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CD209a Expression on Dendritic Cells is Critical for the Development of Pathogenic Th17 Cell Responses in Murine Schistosomiasis[1]

Holly E. Ponichtera[*], **Mara G. Shainheit**[†], **Beiyun C. Liu**[*], **Raktima Raychowdhury**[‡], **Bridget M. Larkin**[*], **Joanne M. Russo**[*], **D. Brenda Salantes**[*], **Chao-Qiang Lai**[§], **Laurence D. Parnell**[§], **Tae J. Yun**[¶], **Cheolho Cheong**[¶], **Stephen C. Bunnell**[*], **Nir Hacohen**[‡], and **Miguel J. Stadecker**[*]

[*]Department of Integrative Physiology and Pathobiology, Tufts University School of Medicine and Sackler School of Graduate Biomedical Sciences, Boston, MA 02111, USA

[†]Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111, USA

[‡]Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

[§]Nutritional Genomics Laboratory, JM-USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA 02111, USA

[¶]Institut de Recherches Cliniques de Montréal. Montréal, Québec, Canada H2W 1R7

Abstract

In murine schistosomiasis, immunopathology and cytokine production in response to parasite eggs is uneven and strain dependent. CBA mice develop severe hepatic granulomatous inflammation associated with prominent T helper 17 (Th17) cell responses driven by dendritic cell (DC)-derived IL-1β and IL-23. Such Th17 cells fail to develop in low-pathology BL/6 mice, and the reasons for these strain-specific differences in antigen (Ag) presenting cell (APC) reactivity to eggs remain unclear. We show by gene profiling that CBA DCs display an 18-fold higher expression of the C-type lectin receptor (CLR) CD209a, a murine homologue of human DC-specific ICAM-3-grabbing non-integrin (DC-SIGN), than BL/6 DCs. Higher CD209a expression was observed in CBA splenic and granuloma APC subpopulations, but only DCs induced Th17 cell differentiation in response to schistosome eggs. Gene silencing in CBA DCs, and over-expression in BL/6 DCs, demonstrated that CD209a is essential for egg-elicited IL-1β and IL-23 production and subsequent Th17 cell development, which is associated with SRC, RAF-1, and ERK1/2 activation. These findings reveal a novel mechanism controlling the development of Th17 cell-mediated severe immunopathology in helminthic disease.

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Correspondence: Miguel Stadecker, Tufts University School of Medicine and Sackler School of Graduate Biomedical Sciences, Boston, MA 02111. Phone: 617 636 6732, Fax: 617 636 2990, miguel.stadecker@tufts.edu.

[2]Abbreviations used in this paper: BMDC, bone marrow-derived dendritic cell; CLR, C-type lectin receptor; DC, dendritic cell; DC-SIGN, DC-specific ICAM-3-grabbing nonintegrin; GC, granuloma cell; PRR, pattern recognition receptor; Tg, transgenic; Th17, T helper 17.

Introduction

Schistosoma mansoni is a trematode helminth that causes extensive disease in the developing world, accounting for over 200 million infections and 200,000 deaths per year. The principal cause of morbidity and mortality in *S. mansoni* infection 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, splenomegaly, ascites, and portal hypertension [1-5].

Similar to human disease, heterogeneity of 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 [6-8]. The severe pathology is largely mediated by T cell IL-17 production induced by egg Ag-stimulated DC secretion of IL-1β and IL-23 [9-12]. In contrast, infected C57BL/6 (BL/6) mice develop mild pathology with significantly smaller liver granulomas in a Th2 polarized environment [13]. IL-17 is largely the product of Th17 cells, a highly proinflammatory subset of $CD4^+$ effector T cells that also produce IL-22, colony stimulating factors (CSFs), CXCL1, CXCL2, and TNF-α [14-17]. Presently, the mechanisms underlying the variation in egg-induced immunopathology and selection of dominant $CD4^+$ T cell phenotype are incompletely understood; however, it is noteworthy that a recent study of *Schistosoma haematobium* infection in humans similarly linked the development of pathology to an increase in Th17 cells [18].

We now demonstrate that genetic differences in pattern recognition receptor (PRR) expression predispose CBA and BL/6 DCs to develop divergent cytokine responses following stimulation with live schistosome eggs. PRRs are innate sensors utilized by APCs to recognize conserved pathogen-associated molecular patterns (PAMPs) [19,20]. C-type lectin receptors (CLRs) are a family of PRRs capable of binding carbohydrates [21,22] such as the glycans Lewis X (Le^X), GalNAcβ1–4GlcNAc (LacdiNAc (LDN)), and fucosylated LDN (LDN-F) typically expressed by schistosome eggs [23-26]. We found overall CLR expression to be higher in CBA than BL/6 cells, and in CBA DCs, there was a striking overexpression of the CLR CD209a, a murine homologue of human DC-specific ICAM-3-grabbing non-integrin (DC-SIGN, CD209). CD209a was shown to facilitate the induction of egg-induced Th17 cells responsible for causing severe immunopathology.

Materials and Methods

Mice, parasites, and infection

5- to 6-week old female CBA and BL/6 mice were obtained from The Jackson Laboratory. Swiss Webster mice were obtained from Charles River Laboratories. A CBA mouse expressing a Tg TCR specific for the Sm-p40 schistosome egg Ag was made in house as previously described [12]. All mice were maintained at the Tufts University School of Medicine Animal Facility in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. For some experiments,

CBA and BL/6 mice were infected with 85 *S. mansoni* cercariae (Puerto Rico strain) by intraperitoneal injection. Cercariae were shed from infected *Biomphalaria glabrata* snails provided to us by BEI Resources, Manassas, VA. All Swiss Webster mice were infected in an identical fashion for the purpose of isolating schistosome eggs. Eggs were isolated from livers of 7- to 8-week infected mice under sterile conditions by a series of blending and straining techniques, as described previously [11].

Cells

BMDCs—Bone marrow was flushed from femurs and tibias of normal CBA and BL/6 mice. Red blood cells (RBCs) were lysed with Tris ammonium chloride buffer and cells were cultured in complete-RPMI 1640 medium (Lonza) containing 10% FBS (Aleken Biologicals) and recombinant GM-CSF at 15ng/ml (Peprotech AF-315-03) or GM-CSF-containing supernatant from the J558L transfectant B cell hybridoma. The medium was changed on day 3 and 5 and cells harvested on day 7. $CD11c^+$ DC purity was >85% by flow cytometric analysis.

$CD4^+$ T cells—Single-cell suspensions were prepared from the spleens of normal CBA and BL/6 mice, RBCs were lysed, and $CD4^+$ T cells were purified by negative selection using $CD4^+$ T cell isolation Kit II for mouse (Miltenyi Biotec). $CD4^+$ T cell purity was >95% by flow cytometric analysis.

Gene expression profiling

CBA and BL/6 BMDCs prepared from individual mice were plated in replicate at 1×10^6 cells/ml in 48-well tissue culture plates (BD Falcon). Replicates were pooled after 4hr and total RNA was obtained by Trizol® (Invitrogen) extraction according to the manufacturer's instructions. Amplified and labeled cRNA was assessed with Affymetrix Mouse Gene 1.0 ST array (Affymetrix, Santa Clara, CA) following manufacturer's instructions. Affymetrix Expression Console 1.1 software was used to generate annotated NetAffx CSV files for analysis. Microarray data were deposited in the NCBI GEO database under accession number GSE55307 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55307). Data from a single chip is representative of gene expression of unstimulated DCs from an individual mouse and two chips were run per mouse strain.

