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DENDRITIC CELL EXPRESSION OF THE C-TYPE LECTIN RECEPTOR CD209a: A NOVEL INNATE PARASITE-SENSING MECHANISM INDUCING Th17 CELLS THAT DRIVE SEVERE IMMUNOPATHOLOGY IN MURINE SCHISTOSOME INFECTION

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Abstract

Following infection with the trematode helminth *Schistosoma mansoni*, CBA mice develop severe parasite egg-induced hepatic granulomatous inflammation as well as prominent CD4⁺ T helper 17 (Th17) cell responses driven by dendritic cell (DC)-derived IL-1 β and IL-23. By comparison, C57BL/6 mice develop mild hepatic immunopathology, egg stimulation of DCs does not result in IL-1 β and IL-23 production, and Th17 cells fail to develop. To investigate the reasons for strain-specific differences in antigen presenting cell (APC) reactivity to eggs, we performed a comparative gene profiling analysis of normal bone marrow-derived DCs (BMDCs) and found that CBA DCs display markedly elevated expression of C-type lectin receptors (CLRs). In particular, expression of CD209a, a murine homologue of human DC-specific ICAM-3-grabbing non-integrin (DC-SIGN, CD209), was strikingly higher in CBA than BL/6 DCs. High CD209a surface expression was observed in various CBA splenic and granuloma APC subpopulations, however, only DCs, and not macrophages, B cells or neutrophils, were able to induce Th17 cell differentiation in response to schistosome eggs. Lentiviral gene silencing in CBA DCs, and over-expression in BL/6 DCs, demonstrated CD209a to be critical for egg-induced DC IL-1 β and IL-23 production necessary for Th17 cell differentiation and expansion. These findings reveal a novel innate parasite-sensing mechanism promoting CD4⁺ Th17 cells that mediate severe immunopathology in schistosomiasis.

Abstract

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Figure 1, Graphical Abstract. Egg-induced, CD209a-dependent induction of pathogenic Th17 cell response. In mice prone to severe pathology, such as CBA, DCs expressing high levels of CD209a recognize schistosome egg Ags and acquire a proinflammatory phenotype (“classical activation”). Although still largely unknown, this activation process involves the phosphorylation of Src, RAF-1 and ERK MAP kinases and results in the secretion of IL-1 β and IL-23. These cytokines are required for the differentiation and expansion of MHC restricted, Ag-specific, Ror γ^+ Th17 cells, which secrete IL-17, CSFs, IL-22, IL-21, TNF- α and the chemokines CXCL1, CXCL2, and orchestrate the development of severe egg-induced hepatic granulomatous inflammation. In mice with mild pathology, such as BL/6, negligible CD209a expression fails to activate DCs, and Th17 cells are not generated (not shown).

Keywords

CD209a; Th17; Schistosomiasis; Dendritic cell; Immunopathology

Schistosomiasis: the human disease and the experimental murine model

Schistosomes are trematode helminths that cause extensive disease in the developing world, accounting for over 200 million infections and more than 200,000 deaths per year. In infection with the species *Schistosoma mansoni* (*S. mansoni*), the principal cause of morbidity and mortality is granulomatous inflammation and subsequent fibrosis around parasite eggs deposited in the liver and intestines [1–5]. Most infected individuals develop mild gastrointestinal disease, but 5–10% develop life-threatening hepatosplenic schistosomiasis, characterized by severe liver fibrosis, portal hypertension, splenomegaly, ascites, gastrointestinal bleeding and death [1–5]. The granulomatous inflammation in schistosomiasis is precipitated and orchestrated by CD4⁺ T lymphocytes sensitized to schistosome eggs, which are highly immunogenic structures capable of secreting a vast array of highly fucosylated glycoproteins [6–9]. Analogous to humans, dissimilar disease severity is also observed in an experimental murine model of schistosomiasis. Infected CBA/J (CBA) mice develop severe hepatic pathology characterized by large poorly circumscribed perioval granulomas [10–12]. Such severe immunopathology in CBA and other high-pathology strains, including C3H, SJL and MOLF [12–14], is largely mediated by Th17 cells [15–18], which are a highly proinflammatory CD4⁺ T cell subset characterized by their production of IL-17A (henceforth termed IL-17), as well as IL-22, IL-21, colony stimulating factors (CSFs), CXCL1, CXCL2, and TNF- α [19–22]. Importantly, Th17 cells were also found to be key mediators of pathology in many autoimmune and infectious diseases [22–27]. Unlike CBA mice, infected C57BL/6 (BL/6) mice develop mild hepatic pathology with

considerably smaller, well-circumscribed liver granulomas and little parenchymal inflammation that arise in a Th2 cell-dominated environment characterized by the presence of IL-4, IL-5, and IL-13 [28]. In both the severe and mild forms of disease, the egg-induced granulomatous inflammation in schistosomiasis is a true example of T cell-mediated “adaptive immunopathology”, as it fails to materialize in the absence of CD4⁺ T cells expressing rearranged $\alpha\beta$ T cell receptors (TCR) [29].

