The Journal of Immunology

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J Immunol 2004; 172:3011-3017; ;
doi: 10.4049/jimmunol.172.5.3011
http://www.jimmunol.org/content/172/5/3011

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

A Type I IFN-Dependent Pathway Induced by *Schistosoma mansoni* Eggs in Mouse Myeloid Dendritic Cells Generates an Inflammatory Signature[1]

François Trottein,[2]* Norman Pavelka,[2†] Caterina Vizzardelli,[†] Veronique Angeli,* Claudia S. Zouain,* Mattia Pelizzola,[†] Monica Capozzoli,[†] Matteo Urbano,[†] Monique Capron,* Filippo Belardelli,[‡] Francesca Granucci,[†] and Paola Ricciardi-Castagnoli[3†]

Schistosomes are helminth parasites that display a dual impact on the immune system of their hosts. Although the larval stage, also known as *schistosomulum*, appears to subvert the host defenses, the egg stage induces strong inflammatory reactions. Given the pivotal role of dendritic cells (DC) in initiating and regulating immune responses, we compared the distinct transcriptional programs induced in immature mouse DC by *S. mansoni* eggs or schistosomula. Although SLA abrogated the transcription of many genes implicated in DC functions, eggs caused myeloid DC to produce IFN-β. Autocrine/paracrine signaling through the type I IFN receptor in response to eggs was necessary for the induction of known IFN-responsive genes and enhanced the synthesis of key inflammatory products. Taken as a whole, our data provide molecular insights into the immune evasion mechanism of schistosomula and suggest an unexpected role for type I IFN in the innate response to helminth eggs. ***The Journal of Immunology*, 2004, 172: 3011–3017.**

Schistosomiasis is a chronic parasitic disease that affects >200 million people in the world (1). The causative agent, a helminth parasite termed *Schistosoma*, has a complex life cycle that is initiated by the transcutaneous penetration of the larvae followed by its rapid transformation into schistosomula (SLA)[4] (2, 3). Once in the skin, SLA closely interact with immunocompetent cells, including dendritic cells (DC), to manipulate the host immune response (4, 5). SLA then begin a long vascular journey to reach the intrahepatic venous system, where they mature into adult male and egg-producing female worms. Eggs that accumulate in the liver, spleen, and lungs induce inflammation and an intense granulomatous hypersensitivity reaction (6).

DC are professional APCs of the immune system characterized by the exclusive capacity to initiate and control both innate and adaptive immune responses (7). Increasing evidence suggests that the extent and the type of immune responses induced by diverse stimuli depend on the effects they have on DC (8, 9). Immature DC are distributed in nonlymphoid organs, where they perform a sentinel function for incoming pathogens (10). Previous global gene expression analyses have showed that after microbial interaction, DC undergo a multistep maturation process (11) and acquire specific immune functions, depending on the type of microbe they have encountered (12).

To understand the molecular bases of the different effects of SLA and eggs on the immune system, given the pivotal role of DC in the control of immune and inflammatory reactions, we have investigated DC-schistosome interactions using a genome-wide expression study. Time-course transcriptional analyses require homogeneous populations of cells to avoid dilution and contamination of data (13). Therefore, although the use of in vitro systems may not completely reflect the interactions that occur in vivo during infections, we have used a near-homogeneous source of mouse DC, the well-defined, long term D1 splenic population (14). The kinetic global gene expression analysis of mouse DC stimulated with eggs or SLA presented in this study indicated that genes encoding inflammatory cytokines, chemokines, and IFN-inducible proteins were oppositely regulated by the two stimuli. Interestingly, eggs, but not SLA, induced the expression of IFN-β that efficiently triggered the type I IFN receptor (IFNAR) expressed on DC, causing phosphorylation of STAT-1 with consequent up-regulation of IFN-induced inflammatory products. Hence, the specific production of IFN-β and its autocrine and/or paracrine function on DC may provide a molecular mechanism to explain the opposite effects exerted by eggs and SLA on the immune response.