Gene ontology analysis

Genes with ≥2 fold difference in expression between CBA and BL/6 DCs and known biological function based on Ensembl release 54 gene archive (http://may2009.archive.ensembl.org/index.html) were defined as differentially expressed. Differentially expressed genes characterized to have putative immunological functions were further selected for GO and pathway analysis using the public web server, g:Profiler with default settings (http://biit.cs.ut.ee/gprofiler/; Institute of Computer Science, University of Tartu, Estonia [27].

Co-cultures

Purified naïve $CD4^+$ T cells from normal spleen and syngeneic BMDCs were cultured (8T: 1DC) in complete-RPMI 1640 medium together with anti-CD3/CD28-coated beads (2×10^5 beads/ml, Dynabeads® Invitrogen) and freshly isolated live schistosome eggs for 96 hr.

Cytokine analysis

ELISA—Supernatants from 96 hr co-cultures were assessed for IL-1β, IL-23, and IL-17A using R&D Systems® ELISA kits.

qRT-PCR—RNA from co-cultures was obtained with Trizol® or TurboCapture 96 mRNA Kit (Qiagen) and cDNA synthesized with High Capacity Reverse Transcription Kit (Invitrogen) or Sensiscript RT kit (Qiagen). TaqMan® probes for *Il17a* (Applied Biosystems Mm00439618) and *Gapdh* (Applied Biosystems 4352339E) were used in combination with TaqMan® Gene Expression Master Mix (Applied Biosystems).

CD209a analysis

Flow cytometry and cell sorting—Spleen cells from individual normal and infected CBA and BL/6 spleens were isolated and RBCs were lysed. Cells were blocked with rat IgG and stained with fluorescently labeled antibodies specific for CD11c (BD Pharmingen 553802), CD19 (BD Pharmingen 553786), Gr-1 (BD Pharmingen 553127), or F4/80 (AbD Serotec MCA497APC) in combination with biotin-conjugated anti-CD209a (BD Pharmingen 558073). Subsequently, cells were stained with Alexa Fluor® 647-conjugated streptavidin (Invitrogen S-21374). Data were acquired with the FACS Calibur Flow Cytometer and CellQuest software version 3.2.1 (Becton Dickinson) and data were analyzed with Summit Software. Spleen and granuloma cells were gated for viability based on forward scatter and side scatter parameters as well as propidium iodide exclusion. Normal CBA splenocytes were sorted at the Tufts Flow Cytometry Core Facility using the Abs listed above.

qRT-PCR—Normal and 7-week infected CBA and BL/6 spleen or liver tissue was homogenized in Trizol® and cDNA was synthesized as described above. A TaqMan probe for *Cd209a* (Applied Biosystems Mm00460067) was used in combination with TaqMan® Gene Expression Master Mix.

Immunohistochemistry—10μm OCT-embedded liver and spleen cryostat sections were fixed in acetone. Liver sections were stained with anti-CD209a Ab (BMD10) for 18 hr at 4°C, followed by mouse-anti-rat IgG2a-HRP for 30 minutes and Tyramide-signal amplification (Invitrogen) or with anti-CD209a plus anti-CD11c (N418) followed by streptavidin-HRP and Tyramide-signal amplification. Spleen sections were additionally stained with anti-B220-Alexa 647. Fluorescent staining was observed using a LSM710 confocal microscope (Zeiss).

RNAi

Knockdown—CBA BMDCs derived with rGM-CSF were infected with lentivirus containing CD209a- or GFP-targeted shRNA (RNAi Platform of the Broad Institute,

Cambridge, MA). Puromycin was used to select for infected cells and BMDCs were harvested for co-culture experiments on day 10.

Over-expression—The open reading frame (ORF) sequence for CD209a [28,50] was inserted into a lentiviral plasmid and packaged into viral particles in HEK293T cells with X-tremeGENE 9 DNA Transfection Reagent (Roche). Concentrated virus was used to infect BMDCs as described above.

Signaling protein analysis

Intracellular Staining—BMDCs were washed, fixed with 3% PFA, blocked with rat IgG, and stained with a fluorescently labeled antibody (Ab) specific for CD11c. Subsequently, cells were washed, permeabilized with methanol, and stained with a primary Ab specific for phospho-ERK (Cell Signaling 4695) followed by a fluorescently labeled secondary anti-rabbit IgG Ab (Invitrogen A11034). Data were acquired with the FACS Calibur Flow Cytometer and CellQuest software version 3.2.1 (Becton Dickinson) and data were analyzed with Summit Software.

Western Blot—BMDCs were washed, lysed, and prepared with Laemmli's SDS-sample buffer (Boston BioProducts). Samples were run on an SDS-PAGE gel and transferred to an Immobilon-P PVDF (Millipore) membrane, which was then blocked in 5% BSA. The activation of MAP-kinases was detected with Abs specific for phospho-SRC Tyr416 (Cell Signaling 2101), phospho-RAF-1 Ser 338 (Cell Signaling 9427), phospho-ERK1/2 Thr202/Tyr204 (Cell Signaling 4695P), phospho-JNK Thr183/Tyr185 (Cell Signaling 9251S), and phospho-p38 Thr180/Tyr182 (Cell Signaling 9215S). Total kinase expression was detected with Abs specific for SRC (Cell signaling 32G6), RAF-1 (Cell Signaling 9422P), ERK1/2 (Cell Signaling 4695), JNK (Cell Signaling 9252), p38 (Cell Signaling 9212), and GAPDH (Cell Signaling 2118S).

Statistical analysis

ANOVA and student's t-tests were used to statistically analyze differences between groups. p-values of < 0.05 were considered significant, (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

Gene profiling reveals elevated C-type lectin receptor expression by CBA vs. BL/6 DCs

CBA and BL/6 DCs were previously shown to induce divergent schistosome egg-specific cytokine profiles [9-12]. To identify genetic differences intrinsic to CBA and BL/6 DCs that may influence the cytokine response to schistosome Ags, we used Affymetrix microarray technology to assess baseline expression of >22,000 genes in CBA and BL/6 bone marrow-derived DCs (BMDCs) (Fig. 1A). Genes of known biological function with a 2-fold or greater difference in expression between CBA vs. BL/6 BMDCs were functionally categorized by gene ontology (GO) analysis using the public web server g:Profiler [27] (Fig. 1B and Table SI). Out of 180 biologically characterized genes that were elevated at least 2-fold in CBA vs. BL/6 BMDCs, 35 had known immunological functions (Table SII). GO analysis revealed elevated expression of genes with predicted roles in cell membrane-

intrinsic immune defense response to external stimuli, carbohydrate binding, and molecular transducer activity (Fig. 1B, Table SI, Table S2). Out of 157 genes that were elevated at least 2-fold in BL/6 vs. CBA BMDCs, 17 had known immunological function (Table SIII). GO analysis revealed that BL/6 BMDCs exhibited a markedly different expression profile punctuated by genes involved in the regulation of immune effector processes and various metabolic processes (Fig. 1B, Table SI).

Among all the genes assessed by microarray technology, the most apparent difference between CBA and BL/6 BMDCs was in PRR expression. In CBA DCs, the majority of over-expressed PRR genes belonged to the CLR family, among which there was a striking 18-fold increase in expression of the CLR *Cd209a* (Fig. 1C). CD209a, also known as mouse DC-SIGN and SIGNR5, is one of eight murine homologues of human DC-SIGN, a CLR known to bind to schistosome egg glycans [25,28,29]. Elevated expression of *CD209a* y CBA DCs was confirmed in BMDCs from individual CBA and BL/6 mice by quantitative RT-PCR (qRT-PCR) (Fig. 1D). These results denote a significant difference in baseline CLR gene expression between CBA and BL/6 BMDCs.