Of note is the recent report that in human infection with *S. haematobium*, a related species that is the causative agent of urinary schistosomiasis, the development of bladder pathology has been similarly linked to an increase in peripheral Th17 cells together with a decrease in Foxp3-expressing regulatory T cells, a pattern akin to that observed in *S. mansoni*-infected CBA mice [30]. These findings imply that Th17 cell responses also occur in human pathology and thus validate the experimental murine model.

Distinct strain-dependent responses to schistosome egg antigens result in divergent CD4⁺ T cell differentiation programs and severity of immunopathology

Presently, the mechanisms underlying the striking strain-dependent differences in egg-induced immunopathology and the selection of opposite dominant CD4⁺ T cell subsets still remain incompletely understood. It is known that the activation of pathogenic Th17 cells in the high-pathology CBA strain is dependent on egg Ag-stimulated DCs that produce the Th17-stimulatory cytokines IL-1 β and IL-23, as well as IL-6 and TGF- β [16,17]. However, in BL/6 mice, egg stimulation of DCs does not result in IL-1 β or IL-23 secretion, and, consequently, Th17 cells fail to develop despite the fact that BL/6 T cells are wholly capable of differentiating into Th17 cells *in vivo* in a surrogate model of severe schistosomiasis that ensues upon concurrent immunization with soluble schistosome egg antigens (SEA) in complete Freund’s adjuvant (SEA/CFA) [15,31], or *in vitro*, when BL/6 T cells are stimulated exogenously with the Th17 cell-stimulatory cytokines IL-6, TGF- β and IL-23 [17]. These findings suggest that dissimilar Ag recognition and handling by DCs can result in disparate cytokine profiles and in the differentiation of T effector cell subsets of variable pathogenicity.

To address the aforementioned paradigm, we set out to elucidate the molecular means by which genetically diverse DCs could instruct distinct T cell differentiation programs following encounter with schistosome products. The initial findings from our studies were presented at the 8th conference on Molecular and Cellular Biology of Helminth Parasites, held on September 1–6, 2014, in Hydra, Greece. These findings constitute the essence of the present article; more details about this work with primary data are provided in a recent publication [32]. Additional information about the murine model of schistosomiasis and the biology of the parasite is available in previous review articles [1–5].

DCs from CBA and BL/6 mice exhibit different C-type lectin receptor expression; the lectin CD209a is significantly elevated in CBA DCs

To evaluate possible intrinsic differences between APCs that instruct divergent T cell differentiation programs in CBA vs. BL/6 mice following encounter with schistosome egg Ags, we subjected normal BMDCs from each strain to comprehensive baseline gene profiling using Affymetrix microarray technology [32,33]. Surprisingly, among the genes with immunological function that were significantly overexpressed in CBA vs. BL/6 DCs were pattern recognition receptors (PRRs), the majority of which belonging to the C-type lectin receptor (CLR) family. CLRs are a large family of calcium-dependent receptors that bind glycans on both pathogen and host cell surfaces, affording recognition of a wide range of glycosylation patterns [34,35]. Prominent among these are fucose-rich schistosome glycans, both O- and N-linked, including Lewis (Le^X), poly Le^X, pseudo Lewis Y (Le^Y), GalNAc β 1–4GlcNAc (LacdiNAc, LDN), and diversely fucosylated LDN (F-LDN, LDN-F, F-LDN-F) [6,7,36]. These glycans are expressed on the surface of eggs and are actively secreted in the form of soluble glycoproteins [8]. In humans, the CLRs DC-SIGN, DC-SIGNR, macrophage galactose-type lectin (MGL), and the mannose receptor (MR) have been previously demonstrated to bind to the egg glycans Le^X, LDN and LDN-F [37–43].