Materials and Methods

DC culture systems and parasite preparation

The murine splenic growth factor-dependent, long term DC line D1 was used as previously described (14), to maintain the homogeneous immature DC phenotype in vitro. Bone marrow-derived DC (BMDC) were generated from the BMs of wild-type or IFNAR$^{-/-}$ mice by culture in GM-CSF-conditioned medium as previously described (15). *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 checked by microscopic analysis. SLA were obtained by the skin penetration procedure from cercariae shed from infected *Biomphalaria glabrata* snails. The absence of endotoxin (<0.015 endotoxin unit/ml

*Centre d'Immunologie et de Biologie Parasitaire, Institut National de la Santé et de la Recherche Médicale, Unité 547, Institut Pasteur de Lille, Lille, France; †Department of Biotechnology and Bioscience, University of Milano-Bicocca, Milan, Italy; and ‡Laboratory of Virology, Istituto Superiore di Sanità, Rome, Italy

Received for publication July 25, 2003. Accepted for publication December 16, 2003.

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[1] This work was supported by grants from the European Community (DC strategies, QLRT-1999-00470) and from the Italian Ministry of Education, University, and Research (FIRB Grant RBNE01Y3N3_003).

[2] F.T. and N.P. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Paola Ricciardi-Castagnoli, Department of Biotechnology and Bioscience, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy. E-mail address: paola.castagnoli@unimib.it

[4] Abbreviations used in this paper: SLA, schistosomula; DC, dendritic cell; BMDC, bone marrow-derived DC; GARG, glucocorticoid-attenuated response gene; IFI, IFN-inducible gene; IFNAR, type I IFN receptor; IP, IFN-inducible protein; ISGF, IFN-stimulated gene factor; MIP, macrophage inflammatory protein.

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according to the manufacturer) in the parasite preparations (10^5 parasite/ml) was checked by a *Limulus* test (Sigma-Aldrich, St. Quentin-Fallavier, France).

RNA isolation, labeling, and array hybridization

D1 cells (10^7) were harvested either before or after 4-, 8-, 12-, or 24-h stimulation with eggs or SLA at a ratio of 1:200 parasites/cell. Total RNA was extracted, purified, and labeled for hybridization to MG-U74Av2 GeneChip oligonucleotide arrays (Affymetrix, Santa Clara, CA) using standard methods (11). Hybridization was conducted overnight with 15 μg of fragmented biotinylated cRNA. Stained microarrays were scanned at a resolution of 7.5 μm on an Affymetrix scanner. Resulting raw images were analyzed with the MicroArray Suite 5.0 program (Affymetrix) for computation of probe set-specific expression parameters.

Data normalization and preprocessing

For the identification of differentially expressed genes, all measurements for each gene were multiplied by a chip-specific scaling factor, computed to reach a target intensity of 100. For clustering and visualization purposes, both per chip and per gene normalizations were performed. Each measurement for each gene was first divided by the median expression level measured over the whole array. Each gene was then normalized to itself by dividing all measurements for that gene by the median expression value over all samples. Finally, data representing replicates of the same experimental condition were averaged.

Identification of differentially expressed genes

The data were analyzed using a modified version (N. Pavelka, M. Pelizzola, C. Vizzardelli, M. Capozzoli, A. Splendiani, F. Granucci, and P. Ricciardi-Castagnoli, manuscript in preparation) of the signal-to-noise statistic (16). Briefly, the reproduction of expression values, measured by 11 distinct chips, all nominally representing the transcriptome of immature D1 cells, was used to fit an empirical global error model describing measurement variability with a confidence of 95%. A gene was called differentially expressed at a given time point in comparison with the immature state if the absolute value of its signal-to-noise ratio, $(\mu_2 - \mu_1)/(\sigma_1 + \sigma_2)$, was higher than 1, where μ_1 and μ_2 represent, respectively, the average signal in the 11 controls or in the given time point, and σ_1 and σ_2 represent the 2 SD directly deduced from the global error model.

Gene expression profile clustering

Agglomerative average-linkage hierarchical clustering (17) of the nine different experimental conditions was obtained with GeneSpring 4.2 software (Silicon Genetics, Redwood City, CA) using Pearson correlation as the similarity metrics, whereas Spearman correlation was chosen for clustering the 283 differentially expressed genes. Supervised clustering was performed in the following way: probe sets were first labeled as enhanced or silenced by a given stimulus if their signal-to-noise ratio was respectively higher than +1 or lower than −1 in at least one time point of the given kinetic or were alternatively designated unchanged. Genes were then clustered according to their transcriptional response to the two stimuli.