CD209a expression is elevated in splenocyte subpopulations from normal and schistosome-infected CBA mice

Cd209a expression is significantly higher in the spleens of both normal and infected CBA vs. BL/6 mice (Fig. 2A). The overall relative expression of *Cd209a* decreases in infected spleen due to the development of pronounced splenomegaly after 7 weeks of infection, primarily caused by clonal T and B cell expansion; however, elevation of *Cd209a* in CBA compared to BL/6 spleen remains intact (Fig. 2A,B).

Several studies have demonstrated that human DC-SIGN is primarily expressed on DCs [22,30-32], but little is known about CD209a expression by APC subpopulations in mice. CD11c, CD19, Gr-1, and F4/80 positive splenocytes from normal and infected CBA and BL/6 mice, largely representative of DCs, B cells, granulocytes, and macrophages, respectively, were assessed for CD209a expression by flow cytometric analysis. The percentage of CD209a-expressing $CD11c^+$ cells within normal and infected splenocytes ($CD209a^+CD11c^+$/bulk spleen and subpopulation-specific-gated $CD209a^+CD11c^+$/total $CD11c^+$) was higher in CBA mice. Moreover, CD209a-expressing $CD11c^+$ cells increased after 7 weeks of infection in CBA spleen, but remained unchanged in BL/6 (Fig. 2C,G). Similarly elevated CD209a expression was seen on $CD19^+$, $Gr\text{-}1^+$, and $F4/80^+$ cells in infected CBA vs. BL/6 spleen (Fig. 2D,E,F). Subpopulation-specific gating revealed that a higher percentage of $CD19^+$ cells ($CD209a^+CD19^+$/total $CD19^+$) and $Gr\text{-}1^+$ cells ($CD209a^+Gr\text{-}1^+$/total $Gr\text{-}1^+$) express CD209a in normal and infected CBA vs. BL/6 spleen (Fig. 2H,I). There was no difference in the percentage of CD209a-expressing $F4/80^+$ cells by subpopulation-specific gating (Fig. 2J). These data indicate that CD209a is expressed by various APC subpopulations and elevated CD209a expression is apparent in normal and schistosome-infected CBA vs. BL/6 APCs.

CD209a expression is higher in granuloma cells from infected CBA vs. BL/6 mice

As the liver is the principal site of egg-induced granulomatous pathology in *S. mansoni* infection, we investigated *Cd209a* expression in livers from normal and infected CBA and BL/6 mice. Although there was no difference in *CD209a* expression in normal livers from these strains when assessed by qRT-PCR, after 7 weeks of infection we detected a significant increase in *CD209a* expression in CBA liver (Fig. 3A). Additionally, elevated CD209a expression was confirmed in bulk CBA liver granuloma cells (GCs) by flow cytometric analysis (Fig. 3B).

Within GCs, the percentage of CD209a-expressing $CD11c^+$ cells ($CD209a^+CD11c^+$/bulk GCs and subpopulation-specific-gated $CD209a^+CD11c^+$/total $CD11c^+$) was elevated in infected CBA vs. BL/6 mice (Fig. 3C,F). Similarly, CD209a expression was also higher in $Gr\text{-}1^+$ and $F4/80^+$ bulk CBA GCs (Fig. 3D,E). Subpopulation-specific gating revealed an increase in the percentage of CD209a-expressing granuloma $Gr\text{-}1^+$ cells ($CD209a^+Gr\text{-}1^+$/ total $Gr\text{-}1^+$), and $F4/80^+$ cells ($CD209a^+F4/80^+$/total $F4/80^+$) within CBA GCs, but not BL/6 GCs (Fig. 3F,G,H). Thus, CD209a expression by CBA GCs, including populations largely representative of DCs, granulocytes, and macrophages, is elevated in comparison to BL/6 GCs.

$CD209a^+$ cells are abundant in infected CBA spleen and liver sections

To assess $CD209a^+$ cell localization and frequency in CBA and BL/6 tissues, infected spleen and liver cryostat sections were stained for CD209a and visualized by confocal microscopy (Fig. 4). In infected CBA spleen, $CD209a^+$ cells were abundant in CD11c-rich interfollicular T cell areas compared to BL/6 spleen (Fig. 4A-D); fewer $CD209a^+$ cells were also present within $B220^+$ B cell-rich follicles in CBA, but not BL/6 spleen (Fig. 4A-B). In the liver sections, marked infiltration of $CD209a^+$ cells was apparent mainly in the egg-induced granulomas (Fig. E,F). In CBA livers, the granulomas were larger and densely infiltrated with $CD209a^+$ cells (Fig. 4E), whereas in BL/6 livers the smaller granulomas contained fewer $CD209a^+$ cells (Fig. 4F); the differences in fluorescence intensity were documented quantitatively as shown in Fig. 4G,H. Considerable cell co-localization of CD209a and CD11c was also evident in CBA granulomas (Fig. 4I). Taken together, these images demonstrate a higher frequency of $CD209a^+$ cells in lymphoid and lesional tissue of CBA mice.

Egg-simulated $CD11c^+$ cells, but not $CD19^+$, $Gr\text{-}1^+$, or $F4/80^+$ cells, elicit Th17 responses in vitro

To determine which CD209a-expressing APC subpopulation induces schistosome-specific Th17 cell responses, splenocytes from normal CBA mice were sorted and co-cultured with naïve $CD4^+$ T cells together with anti-CD3/CD28-coated beads in the presence or absence of eggs. CBA $CD11c^+$ cells induced robust IL-17 production by T cells in response to eggs (Fig. 5A); in contrast, minimal amounts of egg-specific IL-17 was produced by co-cultures containing $CD19^+$, $Gr\text{-}1^+$, or $F4/80^+$ cells (Fig. 5B,C,D). These results indicate that $CD11c^+$ cells, but not $CD19^+$, $Gr\text{-}1^+$, or $F4/80^+$ cells, are the APC subpopulation that most efficiently induces egg-specific IL-17 secretion by $CD4^+$ T cells.

CD209a expression on DCs is necessary for schistosome egg-induced Th17 cell responses

Given the association of CD11c expression with DCs, we examined the role of CD209a on DCs in eliciting Th17 cell responses to eggs. *Cd209a*-targeted short hairpin RNA (shRNA) was delivered into CBA BMDCs via lentiviral infection, resulting in an 86% knockdown as assessed by flow cytometric analysis and qRT-PCR (Fig. 6A,B,C). BMDCs in which CD209a expression was knocked down (shCD209a), or control BMDCs (normal, shCTRL), were subsequently co-cultured with naïve $CD4^+$ T cells and anti-CD3/CD28-coated beads in the presence or absence of live eggs and Th17 cytokine production was assessed by ELISA and qRT-PCR. In response to eggs, co-cultures containing shCD209a DCs produced significantly less IL-1β and IL-23 compared to normal or shCTRL DCs (Fig. 6D,E). Accordingly, there was a substantial decrease in egg-stimulated T cell IL-17 secretion and mRNA expression in co-cultures containing shCD209a DCs compared to co-cultures containing control DCs (Fig. 6F,G). Significantly, shCD209a DCs also elicited lower IL-17 production in egg-stimulated TCR transgenic (Tg) $CD4^+$ T cells specific for the immunodominant peptide of the Sm-p40 major schistosome egg Ag (Fig. 6H) [12].

