Double-stranded RNAs from the helminth parasite *Schistosoma*

activate TLR3 in dendritic cells

Ezra Aksoy¹,², Claudia S. Zouain¹, François Vanhoutte¹, Josette Fontaine¹, Norman Pavelka³, Nathalie Thieblemont⁴, Fabienne Willems², Paola Ricciardi-Castagnoli³, Michel Goldman², Monique Capron¹, Bernard Ryffel⁵, and François Trottein¹

¹Institut National de la Santé et de la Recherche Médicale U547, Institut Pasteur de Lille, Lille, 59019 France; ²Institute for Medical Immunology, Université Libre de Bruxelles, B-1070, Brussels, Belgium; ³Department of Biotechnology and Bioscience, University of Milano-Bicocca, 20126 Milan, Italy; ⁴Centre National de la Recherche Scientifique, 8147 and Université Paris V, Hôpital Necker, 75743 Paris, France; ⁵Laboratoire de Génétique Expérimentale et Moléculaire (GEM2358), Centre National de la Recherche Scientifique, 41500 Orléans, France.

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Address correspondence to François Trottein, Centre d’Immunologie et Biologie Parasitaire, Institut National de la Santé et de la Recherche Médicale U547, 1 rue du Professeur Calmette, Institut Pasteur de LILLE, 59019 Lille, France. Phone: 33-3-20-877-885; Fax: 33-3-20-877-888; email: francois.trottein@pasteur-lille.fr

**Abbreviations used in this paper:** PRR, pattern recognition receptor; TLR, Toll-like receptor; TIR, Toll/IL-1 receptor; MyD88, myeloid differentiation factor 88; IRF, interferon regulatory factor; STAT, signaling transducer activator of transcription; ISG, IFN-stimulated gene; GARG, glucocorticoid-attenuated response gene; p(I:C), polyinosine-polycytidylic acid; PRD, positive regulatory domain; ds, double-stranded; ss, single-stranded.
Running title: Helminth dsRNA is a natural ligand for TLR-3
SUMMARY

Stimulation of dendritic cells (DCs) by the egg stage of the helminth parasite *Schistosoma mansoni* activates a signaling pathway resulting in type I interferon (IFN) and IFN-stimulated gene (ISG) expression. Here, we demonstrate that *S. mansoni* eggs disjointedly activate myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways in DCs. Inflammatory cytokine expression and NF-κB activation in DCs from MyD88 deficient mice were impaired, while signaling transducer activator of transcription (STAT)1(Tyr701) phosphorylation and ISG expression were intact in MyD88 or Toll-like receptor (TLR)4 deficient counterparts. Accordingly, we analyzed distinct TLR members for their ability to respond to schistosome eggs and established that TLR3 resulted in the activation of NF-κB and the positive regulatory domain III-I site from IFN-β promoter. Unexpectedly, egg-derived RNA possessed RNase A resistant and RNase III sensitive structures capable of triggering TLR3 activation, suggesting the involvement of double-stranded (ds) structures. Moreover, DCs from TLR3 deficient mice displayed a complete loss of STAT1 phosphorylation and ISG expression in response to egg-derived dsRNA. Finally, TLR3 deficient DCs showed a reduced response to schistosome eggs relative to wild type cells. Collectively, our data suggest for the first time that dsRNA from a non-viral pathogen may act as an inducer of the innate immune system through TLR3.
INTRODUCTION

DCs are antigen presenting cells that establish an intimate link between innate and adaptive immune responses (1, 2). During a microbial invasion, DCs play a central role in detecting foreign entities in their environment through a wide array of innate pattern recognition receptors (PRRs) and in modulating the succeeding immune/inflammatory responses by polarizing type 1 or type 2 responses or conversely by inducing tolerance to foreign and/or environmental antigen (3).

Pathogenic organisms express evolutionarily-conserved unique motifs that are recognized by distinct PRR families including Toll-like receptors (TLRs) (4). Activation of almost all TLRs results in signaling cascades that utilizes a Toll/IL-1 receptor (TIR) domain-containing adapter molecule myeloid differentiation factor 88 (MyD88)-dependent pathway, that converges on the activation of the transcription factor NF-κB and mitogen-activated protein kinases (5). However, activation of TLR3 engages a MyD88-independent pathway resulting in the expression of a subset of primary response genes. These include type I interferon (IFN)-β, induced by IFN response factor (IRF)-3 (6-9) downstream of TIR-domain-containing adaptor inducing IFN-β (termed as TRIF) and also NF-κB activation (10-13). TLR4 uses a similar type of pathway along with MyD88 (11, 12). Upon TLR-4/TLR-3 triggering, secreted IFN-β instigates an autocrine/paracrine loop and leads to the phosphorylation of signaling transducer activator of transcription (STAT)1 at the Tyr701 residue (14) and the expression of a set of IFN-stimulated genes (ISGs) including members of the glucocorticoid-attenuated response gene (GARG) family, and co-stimulatory molecules (such as CD40 and CD86) (15, 7).