RT-PCR, ELISA, and IFN titration

Total RNA was reverse transcribed using random hexamer primers, and Superscript reverse transcriptase (Life Technologies, Cergy Pontoise, France) and ELISA assays were conducted using ELISA development kits (R&D Systems, Minneapolis, MN) following the manufacturer's recommendations. IFN activity was assessed using standard viral protection assays performed on vesicular stomatitis virus-infected fibroblast cultures.

Determination of STAT-1 phosphorylation

Equal amounts of whole cell lysates (25 μg/lane) were subjected to SDS-PAGE under reducing conditions, and proteins were electrotransferred to polyvinylidene difluoride membranes (Roche, Meylan, France). The membranes were blocked for 1 h at room temperature with 5% milk in 1× TBS plus 0.1% Tween 20 and were incubated overnight at 4°C with Abs that recognize phosphorylated (Zymed Laboratories, San Francisco, CA) and nonphosphorylated (Santa Cruz Biotechnology, Santa Cruz, CA) α and β (91- and 84-kDa) forms of STAT-1 (2 μg/ml). Immunoreactive bands were revealed with HRP-labeled goat anti-rabbit Ig (Sanofi Diagnostics Pasteur, Marnes, France) using the ECL detection method (Pharmacia Biotech, Uppsala, Sweden).

Results

The two developmental stages of schistosome elicit different transcriptional responses in DC

To explore common themes and variations in the response of DC to either *S. mansoni* eggs or SLA, we used high density oligonucleotide microarrays to compare the time-dependent gene expression changes induced by the two parasite forms on D1 cells (supplementary Table I).[5] We identified 283 transcripts that were significantly modulated in at least one time point after treatment (supplementary Table II). Unsupervised clustering of experimental conditions sharply distinguished the two stimuli and outlined the existence of three main groups of samples: cluster A contained all samples of the egg time-course experiment, cluster B contained all time points of the SLA kinetics, and cluster C contained only unstimulated immature DC (Fig. 1*A*). Although SLA stably modulated DC transcriptome within the first 4 h without any major changes in the subsequent time points, eggs progressively modified the DC transcriptional profile during the 24-h stimulation.

A supervised clustering approach, designed to classify the 283 differentially expressed genes on the basis of their behavior after egg or SLA encounter (Fig. 1*B* and supplementary Table III), revealed that only 30 transcripts showed an identical modulation direction in response to the two stimuli (22 down-regulated by both and eight up-regulated by both). The great majority of the remaining 253 transcripts were significantly modulated by just one of the two developmental stages (154 genes were specifically modulated by eggs, and 80 were specifically modulated by SLA). Remarkably, 19 probe sets showed an exactly opposite behavior in response to either stimulus, in particular, the expression levels of all of these 19 genes were enhanced by the egg stage and were silenced by SLA (Fig. 1*B*).

Schistosoma *eggs and SLA induce two functionally distinct gene expression signatures in DC*

To further dissect the molecular program underlying DC-schistosome interaction, we performed a functional classification of the differentially expressed genes that were related to immune transcription (Fig. 2 and supplementary Table IV). The two most abundantly represented families turned out to be that comprising the innate inflammatory response genes (27 probe sets) and that embracing the IFN response genes (22 probe sets). Moreover, the inflammatory response family contained the highest number of genes observed to be oppositely regulated by eggs vs SLA (six probe sets of 19), whereas the IFN response family was dominated (18 probe sets of 22) by members of the expression cluster containing 82 probe sets enhanced by eggs but unchanged by SLA. Therefore, we hypothesized that the differential immune response triggered by the two *S. mansoni* developmental stages in vivo could be related to the specific gene expression signature induced in DC.