Further assessment of transcription factors required for lineage commitment of $CD4^+$ T cells revealed that egg-stimulated co-cultures comprised of T cells and shCD209a DCs exhibited significantly lower expression of the Th17-associated transcription factor *Rorc*. In contrast, the Th1- and Th2-associated transcription factors, *Tbx21* and *Gata3*, were unchanged regardless of the DC population present in the cultures (Fig. 6I,J,K). Collectively, these data indicate that CD209a expression on CBA DCs is necessary for egg-stimulated IL-23 and IL-1β production, which induces Th17 cell differentiation.

CD209a over-expression confers on BL/6 DCs the capacity to induce Th17 cell responses

CD209a expression is significantly lower in BL/6 than in CBA DCs (Fig. 1,2,3), and BL/6 DCs fail to induce Th17 cell responses to eggs *in vitro* [9-12]. We thus investigated whether CD209a might confer on BL/6 DCs the capacity to induce egg-specific Th17 cell responses. A lentiviral vector was utilized to over-express CD209a in BL/6 BMDCs, which was successfully accomplished as determined by flow cytometric analysis and qRT-PCR (Fig. 7A,B,C).

BL/6 CD209a-expressing BMDCs were co-cultured with naïve BL/6 $CD4^+$ T cells and anti-CD3/CD28-coated beads in the presence or absence of live eggs and Th17-associated cytokine production was assessed by ELISA and qRT-PCR. Egg-stimulated CD209a-expressing BMDCs produced higher levels of IL-1β and IL-23 in comparison to control BMDCs (Fig. 7D,E); IL-17 secretion and mRNA expression was also enhanced in CD209a BMDC co-cultures compared to co-cultures containing control BMDCs (Fig. 7F,G). Additionally, *Rorc* expression was elevated in CD209a BMDC co-cultures compared to control BMDCs (Fig. 7H), but there were no significant changes in *Tbx21* or *Gata3* expression (Fig. 7I,J). Overall, these results confirm that CD209a expression enables egg-stimulated DCs to induce IL-1β and IL-23 secretion leading to Th17 cell development.

CD209a expression is associated with schistosome egg-stimulated MAP-kinase activation in DCs

While signaling pathways associated with CD209a are unknown to this date, recent studies have demonstrated that the mitogen-activated protein (MAP) kinases RAF-1 and extracellular signal-related kinases 1 and 2 (ERK1/2), are activated in DCs following ligation of human DC-SIGN [33-36]. We therefore investigated MAP kinase activation in CBA and BL/6 BMDCs. Following stimulation with live eggs, there was a steady increase in phospho-ERK1/2 in CBA BMDCs as determined by intracellular staining, which was not apparent in BL/6 BMDCs (Fig. 8A,B). This striking increase in phospho-ERK1/2 was still clearly evident at 24 hours post egg stimulation in CBA BMDCs by Western blot, at which time little or no phospho-ERK was detectable in BL/6 BMDCs (Fig. 8E). An increase in the phosphorylation of the upstream MAP kinase RAF-1 as well as SRC kinase was also detected in CBA compared to BL/6 BMDCs (Fig. 8C,D). By contrast, there was no significant activation of the MAP kinases p38 or JNK (Fig. 8F,G). These results are consistent with the notion that egg-stimulated CD209a expression on CBA DCs is associated with SRC, RAF-1, and ERK1/2 MAP kinase activation.

Discussion

DCs are potent APCs that sense foreign Ag through PRRs and induce the differentiation of naïve $CD4^+$ T cells into various effector T cell populations. Diverse cellular, environmental, and genetic factors may influence DCs to bias Th cell differentiation towards Th1, Th2, Th17, or T regulatory (Treg) cell lineages [4,20,22,30,37]. In murine *S. mansoni* infection, the development of a Th17 cell response is detrimental to the host, rather than protective, and leads to the development of severe pathology [5,9-12].

In the present study, comprehensive gene profiling revealed major differences in expression of PRRs between CBA and BL/6 mice. We found a strikingly higher expression of the CLR CD209a on CBA than BL/6 DCs. CD209a expression on DCs, but not on B cells, granulocytes, or macrophages, was required for the induction of pathogenic $CD4^+$ Th17 cell responses to live eggs. Absence of CD209a expression, either naturally on normal BL/6 DCs or silenced in CBA DCs, resulted in a marked reduction in IL-1β and IL-23 following egg stimulation and consequent Th17 cell development. Conversely, over-expression of CD209a in BL/6 DCs enabled egg-stimulated IL-1β and IL-23 production and subsequent Th17 cell development. Finally, CD209a expression on egg-stimulated CBA DCs was associated with MAP kinase activation.

Pro-inflammatory cytokine responses to various bacterial, viral, fungal, and parasitic Ag have been characterized downstream of PRR-Ag recognition by APCs [19-22,38,39]. Of the various families of PRRs, CLRs are the best characterized in the context of schistosome Ag recognition [25,26,40,41-43]. CLRs are a large family of calcium-dependent receptors that bind glycans on both pathogen and host cell surfaces, facilitating recognition of a wide range of glycosylation patterns [21,22]. Schistosome-specific glycans, both O- and N- linked, are rich in fucose and include Le^X, polyLe^X, pseudo Lewis Y (Le^Y), LDN, LDN-F, CAA, F-LDN, F-LDN-F, and HexNAc-DF [23,24,26]. Such glycans are heavily expressed on the surface of *S. mansoni* eggs and are actively secreted in the form of soluble glycoproteins

[44]. Human CLRs such as DC-SIGN, DC-SIGNR, macrophage galactose-type lectin (MGL), and the mannose receptor (MR) bind to schistosome egg glycans such as Le^X, LDN, LDN-F [25,40-43,45,46]. Additionally, Ritter et al. demonstrated that components of schistosome soluble egg Ag (SEA) stimulate murine Dectin-2 leading to inflammasome activation and IL-1β production [47]. While two murine homologues of DC-SIGN, CD209b and CD209d, were shown to bind the schistosome-expressed glycan Le^X [29,48], no *in vivo* function has to this date been attributed to these CLRs and their possible role in pathology has not been previously described in murine schistosomiasis. Our novel findings indeed suggest that CD209a plays a critical role in precipitating Th17 cell mediated inflammation following stimulation with egg glycans.

CD209a is one of eight murine homologues of human DC-SIGN, a CLR capable of binding various mannose- and fucose-containing bacterial and viral glycans as well as facilitating endocytosis and cytokine production [22,28,29,49]. To this date, the specific glycan ligand for CD209a has not been identified [29], but some studies do suggest that it may play a role in Ag internalization and presentation [43,50]. 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 [28,29,51]. Such cytoplasmic tail motifs promote receptor internalization and may function in receptor-Ag uptake [51]. As such, DC-SIGN expressed on human DCs was shown to internalize and target SEA to lysosomal compartments containing MHC class II molecules [43]. Along these same lines, $CD209a^+$ monocyte-derived DCs (Mo-DCs) were shown to possess powerful Ag-presenting capability [50]. A more recent study on bacterial sepsis was the first to reveal that leukocyte cell-derived chemotaxin-2 interacts with CD209a on murine macrophages to promote endocytosis, bacterial killing, and cytokine production [52].