Relative to viruses, bacteria and intracellular parasites, only a limited amount of work has been done on the role of TLRs in DC activation in response to extracellular pathogens, including helminths. Using high-density oligonucleotide microarrays, we have recently
demonstrated that the egg stage of the metazoan parasite *Schistosoma mansoni* activates the transcription of many genes in myeloid DCs including those encoding cell surface markers (CD40 and CD86), inflammatory (TNF-α) and immunoregulatory (IL-12p40) cytokines as well as ISGs (16). This signature is highly evocative of a TLR dependency. Although, the effects of *Schistosoma* live eggs on TLR activation have not been studied so far, recent studies reported the involvement of distinct TLRs in DC activation in response to certain egg components. Indeed, two egg-derived components, the glycolipid lyso-phosphatidylserine and the carbohydrate determinant lacto-N-fucopentaose III, have been shown to activate TLR2 and TLR4 respectively in myeloid DCs (17, 18).

In the present work, we aimed to investigate the involvement of TLRs in DC activation in response to live eggs. We showed that schistosome eggs activate a MyD88-dependent and a MyD88-independent pathway in DCs. Screening of distinct TLR members by transfection assays confirmed TLR2 (but not TLR4) activation but also demonstrated that TLR3, a TLR member known to recognize double-stranded (ds)RNA, is activated in response to eggs. In support of this hypothesis, we show evidence that egg-derived, RNase A resistant and RNase III sensitive, RNA structures activate TLR3-expressing cells. Finally, we found that egg dsRNA elicited STAT1 phosphorylation and ISG production in DCs via TLR3 engagement.
EXPERIMENTAL PROCEDURES

Reagents and plasmids—LPS (from Escherichia coli serotype 055:B5) was from Sigma-Aldrich (St Quentin-Fallavier, France). Pam3CSK4 [S-[2,3-bis-(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-Lys4-OH], trihydro-chloride was from EMC Microcollections (Tuebingen, Germany), polyninosine-polycytidylic acid p(I:C) from Amersham Pharmacia Biotechnologies (Roosendaal, The Netherlands), R848 from Cayla (Toulouse, France), CpG-A ODN from Tib Molbiol (Berlin, Germany) and flagellin was kindly provided by J.C. Sirard (Institut Pasteur de Lille). N-[1-(2,3-Dioleoyloxy)propyl]-N,N,Ntrimethylammonium methylsulfate (DOTAP) was purchased from Roche Diagnostics (Brussels, Belgium). The reporter constructs for multimerized NF-κB and the positive regulatory domain (PRD)III-I site from IFN-β promoter have been described. Plasmids encoding human TLR2, TLR3, and TLR4 were gifts from Y. Delneste (Inserm U564, Angers), TLR5 was from J.C. Sirard and TLR7, TLR8 and TLR9 were gifts from H. Wagner (Institute of Medical Microbiology, Munich, Germany).

Mice—Female C57BL/6 mice were purchased from Iffa-Credo (l’Arbesle, France). The generation of MyD88, TLR2, TLR3, and TLR4 deficient C57BL/6 mice has been described earlier (19-21). Mice strains were bred in an animal facility (CDTA, Orléans, France) in pathogen free conditions. All experiments were performed according to institutional guidelines of the animal ethics committee of the Pasteur Institute, Lille.

BM-DC generation—DCs were generated from the BMs of WT or knock-out C57BL/6 mice as described previously (22). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 1% of supernatant from a GM-CSF-expressing cell line (J558-GM-CSF). DCs were used on day 14 of culture (95% pure, as assessed by FACS analysis).
**Preparation of live eggs and egg-derived RNA**—S. mansoni eggs were obtained from the liver of infected golden hamsters after portal vein perfusion. The absence of contaminating hamster tissue fragments in the egg preparation was verified by microscopical analysis. The absence of endotoxin (below 0.015 EU/ml) in the parasite preparations (1 x 10^5 parasites/ml) was analyzed by a Limulus test (Sigma-Aldrich). Total RNA from whole eggs was isolated according to manufacturer’s specification (RNAPlus 3, Qbiogen) and was routinely pretreated with RNase-free DNase I (50 U Dnase I/ 100 µg of RNA) (Roche) at 25°C for 2 h. Total RNA was either pretreated with RNase A (1µg/µg of RNA) or with RNase III (1U/µg of RNA) (New England Biolabs Leusden, The Netherlands) in the buffer containing (150 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM MgCl₂, and 1 mM DTT) for 2 h at 37°C (final volume: 50 µl). As a control, total RNA was also treated with RNaseA in low salt condition (RNase-free H₂O). Enzymatic activities were stopped by heat deactivation at 55°C for 30 min following each treatment.