Transcriptional activation of candidate genes, chosen from the two most copiously represented functional families, was validated by either RT-PCR (Fig. 3*A*) or ELISA (Fig. 3*B*) and compared with the inflammatory stimulus LPS, which was used as a positive control. The results completely confirmed the predictions suggested by the microarray data analysis.

[5] The on-line version of this article contains supplemental material.

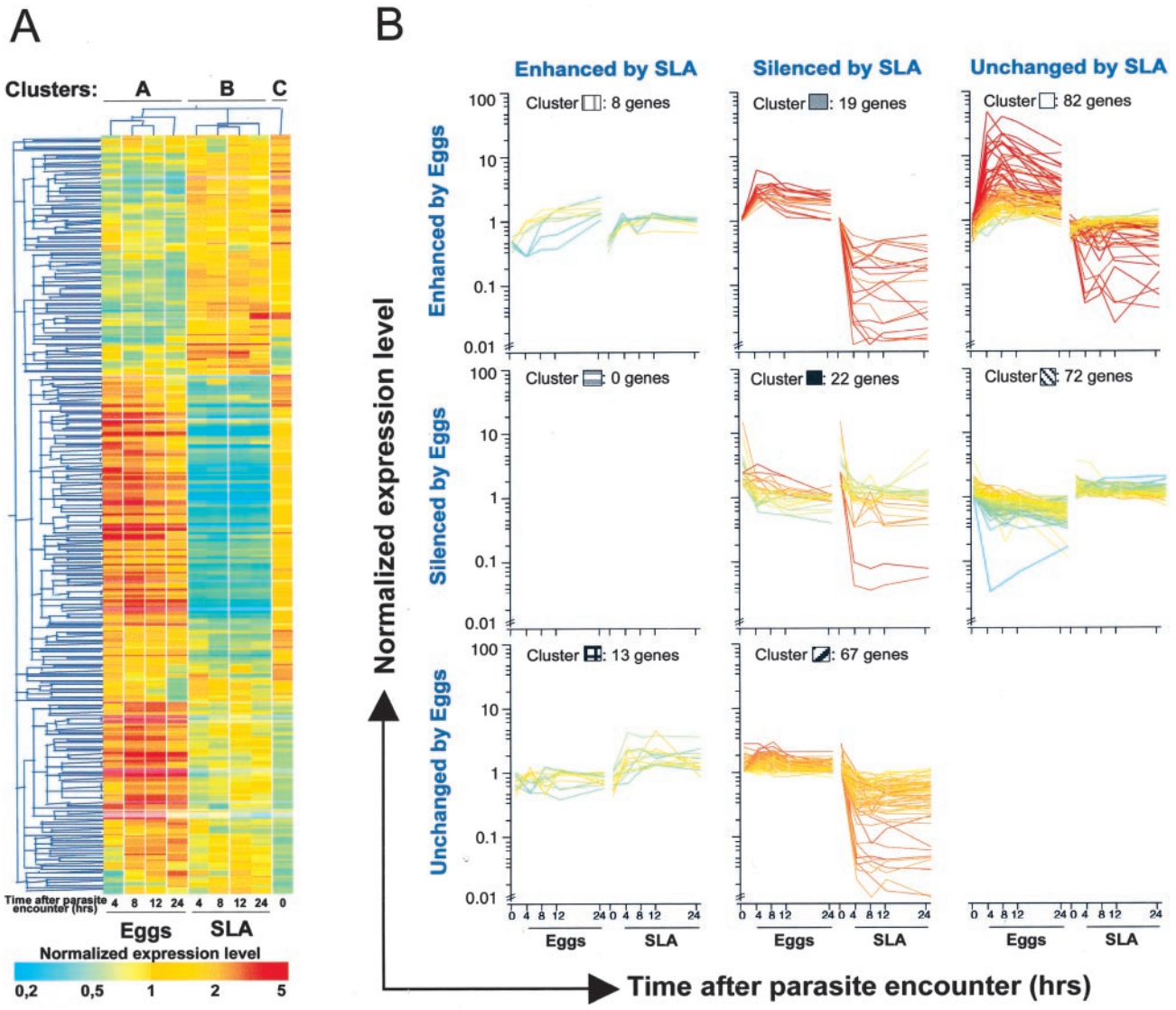

FIGURE 1. Expression profile clustering of 283 genes differentially expressed during DC-schistosome interaction. *A*, Two-way hierarchical clustering of gene expression profiles measured in time-course experiments; normalized expression levels relative to median are displayed in yellow (median expression), red (increased expression), or cyan (decreased expression) according to the color bar. *B*, Supervised clustering of kinetic gene expression profiles. Each panel groups genes that share same transcriptional response (enhanced, silenced or unchanged) relative to the two developmental stages of the parasite. Each line represents the expression profile of a particular gene and is colored according to its normalized expression level 4 h after encountering *S. mansoni* eggs. The data shown are the average of two independent experiments.

Eggs, but not SLA, induce type I IFN production by DC