Signaling events proximal to ligand binding by CD209a have not yet been characterized; however, various human studies have documented MAP kinase activation downstream of DC-SIGN [33-36]. MAP kinases are evolutionarily conserved signaling molecules essential for mediating rapid communication of extracellular signals to the nucleus during diverse cellular processes [53,54]. Gringhuis et al. showed that in the presence of TLR4 stimulation, DC-SIGN ligation by ManLam stimulates the recruitment of a signalosome of scaffolding and signaling proteins that leads to MAP kinase activation, modification of NFκB, and IL-12p40, IL-10, IL-12p35, and IL-6 production [34,35]. Caparrós et al. revealed that ERK1/2 and PI3K, but not p38, are activated proceeding engagement of DC-SIGN, which also co-precipitates with tyrosine kinases Lyn and Syk in human DCs [33]. A similar report documenting interactions between DC-SIGN and syncytial virus glycoprotein G also demonstrated ERK/1/2 stimulation post DC-SIGN ligation [36]. While MAP kinase activation has been reported proceeding cross-linking of CD209b (SIGNR1) [55] as well as CD209d (SIGNR3) [56], the signaling mechanisms critical for Th17 cell associated cytokine production downstream of murine DC-SIGN homologues, particularly in response to schistosome products, are largely unknown.

Our findings demonstrate that CD209a expression on DCs is essential for the induction of egg-stimulated Th17 responses associated with DC ERK1/2 activation. Although little is known of the relationship between ERK1/2 activation and Th17 cell responses, signaling through ERK1/2 has been previously shown to promote IL-23p19 and IL-1β production leading to Th17 cell differentiation in a study of primary human fibroblasts [57]. More relevant to immunity in schistosome infection, ERK1/2 activation, in the absence of significant JNK or p38 activation, has also been reported in response to lacto-*N*-fucopentaose III (LNFPIII) and ES62 from *Acanthocheilonema viteae*; however, these studies focus on the anti-inflammatory properties of helminth-related molecules and the induction of Th2 cell responses [58-62]. Indeed, numerous studies have established the immunomodulatory Th2-promoting properties of helminth products that may signal through receptors such as human DC-SIGN and the MR [61-65]. Prior to this study, the only CLRs known to facilitate Th17 cell responses were Dectin-1 and Dectin-2 which signal via Syk kinase and CARD9 in response to fungal Ags resulting in IL-23 production [21,67,68]. Our present finding that a novel CLR, CD209a, mediates pro-inflammatory Th17 cell responses indicates that the eight murine homologues of DC-SIGN, each of which retains slightly different structural properties of human DC-SIGN, may have evolved to mediate divergent functions.

As reflected in the nomenclature of DC-SIGN, its natural ligand is intercellular adhesion molecule-3 (ICAM-3), one of several transmembrane glycoproteins and intercellular adhesion molecules that facilitate DC – T cell interactions [31]. However, it is currently unknown if murine DC-SIGN homologues also bind adhesion molecules to promote cell-cell interaction. Immune synapse formation between DCs and T cells is required for efficient Ag presentation and DC – T cell intercellular feedback that leads to cytokine production. Future assessment of the ICAM binding capacity of CD209a will clarify whether murine DC-SIGN homologues also have the ability to promote cell adhesion and interaction.

In sum, severe hepatic granulomatous pathology in murine schistosomiasis, as seen in infected CBA mice, is dependent on $CD4^+$ Th17 cell responses. In this report we show that pathogenic Th17 cell cytokine responses to live *S. mansoni* eggs are largely dependent on the expression of CD209a by DCs. Future work investigating the outcome of pathology in *S. mansoni*-infected CD209a deficient CBA mice will clarify the function of CD209a in murine schistosomiasis *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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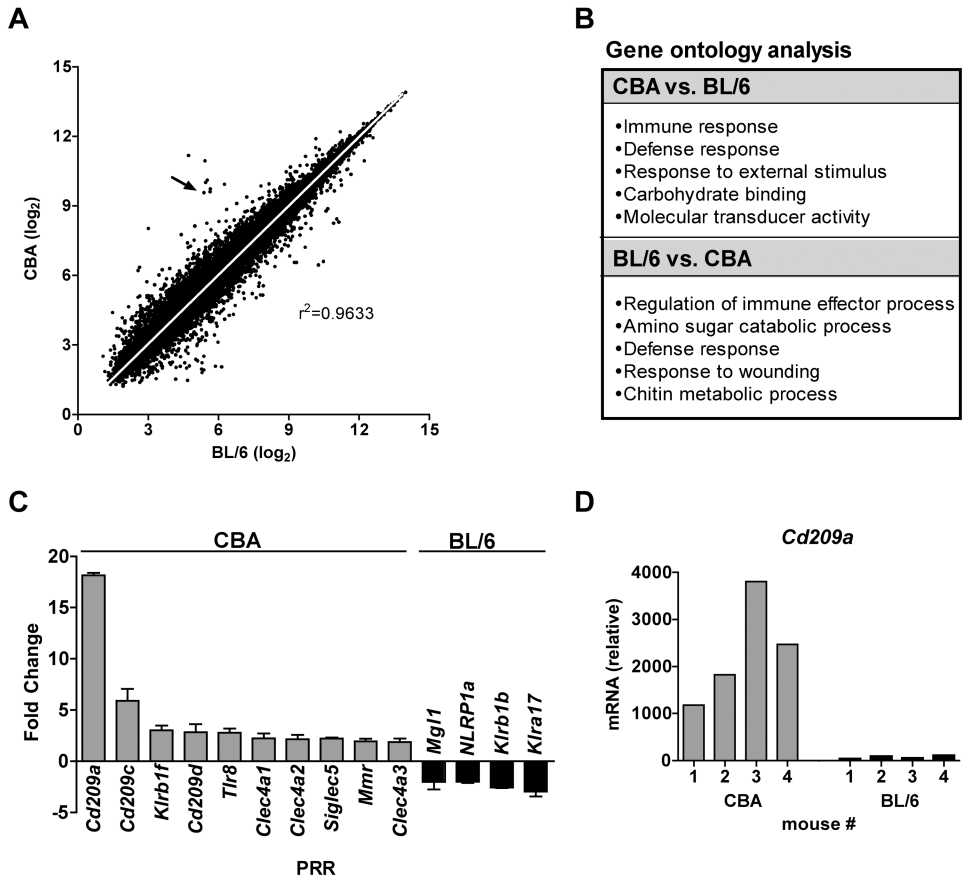

Figure 1. C-type lectin receptor expression is elevated in CBA vs. BL/6 DCs

(A) BL/6 and CBA BMDCs were differentiated in medium containing GM-CSF over 7 days. RNA was purified and gene expression was assessed by Affymetrix Mouse 1.0 ST Gene Array technology. Scatter plot shows log2-transformed gene expression. Arrow points to *CD209a*. (B) Genes with two fold or greater difference in expression between mouse strains with characterized biological function were selected for GO analysis. A functional profile for differentially expressed genes was obtained using the web-server g:Profiler. (C) PRRs with two fold or greater difference in expression by CBA vs. BL/6 DCs are shown. Bars represent means ± S.D. of two independent microarray chips for each strain. (D) *CD209a* expression by BMDCs from four individual CBA and BL/6 mice was determined by qRT-PCR (mRNA relative to GAPDH).