**Cellular activation assays**—BM-DC (1 x 10^6 cells/ml) were stimulated with live eggs (1/200 cells), Pam₃CSK₄ (0.5 µg/ml), p(I:C) (10 µg/ml) or LPS (100 ng/ml) or were left untreated. The conditions for RNA-DOTAP complexation were previously described (23) with a minor modification as 5 x 10^5 human embryonic kidney (HEK) 293T cells were stimulated in 500 µl of complete medium. As controls, DOTAP or p(I:C)-DOTAP complexes were included as described above. After 18-20 h, culture supernatants were collected and IL-12p40 and TNF-α concentrations were measured by ELISA (R&D Systems, Abingdon, UK). Cell death was assessed by trypan blue exclusion.
Transfection assays—HEK 293T parental cell line or cells stably expressing either TLR2, TLR3 or TLR4/MD2 were previously described (11, 24). Cells were seeded in 48-well plates at a density of 5 x 10^5/ml overnight. The following day, cells were transfected with 1 µg of indicated luciferase reporter plasmids or along with the indicated plasmids encoding a specific TLR using FuGENE™-6 (Roche) according to the manufacturer’s specification. All transfections included 40 ng of Renilla luciferase DNA in pRLTK vector (Promega, Leiden, the Netherlands) as an internal control. After cellular activation assays, cells were harvested and promoter activities were analyzed using the Dual Luciferase Reporter Assay System (Promega) in a Packard Topcount NXT (Packard). Promoter activities were normalized to Renilla luciferase activities. Data are expressed as the mean relative stimulation ± SD.

Real-time quantitative RT-PCR—Total RNA from BM-DCs (1 x 10^6 cells/ml) was isolated and cDNA was synthesized from 1 µg of total RNA with random hexamer primers and Superscript reverse transcriptase (Invitrogen, Cergy Pontoise, France) using standard procedures. For real-time RT-PCR, cDNAs were used as templates for PCR amplification using the SYBR® Green PCR Master Mix (Molecular Probes, Leiden, The Netherlands) and the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers specific for GAPDH, IFI204 and GARG39 respectively; GAPDH - forward: TGCCCAGAACATCATCCCTG - reverse: TCAGATCCACGACGGACACA, IFI204 - forward: TGGCAGCTGAGGTCT GTAAGG - reverse: CCAGAGAGGTTCTCCCGACTG and GARG 39 - forward: GCCATTGCGAACTACCGTCT - reverse: ACCCGCGTCAAGCTTCAGTG were designed by the Primer Express Program (Applied Biosystems) and used for amplification in triplicate assays. PCR amplification of GAPDH was performed to control for sample loading and to allow normalization between samples. Data are expressed as fold increase compared to the expression level in unstimulated cells.
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**Immunoblotting**—BM-DC (1 x 10⁶ cells/ml) were collected and directly lysed in Laemmli sample buffer. An equal volume of the total cell lysate from each condition was resolved by 10% SDS-PAGE, and immunoblotted. Membranes were probed with a monoclonal antiphospho-STAT1(Tyr701).antibody (Zymed Laboratories, San Francisco, CA). To verify loading, immunoblots were reprobed with an antibody that recognizes nonphosphorylated STAT1 (Santa Cruz Biotechnologies, Boechout, Belgium). Immunoreactive bands were revealed using the ECL detection method (Amersham Pharmacia).

**EMSA**—Nuclear extracts from BM-DCs (1 x 10⁶/ml cells) were prepared as described earlier (25). The ds binding oligonucleotides for consensus NF-κB were from Promega. The EMSA for NF-κB was carried out as reported previously (26).

**Statistical analysis**—The statistical significance of differences between experimental groups was calculated using the Student’s t test. p < 0.05 was considered as significant.

**Online supplemental material**—Figure S1 shows egg-derived RNA fractions do not activate TLR2 or TLR4 stably expressing HEK 293T cells.
RESULTS

MyD88 is in part involved in DC activation in response to S. mansoni eggs.

The kinetic global gene expression analysis of mouse DCs suggested that *S. mansoni* live eggs activate DCs through TLR engagement (16). We first aimed to analyze the contribution of MyD88, a critical adaptor molecule used by all TLRs (with the exception of TLR3) (27, 28), in the egg-induced DC activation. In addition, we investigated the role of TLR2 and TLR4, two TLR members recently described to be involved in the response to distinct *S. mansoni* egg components (17, 18).