As the expression of a large number of validated genes was reported in the literature to be mainly dependent on IFN, we hypothesized that, in contrast to DC-SLA interaction, DC-egg interaction may lead to the production of some type of IFN. Indeed, microarray data revealed a 3-fold up-regulation of IFN-β transcripts as early as 4 h after egg encounter, and no expression in the SLA kinetics (Fig. 4*A*). This finding was confirmed by RT-PCR on D1 cells as well as on BMDC. As shown in Fig. 4*B*, *S. mansoni* eggs and LPS (positive control) (18), but not SLA, induced transcription of the IFN-β gene in DC. Notably, IFN activity was detected in the supernatants of both D1 cells and BMDC stimulated with either *S. mansoni* eggs or LPS (Fig. 4*C*), suggesting that the accumulation of IFN-β mRNA observed in the treated cultures can result in the secretion of the corresponding biologically active protein.

Type I IFN autocrine/paracrine stimulation of DC in response to inflammatory stimuli

We next investigated whether IFN, secreted by DC after interaction with *S. mansoni* eggs, was capable of efficiently triggering the IFNAR, thus inducing phosphorylation of STAT1 protein, one of the major targets of IFN receptor signaling (19). Preliminary kinetic experiments indicated a peak of phosphorylation of STAT-1 protein at 4 h after egg encounter or LPS stimulation in both D1 cells and wild-type BMDC (data not shown). Eggs and LPS induced phosphorylation of STAT-1 in IFNAR-competent ($IFNAR^{+/+}$) DC, whereas neither was capable of triggering activation of STAT-1 in IFNAR-deficient ($IFNAR^{-/-}$) DC (Fig. 5*A*), demonstrating that STAT-1 phosphorylation in response to eggs is mediated by autocrine/paracrine activation of IFNAR by type I IFN.