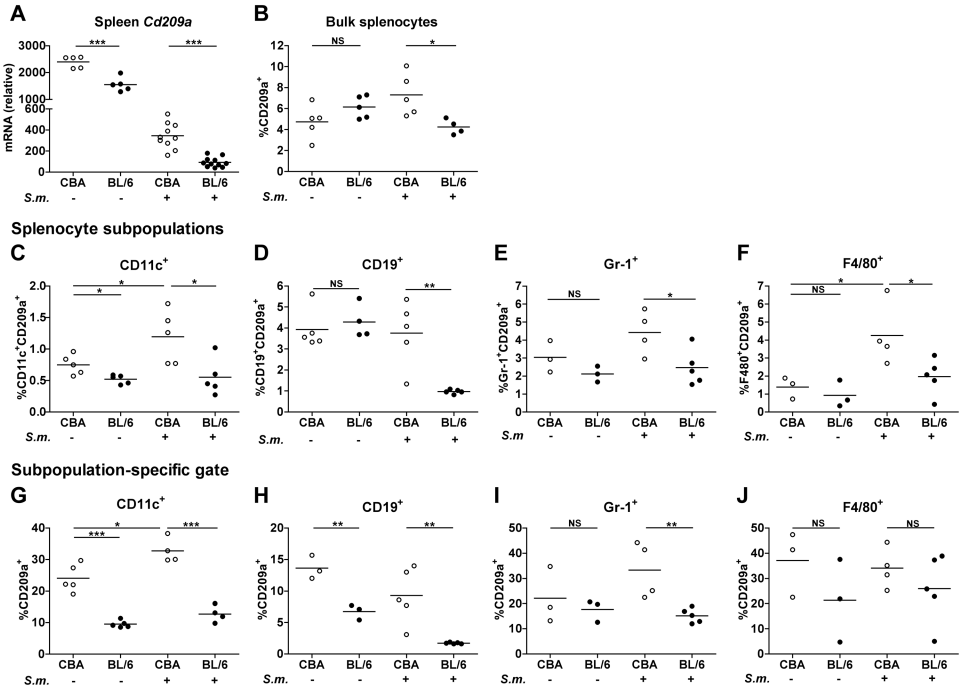

Figure 2. CD209a expression is elevated in splenocyte subpopulations from normal and infected CBA mice

(A) CBA and BL/6 mice were infected with *S. mansoni* for 7 weeks. RNA was purified from whole spleen tissue of individual mice and *CD209a* expression was assessed by qRT-PCR (mRNA relative to GAPDH). Data are from one representative experiment of two. (B-J) Splenocytes were isolated from normal and 7-week infected CBA and BL/6 mice. Surface expression of CD209a was assessed by flow cytometric analysis. Splenocytes were additionally stained for cell subpopulation markers CD11c, CD19, Gr-1, and F4/80 (C-F). The percentage of each individual splenocyte subpopulation that expresses CD209a was assessed by separately gating for $CD11c^+$, $CD19^+$, $Gr\text{-}1^+$, or $F4/80^+$ cells (G-J). Data are from one representative experiment of three or four. *p<0.05, **p<0.01, ***p<0.001, NS = not significant.

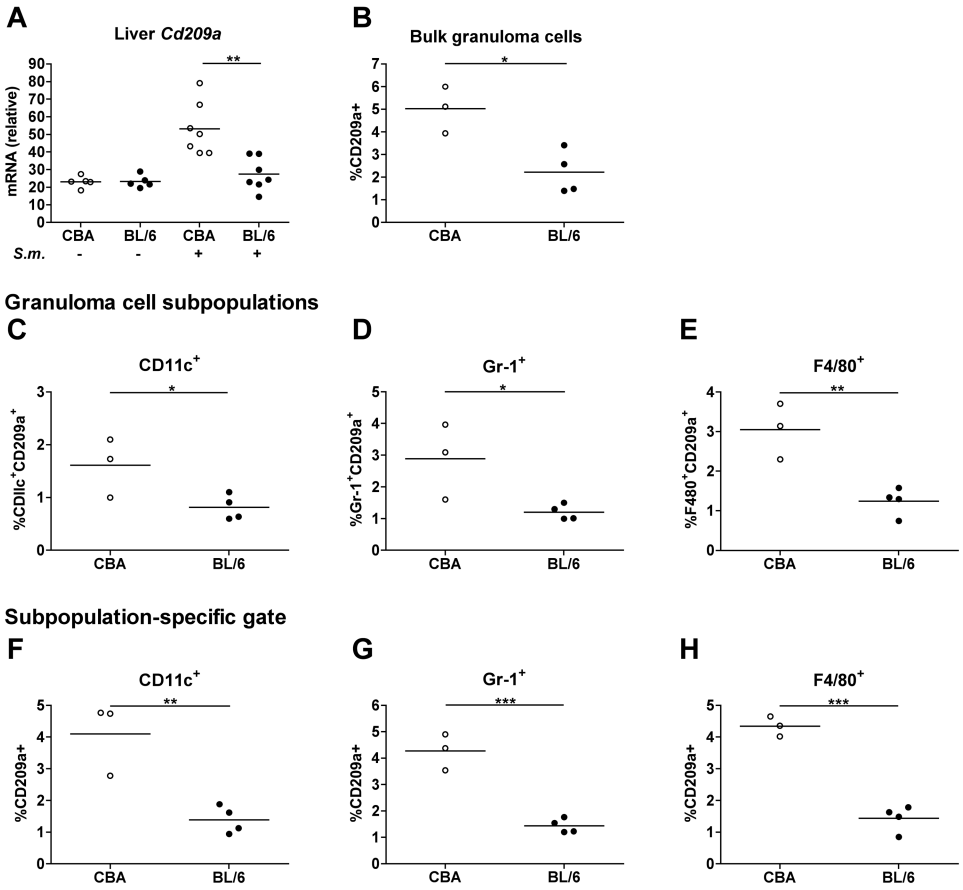

Figure 3. CD209a expression is higher in granuloma cells from infected CBA vs. BL/6 mice

(A) CBA and BL/6 mice were infected with *S. mansoni* for 7 weeks. RNA was purified from whole liver tissue of individual mice and *CD209a* expression was assessed by qRT-PCR (mRNA relative to GAPDH). Data are from one representative experiment of two. (B-H) Liver granuloma cells were isolated from livers by collagenase digestion followed by Lympholyte® cell separation. Surface expression of CD209a was assessed by flow cytometric analysis. Granuloma cells were additionally stained for cell subpopulation markers CD11c, Gr-1, and F4/80 (C-E). The percentage of each individual granuloma subpopulation that expresses CD209a was assessed by separately gating for $CD11c^+$, $Gr\text{-}1^+$, or $F4/80^+$ cells (F-H). Data are from one representative experiment of three. *p<0.05, **p<0.01, ***p<0.001.