For this purpose, BM-DCs generated from wild-type (WT), TLR2, TLR4 or MyD88 deficient (-/-) mice were exposed to live eggs and the production of IL-12p40 and TNF-α, two classical cytokines described to be produced in response to TLR engagement, was quantified. As depicted in Fig. 1A, LPS (a potent TLR4 agonist), Pam3CSK4 (a potent TLR2 agonist) and live eggs induce the secretion of both IL-12p40 and TNF-α. As previously demonstrated, compared to WT DCs, IL-12p40 and TNF-α production was abolished in TLR4-/- and TLR2-/- DCs in response to their cognate ligands. Similarly, LPS and Pam3CSK4 failed to induce IL-12p40 and TNF-α production in MyD88-/- DCs. In response to eggs, compared to WT DCs, the production of IL-12p40 and TNF-α by TLR2-/- DCs was not significantly modified whereas MyD88 deficiency resulted in approximately 75% decreased IL-12p40 and TNF-α production. In contrast, TLR4 deficiency did not influence the egg-induced production of these cytokines.

We next analyzed the activation of NF-κB (29) since it represents a common signaling pathway shared by all TLR members (27). As depicted in Fig. 1B, LPS, Pam3CSK4 and live eggs induce NF-κB nuclear translocation in WT DCs, within 1 h post-stimulation. In comparison to their WT counterparts, NF-κB activation was abolished in TLR4-/- and TLR2-/- DCs in response to LPS and Pam3CSK4, respectively. In contrast, the lack of TLR2 or TLR4...
Helminth dsRNA is a natural ligand for TLR-3 in DCs did not modify NF-κB DNA-binding mediated by schistosome eggs (Fig. 1B). In MyD88−/− DCs, LPS activated NF-κB, albeit with delayed kinetics, whereas Pam3CSK4-mediated NF-κB activation was abolished. After schistosome egg stimulation, NF-κB activation was strongly down-regulated at 1 h but a substantial activation was still observed at 2 h in MyD88−/− DCs. As a whole, these data revealed that MyD88 is in part involved in the egg-induced NF-κB activation and IL-12p40 and TNF-α production by DCs.

S. mansoni eggs activate a MyD88-independent pathway in DCs.

We have previously reported that DC activation by schistosome egg exposure is characterized by a rapid production of bioactive IFN-β (16), a key mediator necessary for DC maturation in response to certain TLRs including TLR3 and TLR4 (by a MyD88-independent pathway) and TLR7-9 (by a MyD88-dependent pathway) (27). We have also demonstrated that IFN-β production in response to eggs efficiently triggered the type I IFN receptor expressed on DC causing phosphorylation of STAT1 (Tyr701) with the consequent up-regulation of ISGs. Therefore, we investigated the contribution of MyD88 and TLR4 in STAT1 phosphorylation and ISG expression upon egg exposure.

First, we analyzed the phosphorylation state of STAT1 in WT, MyD88−/−, TLR2−/− and TLR4−/− DCs upon egg stimulation. As represented in Fig.2A, eggs and LPS induced STAT1 phosphorylation in WT DCs while, as previously demonstrated, Pam3CSK4 was ineffective (not shown). Although TLR2 and MyD88 deficiency did not modify the level of STAT1 phosphorylation induced by either eggs or LPS, TLR4−/− cells failed to trigger STAT1 phosphorylation in response to LPS, but not in response to eggs. Thus, the egg-mediated STAT1 phosphorylation does not involve TLR4 and MyD88 dependent pathways.
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Next, we examined the role of MyD88 and TLR4 in the egg-induced expression of two typical ISGs by real-time RT-PCR. As shown in Fig. 2B, relative to untreated WT DCs, IFI204 and GARG39 mRNA levels were markedly increased 4 h after LPS or egg stimulation. In contrast, LPS failed to induce IFI204 and GARG39 mRNA synthesis in TLR4\(^{-/-}\) but not in MyD88\(^{-/-}\) DCs. Upon egg stimulation, DCs from either mouse strain were able to induce IFI204 and GARG39 mRNAs synthesis at a level similar to that observed in WT DCs (Fig. 2B). Taken as a whole, these data clearly demonstrate that eggs activate a MyD88-independent pathway in DCs that culminates in ISG production, independently of TLR4 engagement.

*S. mansoni* eggs activate TLR3.

In order to characterize the potential receptor(s) involved in schistosome egg recognition, HEK 293T cells were transiently transfected with plasmids encoding either TLR2, 3, 4, 5, 7, 8 or 9 and their ability to activate an NF-κB-containing promoter in response to eggs was analyzed. As shown in Fig. 3A, stimulation of TLR-transfected cells with their cognate ligands promoted NF-κB-mediated luciferase expression. Following schistosome egg exposure, a substantial NF-κB activation was detected in TLR2- and TLR3-transfected cells, but not in the other tested transfectants. To confirm these results, and because the efficiency of stable transfectants is higher than that of transient transfectants, stable TLR2-, TLR3- and TLR4-expressing HEK 293T cells were used. As shown in Fig. 3B (upper panel), eggs induced a strong NF-κB-dependent luciferase expression in TLR2- (15-fold) and TLR3- (5-fold), but not TLR4-, expressing cells. In addition, the ability of eggs to activate the PRDIII-I site from type I IFN-β promoter, which requires IRF-3 binding (30, 31), was further investigated. In accordance with the previous reports, stimulation of TLR3- and TLR4-, but not TLR2-, expressing cells by their cognate ligands resulted in PRDIII-I activation (Fig. 3B,
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lower panel). Compared to unstimulated cells, egg exposure induced a strong activation (15-fold) of the PRDIII-I element in TLR3- (but not TLR2- and TLR4-) transfected cells. These results indicate that live eggs selectively activate TLR2 and TLR3, but not the other TLR members tested.