Closer gene profiling analysis revealed that among the elevated CLRs in CBA DCs there was a striking 18-fold increase in the expression of *Cd209a*, which was confirmed by quantitative RT-PCR (qRT-PCR) in BMDCs obtained from individual CBA and BL/6 mice. CD209a, also known as mouse DC-SIGN and SIGNR5, is one of eight murine homologues of human DC-SIGN, a well-characterized CLR capable of binding various mannose- and fucose-containing glycans including those present in schistosomes as well as other viral and bacterial pathogens [35,44–46]. To this date, a specific pathogen-expressed glycan ligand for the murine CD209a homologue has not been identified [45]. Nonetheless, peptide sequence analysis has shown that CD209a retains many structural properties of human DC-SIGN including one carbohydrate- and calcium-binding domain in the extracellular peptide sequence, one conserved 23-amino acid sequence in the neck region, and tri-acidic cluster and dileucine motifs in the cytoplasmic tail [44,45,47]. Such cytoplasmic tail motifs promote receptor internalization and may function in receptor-Ag uptake [47]. While minimal information on the function of CD209a was available prior to this work, a recent study by Lu et al. showed that leukocyte cell-derived chemotaxin-2 (LECT2) interacts with CD209a on murine macrophages to promote endocytosis, bacterial killing, and cytokine production [48].

Among the other CLR genes that were upregulated in CBA compared to BL/6 BMDCs were *CD209c* and *CD209d*, two other murine DC-SIGN homologues, as well as *Clec4a1*, *Clec4a2*, *Mmr*, and *Clec4a3* [32]. Notably, the only CLR gene that was up-regulated in BL/6 BMDCs was *Mgl1*, which encodes for Macrophage galactose N-acetylgalactosamine specific lectin 1 (MGL1), a CLR shown to have anti-inflammatory properties [32,49]. Ultimately, we chose to focus our study on *CD209a* because of its dramatic 18-fold increase in CBA DCs.

CD209a expression is increased in spleen and granuloma cell populations from infected CBA mice, but only egg-stimulated DCs elicit Th17 cell responses *in vitro*

As in the case of BMDCs, CD209a expression was also significantly elevated in the spleens of CBA vs. BL/6 mice, both normal as well as 7-week schistosome-infected. CD209a was detected on the surface of various splenic immune cell populations with APC capabilities, including DCs, B cells, granulocytes as well as macrophages. In all cases, CD209a expression was significantly higher in CBA cells compared to BL/6. A markedly higher expression of CD209a, both in bulk cell populations as well as individually on DCs, granulocytes and macrophages, was also observed in the egg-induced hepatic granulomatous lesions of the CBA mice. By confocal immunofluorescence microscopy, CD209a⁺ cells were abundantly represented within CD11c-rich interfollicular T cell areas in the spleen, while fewer CD209a⁺ cells were present within B220⁺ B cell-rich follicles. In liver sections, CD209a⁺ cells localized within the egg granulomas, where the frequency as well as fluorescence intensity were higher in granulomas from CBA mice. Considerable cell co-localization of CD209a and the classic DC marker CD11c was visible in these lesions [32].

Of particular significance was our observation that following exposure to live schistosome eggs, the CD11c⁺ splenic DCs from normal CBA mice induced naïve, anti-CD3/CD28 ab-costimulated T cells towards Th17 cell differentiation *in vitro*; neither macrophages, granulocytes nor B cells from CBA mice were capable of the same effect. In contrast, there was no evidence of Th17 cell differentiation in the corresponding DC-T cell-egg co-cultures from BL/6 mice [32].

CD209a expression on DCs is necessary for schistosome egg-induced IL-23 and IL-1 β secretion and subsequent Th17 cell subset differentiation

The finding that CD11c⁺ DCs are the most efficient APC in inducing Th17 cells prompted the question of whether CD209a itself plays a key role in this function. This issue was first approached by delivering a *Cd209a*-targeted short hairpin RNA (shCD209a) into CBA BMDCs via lentiviral infection, which resulted in efficient (84%) knockdown. In co-culture with CD4⁺ T cells, and upon stimulation with schistosome eggs, such shCD209a DCs produced significantly less IL-1 β and IL-23 in comparison with control DCs, and, in the presence of anti-CD3/CD28 costimulatory abs, elicited significantly lower IL-17 secretion by T cells as well as lower expression of the Th17 cell-associated transcription factor *Rorc*. Of note is that similarly inhibited IL-17 production was also found in co-cultures of egg-stimulated shCD209a DCs with syngeneic CD4⁺ T cells expressing a transgenic TCR specific for the major schistosome egg Ag Sm-p40, in which the addition of anti-CD3/CD28 abs was unnecessary and thus omitted [32,18].