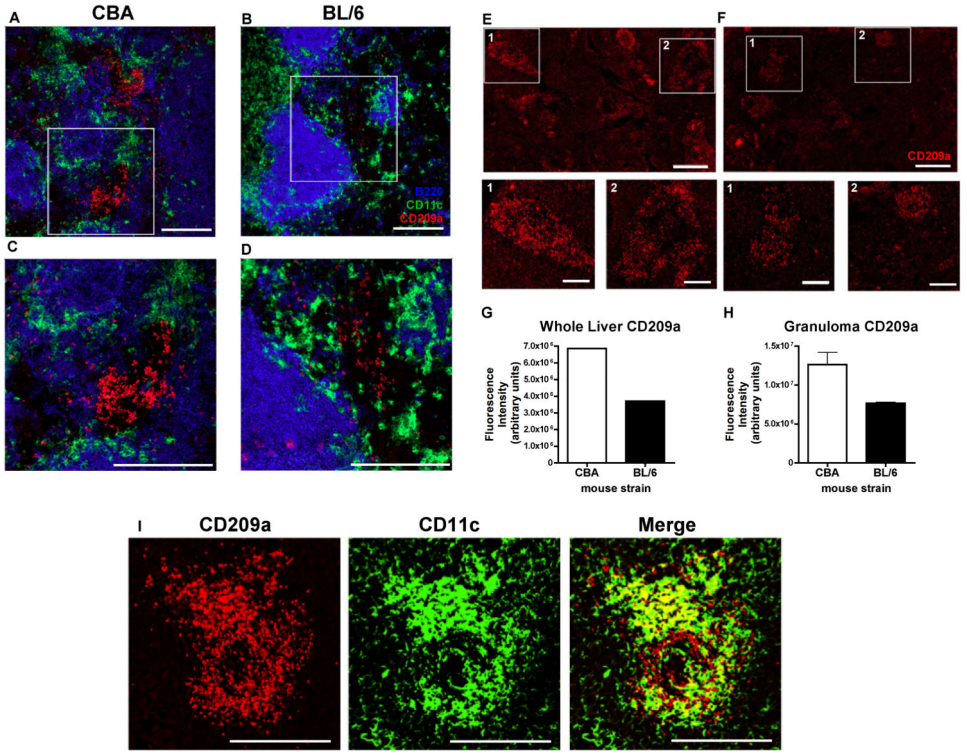

Figure 4. CD209a$^+$ cells are abundant in infected CBA spleen and liver sections

CBA and BL/6 mice were infected for 7 weeks. OCT-embedded frozen spleen (A-D) and liver (E-F) cryostat sections were stained for CD209a (red) and imaged by confocal microscopy. CBA (A,C) and BL/6 (B,D) spleen sections were counterstained for CD11c (green) and B220 (blue). Panels A-D demonstrate CD209a localization in spleens from CBA and BL/6 mice. Scale bars represent 200 μm for both 100× and 200× magnifications. For CD209a imaging in infected CBA (E) and BL/6 (F) livers, scale bars represent 500 μm for panoramic view and 200 μm for 100× magnifications. (G-H) Average CD209a fluorescence intensity in whole liver (E,F) and granulomas (E1,2, F1,2) was quantified by Volocity 6.0 Software (PerkinElmer). (I) A CBA liver section was stained for CD209a and CD11c to demonstrate co-localization in a granuloma after merge. Scale bars represent 200μm. Images are representative of 5 mice examined per strain.

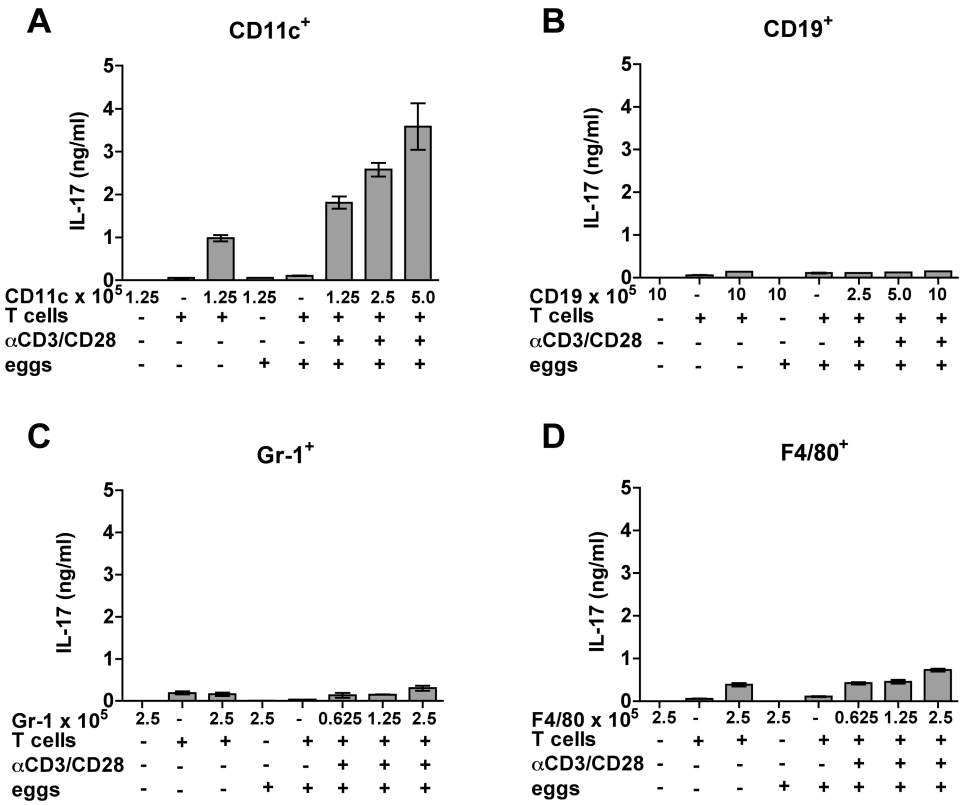

Figure 5. CD11c$^+$, but not CD19$^+$, Gr-1$^+$, or F4/80$^+$ cells, elicit Th17 cell responses

Splenocytes from normal CBA mice were stained for CD11c, CD19, Gr-1 or F4/80 subpopulation markers and separated by fluorescence-activated cell sorting (FACS). The indicated concentrations of (A) CD11c$^+$, (B) CD19$^+$, (C) Gr-1$^+$, and (D) F4/80$^+$ cells were individually co-cultured with 1 × 10^6 CD4$^+$ T cells and anti-CD3/CD28-coated beads ± schistosome eggs. IL-17 production in 96 hr culture supernatants was assessed by ELISA. Additional single-cell controls are shown. Data are from one representative experiment of two.

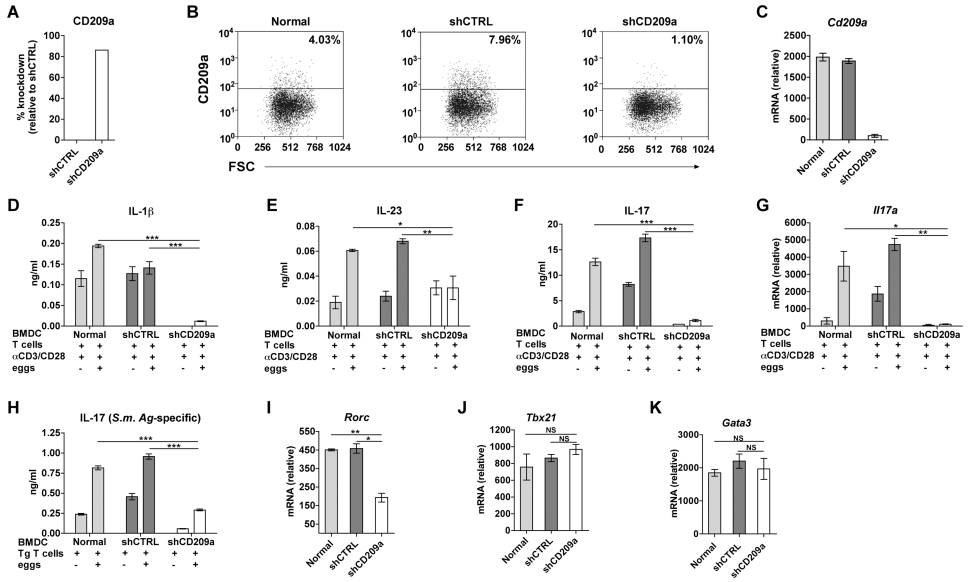

Figure 6. CD209a expression on CBA DCs is necessary for the induction of Th17 cell responses