Double-stranded RNA from S. mansoni eggs is a natural ligand for TLR3.

TLR3 was originally described as a PRR recognizing viral dsRNA (21) and certain single-stranded (ss) mRNA structures (32, 33). Therefore, we tested the hypothesis that egg-derived RNA could activate TLR3. To do so, total DNase-treated RNA was prepared from eggs and tested for its capacity to activate NF-κB in HEK 293T cells stably expressing TLR3. Total RNA was delivered in the presence or absence of the cationic liposome DOTAP, which facilitates the uptake of RNA (23). As shown in Fig. 4A, egg RNA complexed to DOTAP dose-dependently activated NF-κB transcriptional activity with a maximal capacity at 10 µg/ml. In addition, egg RNA alone also induced TLR3-specific NF-κB reporter activity, albeit with a lesser efficiency (Fig. 4A). It should be noted that the lack of responsiveness of cells expressing either TLR2 or TLR4 confirmed that the RNA fractions were free of endotoxin and other carryover contaminations resulting during RNA isolation (supplemental Fig. S1).

We next attempted to determine the nature of the RNA species responsible for TLR3 stimulatory ability by analyzing the effects of different types of Rnases on total egg-derived RNA. To this end, total egg RNA was subjected to treatments either by E. coli RNase III, a dsRNA specific enzyme homologous to Dicer that effectively cleaves and processes ds-containing RNA structures or by RNase A, an endoribonuclease which degrades primarily ssRNA but can as well process dsRNA structures in low salt concentrations (34-37). Since under physiological salt conditions dsRNA is resistant to the action of RNase A (38), we have taken advantage of this phenomenon to investigate the susceptibility of egg RNA to Rnases.
under varying salt concentrations. Treated egg RNA was next analyzed for its ability to activate NF-κB promoter in cells stably expressing TLR3. As a control, p(I:C), a synthetic mimic of dsRNA, was used. As shown in Fig. 4B, stimulation of TLR3 expressing cells with p(I:C) or p(I:C)-pretreated with RNase A (in high salt) resulted in NF-κB activation, demonstrating that this treatment conserves dsRNA structures. In contrast, RNase A (in low salt) or RNase III (in high salt) diminished the ability of p(I:C) to activate cells. Similarly, treatment with RNase A (in high salt) did not affect the ability of total egg RNA to activate TLR3 transfected cells, albeit with slightly decreased efficacy compared to total RNA. In contrast, RNase A (in low salt) or RNase III (in high salt) markedly impaired the capacity of egg RNA to activate cells. We therefore conclude that potential dsRNA-like structures pre-existing in egg-derived RNA can activate TLR3.

*S. mansoni* egg-derived dsRNA activate DCs via TLR3.

We next investigated whether *S. mansoni* eggs and egg-derived dsRNA structures activate DCs via TLR3. To achieve this, WT and TLR3−/− DCs were stimulated with live eggs or with egg-derived RNA treated with RNAsAse A (in high salt) (referred as dsRNA), and the level of STAT1 phosphorylation was measured. As observed in Fig. 5A, p(I:C), eggs and egg-derived dsRNA triggered phosphorylation of STAT1 (Tyr701) in WT DCs. In contrast, the response to p(I:C) and egg-derived dsRNA was ablated in TLR3−/− DCs. Under the same conditions, egg-mediated STAT1 phosphorylation was strongly reduced, but not fully abrogated, in TLR3−/− DCs as compared to WT counterparts.

To validate this finding, IFI204 and GARG39 mRNA levels were quantified by real time RT-PCR. As represented in Fig. 5B, relative to untreated cells, p(I:C) induced a dramatic increase in IFI204 and GARG39 transcripts in DCs from WT but not from TLR3−/− mice. Similarly, levels of these mRNAs were markedly increased in WT DCs after egg dsRNA or
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egg exposure. Interestingly, TLR3 deficiency resulted in a complete loss of ISG mRNA up-regulation upon egg dsRNA stimulation, similar to that observed with p(I:C)-activated TLR3−/− DCs. On the other hand, IFI204 and GARG39 mRNA up-regulation was strongly reduced, but not fully abrogated, in TLR3−/− DCs, in response to live eggs.