To further confirm the role of CD209a in the development of Th17 responses, we took the reverse approach, which consisted of overexpressing CD209a in BL/6 BMDCs via a lentiviral vector. Egg-stimulated BL/6 CD209a-expressing BMDCs indeed produced significantly more IL-1 β and IL-23 than control DCs; moreover, in the presence of anti-

CD3/CD28 costimulatory abs, BL/6 CD209a-expressing DCs induced syngeneic naïve T cells to secrete IL-17. CD209a⁺ BMDCs also expressed significantly higher *Rorc* in comparison with controls. Taken together, these findings support the notion that CD209a expression critically enables egg-stimulated DCs to induce IL-1 β and IL-23 secretion leading to Th17 cell development [32].

Possible CD209a-dependent signaling pathways promoting DC proinflammatory cytokine production

To date, the signaling mechanisms involved in Th17 cell associated cytokine production downstream of murine DC-SIGN homologues, particularly in response to schistosome products, are largely unknown; however, various studies have documented MAP kinase activation downstream of DC-SIGN [50–53]. MAP kinases are evolutionarily conserved signaling molecules that mediate rapid communication of extracellular signals to the nucleus during diverse cellular processes [54,55]. Previous studies showed that during simultaneous TLR4 stimulation, DC-SIGN ligation by ManLam induces the recruitment of a signalosome of scaffolding and signaling proteins that results in MAP kinase activation, modification of NF κ B, and IL-12p40, IL-10, IL-12p35, and IL-6 production [51,52]. Furthermore, it was also demonstrated that ERK1/2 and PI3K, but not p38, are phosphorylated following ligation of human DC-SIGN, which has also been shown to co-precipitate with tyrosine kinases Lyn and Syk [50]. A comparable study documenting the interactions between DC-SIGN and syncytial virus glycoprotein G also demonstrated that ERK1/2 is stimulated after DC-SIGN ligation [53], and signaling through ERK1/2 was additionally shown to promote proinflammatory IL-23p19 and IL-1 β production leading to Th17 cell differentiation in a study of primary human fibroblasts [56]. MAP kinase activation has been reported after cross-linking of murine CD209b (SIGNR1) [57] and CD209d (SIGNR3) [58], both of which capable of binding to Le^X [45,59], yet no *in vivo* function has been attributed to these CLRs and their possible role in pathology has not been previously described in murine schistosomiasis. Of interest is that a number of studies involving helminth products have observed DC-SIGN or ERK to be associated with antiinflammatory Th2 and regulatory responses, largely to counteract the effect of Toll-like receptor agonists [60–66].

Based on these studies, we initially focused on assessing MAP kinase activation in CD209a-expressing DCs and found that following stimulation of CBA BMDCs with live eggs, there was a progressive and lasting increase in phospho-ERK1/2, which was not seen in BL/6 cells. An increase in the phosphorylation of the upstream MAP kinase RAF-1 as well as SRC kinase was also detected in the CBA BMDCs, but there was no significant activation of the MAP kinases p38 or JNK. Preliminary findings indicate that inhibition of ERK1/2 activation in egg-stimulated CD209a-expressing DCs renders these cells unable to secrete IL-1 β and IL-23, and to induce IL-17 production in CD4⁺ T cells (HEP and MJS, unpublished observation). These results suggest that egg stimulation of CD209a-expressing DCs is associated with SRC, RAF-1, and ERK1/2 MAP kinase activation, and that ERK is involved in the signaling cascade leading to proinflammatory cytokine production and Th17 cell development.

The above represent early findings of what will likely turn out to be a complex CD209a-activated signalosome leading to proinflammatory IL-23 and IL-1 β production. Of particular relevance, however, are observations on related CLRs. For example, dectin-1 and dectin-2 were shown to mediate IL-23 production in response to fungal antigens by signaling via Syk kinase and CARD9 [34,67,68], and schistosome egg components stimulated murine dectin-2 leading to inflammasome activation and IL-1 β secretion [69]. Examination of these and other pathways will be necessary for a more thorough understanding and control of CD209a-related inflammation.

The significance of the CD209a lectin in Th17 cell-mediated immunopathology

Of the many proinflammatory cytokine responses to bacterial, viral, fungal, and parasitic Ags operating downstream of PRRs on APCs [34,35,70–73], the CLRs are the best characterized in the context of responses to schistosomes [7,37,38–41]. Our findings described herein reveal a novel pathway whereby the CLR CD209a expressed on DCs precipitates a proinflammatory cytokine response resulting in the development of pathogenic Th17 cells that mediate severe immunopathology in murine schistosomiasis (**Figure 1, Graphical Abstract**). It is currently unknown whether DC-SIGN is in any way involved in mediating severe disease in humans; equally unclear is whether murine CD209a *per se* represents a cellular adhesion molecule that facilitates DC – T cell interactions [74] or is involved in immune synapse formation, in consideration of the fact that the endogenous ligand of human DC-SIGN is ICAM-3. Future assessment of the ICAM binding capacity of CD209a will clarify whether this or other murine DC-SIGN homologues also have the ability to promote cell adhesion and interaction.

