells (7, 12, 23, 24, 29). Thus, the absence of alterations in schistosome egg-induced pulmonary response in SIGNR1$^{-/-}$ mice (Fig. 2) could be due to the lack of expression of SIGNR1 on lung alveolar macrophages. In view of the essential role for macrophages in *S. mansoni* infection (8), SIGNR1$^{-/-}$ and wild-type mice were infected with *S. mansoni*, and the immunological response and pathology were compared between groups. Following infection, parasitological and immune responses of SIGNR1$^{-/-}$ and wild-type animals were analyzed at the acute 8-week stage of infection and the chronic down-modulated stage of infection at 16 weeks.

There were no statistical differences in the range of parasitological and pathological responses between SIGNR1$^{-/-}$ and wild-type mice following schistosome infection. Worm fecundity and fecal egg excretion were unaltered in deficient mice (Fig. 3A and B), and the size and eosinophil content of granulomas surrounding eggs in the liver were comparable, as were the levels of hepatic fibrosis (Fig. 3C to E). Consistent with normal liver pathology in schistosome infection and intact egg excretion, there were no differences in plasma levels of AST (Fig. 3F), a marker for hepatocyte damage in *S. mansoni* infection of mice (4). Furthermore, the deficient mice developed the normal downmodulation in inflammatory responses seen in chronic stages of infection, with smaller-sized granulomas in both groups of mice in the chronic stages versus acute stages of infection (Fig. 3C). A characteristic of the hepatic pathology in *S. mansoni*-infected mice is the upregulation of Arg-1 in the liver, consistent with an increase in alternatively activated macrophages in the liver (9). *S. mansoni*-infected wild-type and SIGNR1$^{-/-}$ mice had marked elevations in Arg-1 mRNA levels in the livers relative to uninfected mice, with no differences in Arg-1 expression between groups (data not shown). Indeed, IL-4-induced expression of alternatively activated genes Fizz-1, Ym-1, and Arg-1 by peritoneal macrophages was comparable between wild-type and SIGNR1$^{-/-}$ mice (data not shown), indicating no in vivo or in vitro defect in alternative activation of SIGNR1$^{-/-}$ macrophages.

Spleens and mesenteric lymph nodes were removed from uninfected mice and animals with an acute or chronic infection. Granulomas were separated from livers, and granuloma cells were isolated. Cells were simulated in vitro with PMA/ionomycin, SEA, or AWA. The secretion of IL-4, IFN-γ, IL-10, and TNF-α were detected by ELISA as these cytokines are

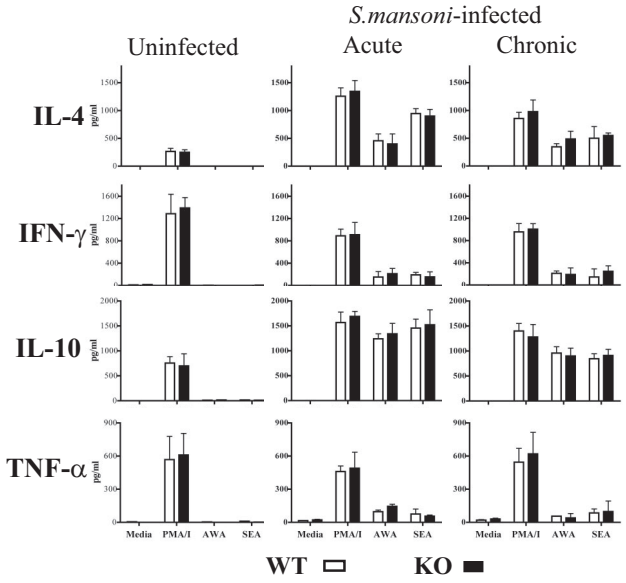

FIG. 4. Production of cytokines from spleen cells from uninfected and *S. mansoni*-infected wild-type (WT) and SIGNR1$^{-/-}$ (KO) mice. Spleens were removed, and prepared cell suspensions were cultured in vitro in medium alone, PMA/ionomycin, AWA, or SEA. After 72 h supernatants were assayed by ELISA for IL-4, IFN-γ, IL-10, and TNF-α. Data shown are means ± standard error of the means from 2 to 4 individual spleens and are representative of at least three experiments per time point.

implicated in altered immunopathology in schistosome infection of mice (39). Following polyclonal stimulation with PMA/ionomycin, there were no differences in cytokine production between wild-type and SIGNR1$^{-/-}$ mice (Fig. 4). Furthermore, even though SIGNR1 bound SEA and AWA in vitro (Fig. 1), spleen cells from schistosome-infected SIGNR1$^{-/-}$ mice had intact cytokine responses to these antigens upon restimulation (Fig. 4). We also observed no differences in cytokine production by cells from the mesenteric lymph nodes or granuloma (data not shown). In addition, there was no difference in the production of SEA-specific IgG and IgE antibodies between wild-type and schistosome-infected SIGNR1$^{-/-}$ mice (data not shown).