The secretion of TNF-α and IL-12p40 production was also determined in WT and TLR3−/− DCs in response to eggs and egg-derived dsRNA. As observed in Fig. 5C, IL-12p40 and TNF-α production upon p(I:C) and egg-derived dsRNA stimulation was inhibited in TLR3−/− DCs compared to WT counterparts. Under the same conditions and compared to WT cells, TLR3−/− DCs exhibited a reduced capacity to elicit TNF-α and IL-12p40 production upon egg contact. Taken as a whole, our results demonstrate that egg-derived dsRNA activates DCs through a fully TLR3-dependent pathway. Moreover, live eggs also activate DCs, in part via TLR3.
DISCUSSION

TLR activation by pathogens is one of the main pathways through which DCs become activated during infections (39). In vivo studies clearly show that myeloid DCs become activated after encountering Schistosoma (40, 41), including egg components (42). While this phenomenon is presumably due to innate recognition of certain schistosomal products, the molecular mechanisms leading to DC activation as well as the consequences they have in the inflammatory/immune responses are still unclear. Although until now, the effects of live eggs on TLR activation have not been investigated, two independent studies have shown the implication of TLR2 and TLR4 in the recognition of certain egg components (17, 18). Recently, using a genome-wide expression study, we found that live eggs induce a transcriptional program in myeloid DCs that culminates in the production of many IFN-induced inflammatory gene products (16). In the present study, we have taken a genetic and molecular approach to identify the TLR members involved in this phenomenon. Remarkably, our data show for the first time that TLR3 can be activated by an extracellular pathogen and that ds-containing RNA structures from the helminth parasite Schistosoma may act as an inducer of the innate immune system.

First, we showed that MyD88, an important molecule involved in TLR signaling, is implicated, but not essential in DC activation by live eggs. Indeed, the kinetics of NF-κB activation was delayed but not abrogated in MyD88−/− DCs in comparison to that observed in WT counterparts. Moreover, although MyD88 deficiency did not affect the egg-induced phenotypic maturation of DCs, as assessed by CD86 or CD40 surface expression (data not shown), it reduced the production of IL-12p40 and TNF-α. This suggested that, along with MyD88, eggs might also recruit a MyD88-independent pathway in DCs. Since the MyD88-independent pathway triggered by some TLRs (TLR3 and TLR4) is important in ISG
Helminth dsRNA is a natural ligand for TLR-3 synthesis, we decided to investigate the consequences of MyD88 deficiency on the egg-induced IFN/STAT1-dependent pathway. Compared to WT DCs, egg-mediated STAT1 phosphorylation and ISG synthesis were unaffected in MyD88−/− and TLR4−/− DCs. Among TLRs able to induce ISG expression are TLR3, TLR4 and TLR7-9. Since, as stated above, TLR4 deficiency does not influence the egg-induced ISG synthesis and since TLR7-9 fully depend on MyD88 (27), we postulated that TLR3, a TLR known to be associated with viral dsRNA recognition, may be a good candidate. Transfection assays carried out on cells expressing distinct TLR members confirmed this hypothesis (Fig. 3). On the other hand, eggs do not activate TLR4 in HEK 293T transfected cells nor in DCs, a finding that is not in agreement with recent data reported by Thomas et al. (18). These observed differences could be attributable to a lack of accessibility of the lacto-N-fucopentaose III determinant to DCs, when they are exposed to live eggs. Another possibility is that live eggs express components capable of interfering with TLR4 signaling, as recently suggested (43, 44). In accordance with van der Kleij et al. (17), we however confirmed that eggs activate TLR2 (at least in transfected cells, Fig. 3), although this pathway does not appear to account for the egg-mediated IL-12p40 and TNF-α production by murine DCs.

Therefore, TLR2 and TLR3, but not the other TLR members tested, appear to be selectively activated by live eggs. To the best of our knowledge, this is the first time that TLR3 has been described to be activated by a non-viral pathogen. Since ligands for TLR3 include ssRNA (32, 33) and dsRNA (21, 27) structures, we attempted to validate our finding by testing the ability of egg-derived RNA to activate TLR3 in transfected cells. By the use of different types of RNases, we provided evidence for the existence of dsRNA structures that are necessary for activating TLR3, whereas ssRNA structures appear dispensable. More importantly, we found that the exogenous addition of egg dsRNA activated not only ISG
Helminth dsRNA is a natural ligand for TLR-3 expression (at least in part via STAT1 phosphorylation) but also classical cytokines produced during DC activation, through a fully TLR3 dependent pathway. This indicates that other molecules known to trigger immune responses to intracellular dsRNA such as protein kinase R, a cytosolic kinase activated by autophosphorylation on binding to dsRNA (45-47) or the RNA helicase retinoic acid inducible gene I (48) are not required for this response. Our data also show that TLR3 is in part involved in DC activation (STAT1 phosphorylation, cytokine production) when they encounter live eggs. However, it is clear from our studies that TLR3 does not fully account for DC activation in response to live eggs since TLR3\(^{-/-}\) DCs still produce substantial amounts of IL-12p40, TNF-\(\alpha\) and ISGs. The differences in TLR3 dependency observed between egg dsRNA versus whole eggs can be attributed to the engagement of additional PRRs upon egg exposure.