CBA BMDCs were differentiated in rGM-CSF-containing medium over 10 days. shRNA delivered by a lentiviral vector was used to knock down CD209a expression (shCD209a). Infected DCs were enriched by puromycin-selection and CD209a knockdown efficiency was assessed by flow cytometric analysis relative to CBA BMDCs receiving a GFP-targeted control shRNA (shCTRL) or normal BMDCs (A,B). *CD209a* expression knockdown was also assessed by qRT-PCR (mRNA relative to GAPDH) (C). (D-F) Normal CBA, shCTRL, or shCD209a DCs were co-cultured with naïve CBA $CD4^+$ T cells and anti-CD3/CD28-coated beads ± schistosome eggs for 96 hr. IL-1β, IL-23, and IL-17 in supernatants was assessed by ELISA. *Il17a* (G) was assessed by qRT-PCR. (H) Normal CBA, shCTRL, or shCD209a DCs were co-cultured with *S. mansoni* major egg Ag Sm-p40-specific Tg T cells (Tg T) ± schistosome eggs. IL-17 was assessed by ELISA. (I-K) Transcription factors *Rorc*, *Tbx21*, and *Gata3* were assessed by qRT-PCR in egg-stimulated co-cultures. Bars represent the mean ± S.D. of three biological replicates of one representative experiment of five. *p<0.05, **p<0.01, ***p<0.001, NS = not significant.

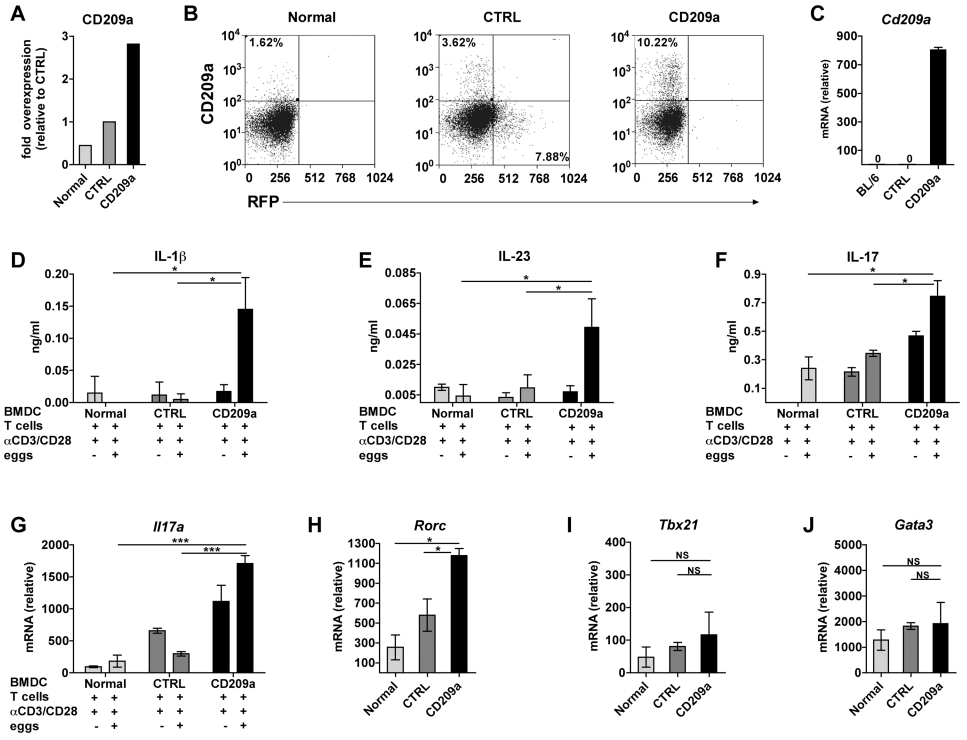

Figure 7. CD209a over-expression confers on BL/6 DCs the capacity to induce Th17 cell responses

BL/6 BMDCs were differentiated in rGM-CSF-containing medium over 10 days. CD209a over-expression was achieved using a lentiviral vector and confirmed by flow cytometric analysis relative to BL/6 control BMDCs that over-expressed RFP (CTRL) or normal BMDCs (A,B). *CD209a* over-expression was also assessed by qRT-PCR (mRNA relative to GAPDH) (C). (D-F) Normal BL/6, CTRL, or CD209a DCs were co-cultured with naïve BL/6 $CD4^+$ T cells and anti-CD3/CD28-coated beads ± schistosome eggs for 96 hr. IL-1β, IL-23, and IL-17 in supernatants was assessed by ELISA. *Il17a* was assessed by qRT-PCR (G). (H-J) Transcription factors *Rorc*, *Tbx21*, and G*ata3* were assessed by qRT-PCR in egg-stimulated co-cultures. Bars represent the mean ± S.D of three biological replicates of one experiment representative of three. *p<0.05, **p<0.01, ***p<0.001, NS = not significant.

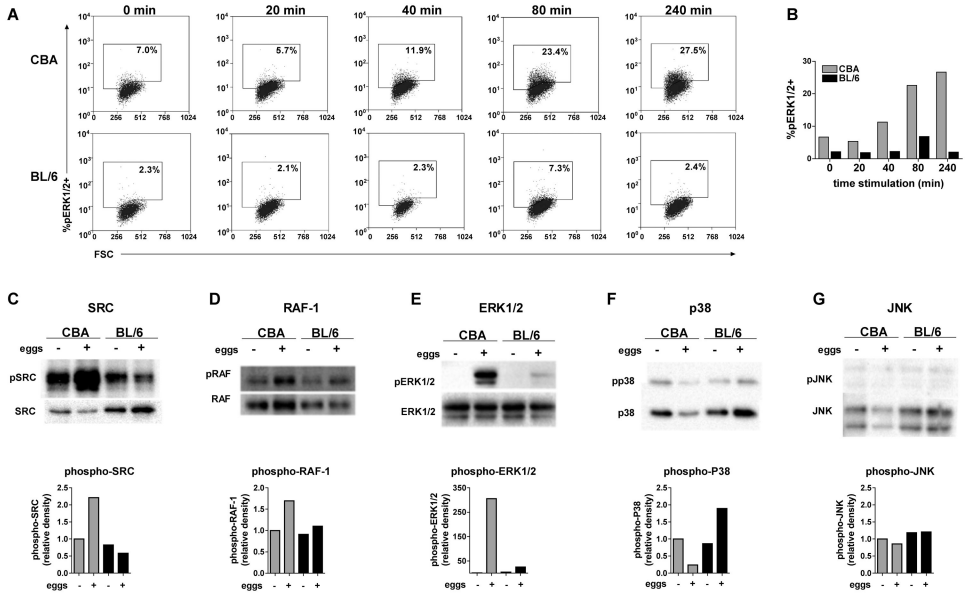

Figure 8. Egg stimulation of CBA DCs is associated with greater MAP kinase activation

(A-B) CBA and BL/6 BMDCs were differentiated in GM-CSF-containing medium over 7 days and cultured with schistosome eggs for the indicated time periods. Cells were fixed, permeablized and analyzed by intracellular staining for phospho-ERK1/2. Flow cytometry plots were gated for viability and $CD11c^+$ cells, which were > 80% for both strains. Data are from one representative experiment of three. (C-G) CBA and BL/6 BMDCs were cultured ± schistosome eggs for 24 hr and phosphorylated vs. total SRC, RAF-1, ERK1/2, JNK, and p38 expression was assessed by Western blot and relative band density was quantified. Data are from one representative experiment of four.