DISCUSSION

Since schistosome SEA has been shown to bind human DC-SIGN (1) and since the receptor is similar to the murine DC-SIGN homologue SIGNR1, we explored whether *S. mansoni* SEA and AWA were also recognized by SIGNR1. We show using an in vitro cell transfection assay that glycans from both SEAs and AWAs bind SIGNR1 in a dose-dependent manner, thus illustrating recognition of glycoconjugates expressed on two different stages of the parasite life cycle by murine SIGNR1. This is the first report of the binding of schistosome antigens by SIGNR1.

To investigate the role of SIGNR1 recognition of schistosome antigens in vivo, we used SIGNR1$^{-/-}$ mice (16). We demonstrated that SIGNR1 has no role in a primary or secondary pulmonary granuloma model induced by schistosome eggs, with deficient mice exhibiting an intact capacity to generate an immune response toward egg antigens. Considering that, to date, SIGNR1 expression has been attributed almost exclusively to macrophage populations of the spleen MZ, lymph nodes, and the peritoneum (7, 12, 29), an absence of its expression on alveolar macrophages could have accounted for the lack of an altered immune response in the pulmonary granulomatous model. However, in pulmonary challenge models with *S. pneumoniae*, SIGNR1 recognizes *S. pneumoniae* polysaccharides in in vitro assays, and the SIGNR1$^{-/-}$ mice used here have been shown to be more susceptible than wild-type mice to infection (15, 16). In contrast, although SIGNR1 binds *Mycobacterium tuberculosis* mannosylated lipoarabinomannan (14), infection of SIGNR1$^{-/-}$ mice indicated a limited role for the molecule during infection (38). Indeed, discrepancies between in vitro recognition of pathogen PAMPs by CLRs and the functional role for the CLRs during in vivo infection are features of a number of bacteria (27).

In order to fully elucidate any potential immunological role for SIGNR1 during *S. mansoni* infection, we infected SIGNR1$^{-/-}$ mice. Analysis of a range of immunological, parasitological, and pathological infection-related parameters demonstrated that SIGNR1$^{-/-}$ mice are comparable to wild-type animals. There was no difference between groups at the acute (8 week) and chronic (16 week) stages of infection, with SIGNR1$^{-/-}$ mice developing the normal downmodulation of granulomatous inflammation that occurred in wild-type mice during chronic stages of infection. Furthermore, while SIGNR1-transfected cells bound SEA and AWA in an in vitro assay, restimulation of spleen, mesenteric, or granuloma cells from schistosome-infected SIGNR1$^{-/-}$ mice with SEA or AWA showed intact production of IFN-γ, IL-4, IL-10, and TNF-α. These are cytokines implicated in altered immunopathology in schistosome infection of mice (3, 39). Consistent with no alterations in infection-induced immunity, the levels of SEA-specific IgE and IgG and also anti-AWA (data not shown) in schistosome-infected SIGNR1$^{-/-}$ mice were no different from those of the wild-type group. Alternatively activated macrophages are induced in schistosome infection of mice (9) in part via the egg glycan LNFPIII (2), with alternatively activated macrophages having an essential biological role in infection (8). Consistent with the absence of exacerbated pathology, there were no differences between wild-type and SIGNR1$^{-/-}$ mice in the in vitro or in vivo expression of alternatively activated macrophage markers. Even in heavier, more acute experimental infections (using 100 cercariae), there were also no differences in mortality or inflammatory responses between wild-type and SIGNR1$^{-/-}$ mice (data not shown). These data indicate that in the absence of SIGNR1, mice develop a normal *S. mansoni* infection.

In a recent in vitro study, multiple CLRs were shown to be involved in the binding and internalization of SEA by DCs, suggesting that DC-SIGN functions in tandem with other CLRs for antigen processing and presentation to occur in response to SEA (35). In our studies the absence of an in vivo effect in SIGNR1$^{-/-}$ mice may be due to the redundancy of this molecule, with other macrophage CLRs, such as other SIGNR molecules, the mannose receptor, or macrophage galactose-type lectin, compensating in recognition of schistosome glycans. Consequently, a number of in vivo studies with mice deficient in CLRs, such as the mannose receptor (17), would

undoubtedly prove informative as to which molecules schistosomes target in order to modulate the host toward a type 2 immune response. As shown here with SIGNR1, due to the potential for redundancy and compensatory recognition of glycans, mice with multiple CLR depletions may be required. Ultimately, in the context of the biological function of CLRs in human schistosome infection, studies on the role of DC-SIGN are required. Such studies may be helped by a recent transgenic mouse model designed to express human DC-SIGN. Transgenic mice have been generated that express human DC-SIGN under the control of the CD11c promoter; these revealed a new perspective on the role of DC-SIGN in *M. tuberculosis* infection (28).

In summary, this study demonstrates that although there was binding of SIGNR1 to immunogenic factors produced in the *S. mansoni* life cycle, this does not translate to the receptor having in vivo function during *S. mansoni* infection or egg-induced pulmonary responses. These data illustrate the need for caution when extrapolating from in vitro binding of PAMP in transfected cell assays to biological function during infection.

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