There are several possibilities that may explain the occurrence of dsRNA structures in *Schistosoma* eggs. First, recent genome analysis (49) suggests that *Schistosoma* express the components of the protein machinery necessary for RNA interference, a dsRNA-dependent posttranscriptional gene silencing process known to inhibit the expression of unwanted genes (35, 50, 51). Although it is still unknown whether these helminths exploit such RNA-processing mechanisms, the effectiveness of RNA interference using exogenous dsRNA introduced to silence gene expression in *S. mansoni* is strongly indicative of an active posttranscriptional gene silencing mechanism in schistosomes (52). Another possibility, among others, is the existence of transposon-derived dsRNA in *Schistosoma* eggs. It is indeed demonstrated that there are large numbers of repetitive elements in the *S. mansoni* genome in particular the retroposon Sm\(\alpha\), a hammerhead ribozyme which forms transcripts with a high degree of secondary structures containing a large proportion of ds regions (53, 54).
Understanding how these dsRNA structures become available to DCs (*in vitro* and *in vivo*) however requires further study.

In conclusion, we propose that eggs from the extracellular parasite *S. mansoni* may activate innate immunity through TLR2 and TLR3. During DC contact with eggs, TLR2 and TLR3 may act in concert and impact innate and adaptive immune responses in a cumulative manner. Therefore, future work is required to clarify the respective role of TLR2 and TLR3 on the ensuing immune response during schistosomiasis. Finally, the existence of dsRNA structures in helminths exhibiting strong immunostimulatory potential to promote DC maturation through TLR3 further implicates the adjuvanticity of dsRNA from eukaryotic pathogens in general.
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FIGURE LEGENDS

FIG.1. Effects of TLR2, TLR4 and MyD88 deficiency on BM-DC activation in response to *S. mansoni* live eggs. *A-B*, BM-DCs from WT, TLR4<sup>−/−</sup>, TLR2<sup>−/−</sup> or MyD88<sup>−/−</sup> mice were stimulated with LPS (100 ng/ml), Pam3CSK<sub>4</sub> (0.5 µg/ml) or *S. mansoni* eggs (1/200 cells). *A* After 20 h, IL-12p40 and TNF-α levels in culture supernatants were analyzed by ELISA. Data represent means ± SEM of 5 independent experiments. *p < 0.05 or **p < 0.005 as compared to cytokine production from WT DCs stimulated by corresponding TLR agonist or live eggs. *B*, At indicated time intervals, cells were collected, washed and nuclear extracts were prepared. Ten µg of nuclear extract from each condition was analyzed by EMSA for NF-κB DNA-binding activity. Arrows indicate NF-κB complexes. One representative experiment out of 4 is shown.

FIG.2. Effects of TLR4 or MyD88 deficiency on STAT1 phosphorylation and ISG synthesis by BM-DC in responses to live eggs. *A*, BM-DC from WT, TLR4<sup>−/−</sup>, TLR2<sup>−/−</sup> and MyD88<sup>−/−</sup> mice were activated by LPS (100 ng/ml) or *S. mansoni* eggs (1/200 cells). At indicated time intervals, cells were collected and lysed in Laemmlli buffer. Protein extracts were analyzed by direct western blotting with a specific antibody directed against the phosphorylated form of STAT1(Tyr<sup>701</sup>). Protein loading was controlled by reprobing the membrane with an Ab directed against unphosphorylated STAT1. One representative out of 3 experiments is shown. *B*, Quantification of IFI204 and GARG39 mRNA expression. RNA was harvested after 4 h stimulation and IFI204 and GARG39 mRNA accumulation were measured by real-time RT-PCR. The level of IFI204 and GARG39 mRNA is given as a fold increase relative to the mRNA levels in unstimulated cells. *p < 0.05 or **p < 0.005 as compared to mRNA fold induction from WT DCs stimulated by LPS or live eggs.
FIG. 3. **Schistosome eggs activate TLR3.**  
A, HEK 293T cells were transiently transfected with 1 µg of NF-κB reporter plasmid along with TLR2, 3, 4, 5, 7, 8 or 9 expression vectors. After 18 h, cells were activated by the corresponding TLR ligands; Pam3CSK4 (10 µg/ml, for TLR2), p(I:C) (50 µg/ml for TLR3), LPS (100 ng/ml for TLR4), flagellin (1 µg/ml for TLR5), R848 (1 µM or 10 µM for TLR7 or TLR8, respectively) and CpG (50 µg/ml for TLR9) or were exposed to *S. mansoni* eggs (1/200 cells). Luciferase reporter gene activity was measured 18 h after cellular stimulation. Data represent mean relative stimulation ± DB of 3 independent experiments. *p < 0.005 as compared to unstimulated cells. 