The evolutionary history of DC-SIGN also provides insight into the function of this gene family. *CD209* family genes have been described in humans and non-human primates in addition to a range of other species in the animal kingdom including mice, rats, birds, horses, squirrels, cattle, bats, and dogs. Evolutionary studies indicate that several *CD209* genes originated from duplication events in early anthropoid ancestors around 40 million years ago [75]. The human *CD209* genes encode for DC-SIGN and the related receptor L-SIGN (DC-SIGNR, *Cd209l*), which both cluster on chromosome 19. In primates, DC-SIGN and L-SIGN are primarily expressed by phagocytic cells and play a crucial role in host defense responses. L-SIGN is most highly expressed in the lymph node and liver sinus endothelia, and thus termed “L”-SIGN. Non-human primates also possess a third *CD209* family member gene, *Cd209l2* (CD209L2), a *CD209* homologue that was likely deleted in humans due to selective pressure [58,75–77]. Although *CD209* gene function in most other species remains unclear, sequence variation provides insight into the evolutionary history of this family of CLR genes. It is especially intriguing that mice evolved to express as many as eight *CD209* homologues, all encoded by genes in chromosome 8, and each retaining different structural properties amongst themselves and with respect to human DC-SIGN [45,75]. Yet, based on our present findings, CD209a appears to play a unique role in the schistosome infection as none of the other homologues is able to compensate for its absence [32].

Our findings demonstrating a proinflammatory function of CD209a in the pathogenic Th17 cell response to schistosome eggs is seemingly at odds with the widespread belief that helminths induce Th2 and regulatory T cell subsets. This concept largely derives from studies performed in BL/6 mice - the strain studied by the majority of investigators in this field - but it is not universally applicable to all mouse strains. For example, SJL [13] and wild-derived MOLF mice [14] develop Th17 cell-mediated severe immunopathology which, in the latter case, is associated with increased DC CD209a expression [HEP and MJS, unpublished observation]. Moreover, in murine infection with *S. japonicum*, severe hepatic inflammation has similarly been associated with Th17 cells [78–80]. Most likely, the use of genetically diverse mouse strains will reveal additional examples of severe inflammation induced by schistosomes and other helminths. In regards to the murine CD209 family, low DC CD209a expression in BL/6 mice appears to be evolutionarily advantageous to avert severe pathology, whereas its persistence in other strains is harmful, even though ancestrally it could have played a useful role against pathogens. In any case, for situations in which expression of CD209a (or possibly human DC-SIGN) are proven or suspected to be deleterious, elucidation of downstream pathways may reveal targets amenable to therapeutic intervention to curtail severe disease.

Conclusions

Here we report that the unsuspected CLR CD209a, one of eight murine homologues of human DC-SIGN (CD209), plays a novel and significant role in stimulating pathogenic Th17 cell responses that mediate severe inflammation in murine schistosomiasis. Our conclusions are based on the results of a comprehensive gene profiling analysis that revealed major differences in PRR expression between CBA and BL/6 mice developing severe vs. mild forms of hepatic egg-induced immunopathology. Salient and most consequential was the strikingly high expression of CD209a in CBA DCs, which was critical for the induction of schistosome egg-stimulated proinflammatory IL-1 β and IL-23 secretion leading to the development of pathogenic Th17 cells. Our study makes a significant contribution towards understanding the paradigm of CLR-mediated innate immunity, as it is the first report implicating a murine CD209 homologue in Th17 cell-mediated disease. Considering the promiscuous carbohydrate-binding behavior of CD209 proteins [37,53,76,81,82], the investigation of a proinflammatory role for DC-SIGN and/or L-SIGN could be relevant to various human infectious and/or inflammatory conditions.

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Highlights

- CBA mice develop severe Th17 cell-mediated pathology in schistosomiasis.
- The Th17 cells are induced by parasite egg-stimulated dendritic cells (DCs).
- CBA DCs express high levels of the c-type lectin receptor CD209a.
- Th17 cell development depends on DC CD209a expression.
- DC CD209a expression represents a novel proinflammatory parasite sensing mechanism.