B, HEK 293T cells expressing TLR2, TLR3 or TLR4/MD2 were transfected with 1 µg of NF-κB (upper panel) or PRDIII-I (lower panel) reporter plasmids. Following 18 h post-stimulation with cognate ligands or *S. mansoni* eggs (1/200 cells), luciferase reporter gene activity was measured (one triplicate representative out of 3 experiments is shown).

FIG. 4. **Egg-derived dsRNA is a natural ligand for TLR3.**  
A, TLR3 mediates NF-κB activation in response to egg-derived RNA. TLR3 stably expressing HEK 293T cells were transfected with 1 µg of NF-κB reporter plasmid. After 18 h, cells were activated by graded concentrations of total egg-derived RNA-complexed to DOTAP, egg RNA (10 µg/ml) alone or were left untreated for a further 18 h. Luciferase reporter gene activity was measured (one triplicate representative out of 3 experiments is shown). 

B, Egg-derived RNA contains dsRNA structures that activate TLR3. HEK 293T cells expressing TLR3 were activated either with egg-derived RNA (10 µg/ml) or p(I:C) (50 µg/ml) complexed to DOTAP following pretreatments with RNase A (+ salt), RNase A (-salt) or RNase III (+ salt) (described under Experimental Procedures) or were left untreated for further 18 h. Luciferase reporter gene activity was measured (one triplicate representative out of 3 experiments is shown).
Helminth dsRNA is a natural ligand for TLR-3

FIG.5. Eggs and egg-derived dsRNA activate BM-DC via TLR3. A-C, BM-DCs from WT and TLR3−/− mice were activated by p(I:C) (50 µg/ml), egg-derived dsRNA (10 µg/ml) or live eggs (1/200 cells). A, TLR3 is required for egg and egg-derived dsRNA mediated STAT1 phosphorylation. At indicated time intervals, cells were collected and lysed in Laemmli buffer. Protein extracts were analyzed by direct western blotting with antibodies directed against phosphorylated (Tyr701) or nonphosphorylated forms of STAT-1. One representative out of 3 experiments is shown. B, Egg dsRNA induces ISG expression through TLR3. At 4 h following cellular stimulation, mRNA was extracted, reverse transcribed and amplified by quantitative real time PCR using primers specific for IFI204, GARG39 or GAPDH. The level of IFI204 and GARG39 mRNA is given as a fold increase relative to the mRNA levels in unstimulated cells. One representative out of 3 independent experiments is shown. *p < 0.05 or **p < 0.005 as compared to mRNA induction from WT DCs stimulated by eggs, egg-dsRNA or p(I:C). C, TLR3 mediates cytokine production by egg dsRNA or eggs. IL-12p40 and TNF-α production in culture supernatants were analyzed by ELISA. Data represent means ± SEM of 3 independent experiments. *p < 0.05 as compared to cytokine production from WT DCs stimulated by eggs, egg-dsRNA or p(I:C).
FIG.S1. Egg-derived dsRNA does not activate TLR2 and TLR4. TLR2- or TLR4-expressing HEK 293T cells were transfected with 1 µg of NF-κB reporter plasmid. After 18 h, cells were activated by; LPS (100 ng/ml, cognate ligand for TLR4), PGN (10 µg/ml, cognate ligand for TLR2), egg-derived RNA (10 µg/ml) alone or following pretreatments with RNase A (+ salt), RNase A (-salt) or RNase III (+ salt) (described under Experimental Procedures), or were left untreated for further 18 h. Luciferase reporter gene activity was measured (one triplicate representative out of 3 experiments is shown).
Figure 1

A. 

![Graph showing IL-12 p40 levels in different conditions](image)

B. 

![Graph showing TNF-α levels in different conditions](image)

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Figure 2

Table A:

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Figure B:

A. Bar graph showing the fold index of IFI1204 in WT, TLR4Δ/Δ, and MyD88Δ/Δ cells treated with LPS and Egg.

B. Bar graph showing the fold index of GARG30 in WT, TLR4Δ/Δ, and MyD88Δ/Δ cells treated with LPS and Egg.
Figure 3

A. 

B. 

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Figure 4

A.

B.
Figure 5

A. 

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Figure S1

Helminth dsRNA is a natural ligand for TLR-3

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Double-stranded RNAs from the helminth parasite Schistosoma activate TLR3 in dendritic cells

Ezra Aksoy, Claudia Zouain, Francois Vanhoutte, Josette Fontaine, Norman Pavelka, Nathalie Thieblemont, Fabienne Willems, Paola Ricciardi-Castagnoli, Michel Goldman, Monique Capron, Bernard Ryffel and Francois Trottein

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