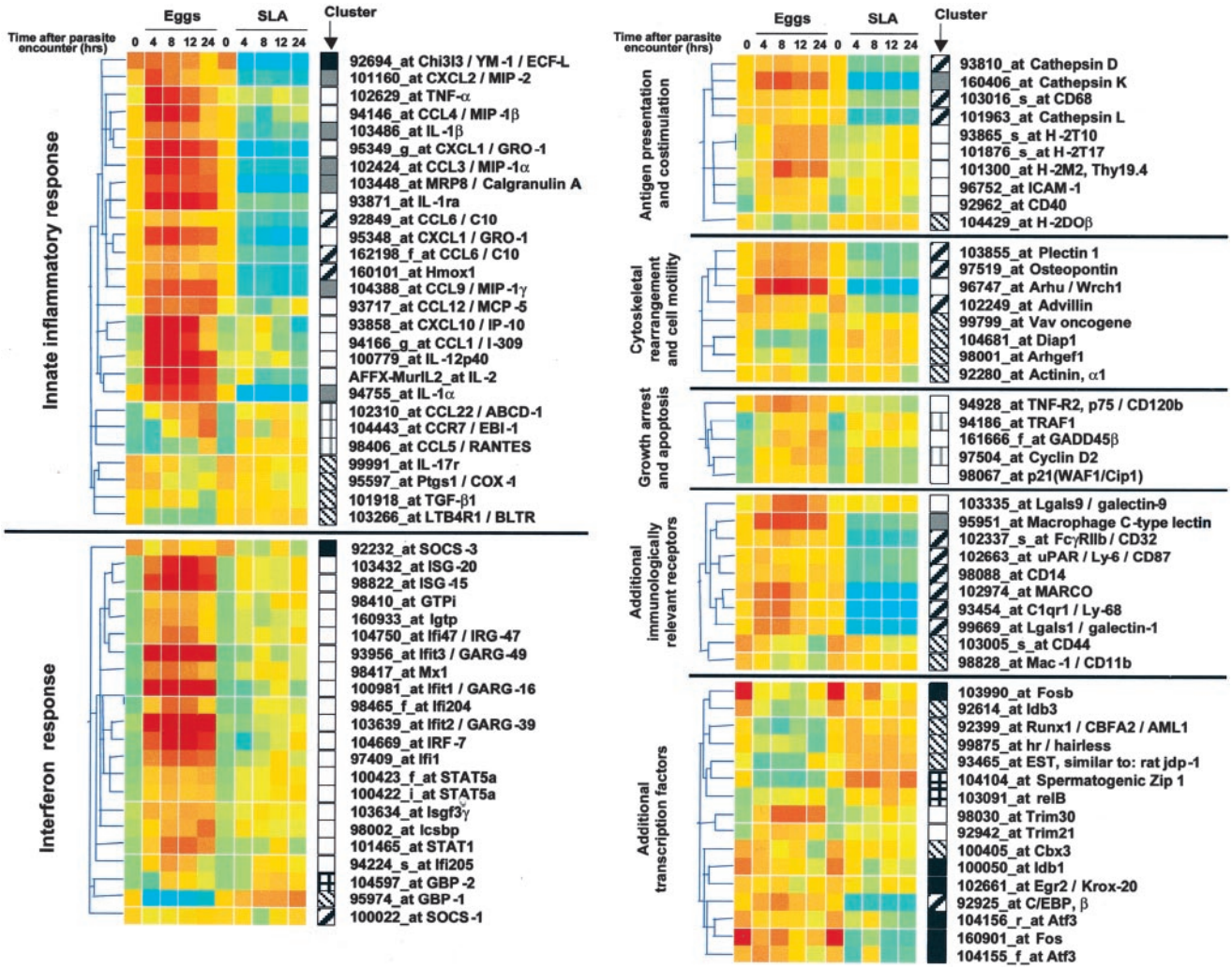

FIGURE 2. Functional classification of 98 selected, differentially expressed genes. Genes are divided into immunologically relevant functional families and then hierarchically clustered. Each colored box represents the normalized expression level of a given gene in a particular experimental condition and is colored according to the color bar in Fig. 1. Black and white boxes next to each gene expression profile represent membership to clusters in Fig. 1*B*. The data shown are the average of two independent experiments.

Finally, we verified whether this IFNAR-dependent pathway was relevant in inducing the expression of IFN and/or inflammatory response genes in DC, as initially suggested by the microarray analysis. As expected, up-regulation of genes known to encode IFN-inducible products, such as glucocorticoid-attenuated response gene (GARG)-16, GARG-39, GARG-49, IFN-inducible gene (IFI)-1, IFI47, IFN-stimulated gene factor-3γ (ISGF3γ), and IFN-inducible protein (IP)-10 (CXCL10), was abrogated in IFNAR$^{-/-}$ DC (Fig. 5*B*) after either egg or LPS stimulation, indicating that the IFN-β-mediated autocrine stimulation mechanism was required for the expression of these genes. Surprisingly, the expression of IFN-induced antiviral GTPase Mx-1, an antiviral gene whose promoter is commonly exploited for highly specific and sensitive type I IFN detection assays (20), was only partially reduced in egg- or LPS-stimulated IFNAR-deficient DC, suggesting the existence of a type I IFN-independent pathway involved in the augmentation of Mx-1 mRNA levels in the response of DC to inflammatory stimuli.

Lack of IFNAR expression did not significantly impact the egg- (and LPS-) induced mRNA and protein synthesis of IL-2, IL-12p40, macrophage inflammatory protein-2 (MIP-2; Fig. 5, *B* and *C*) and TNF-α (data not shown), confirming the existence in DC of a core response to inflammatory stimuli (21) that, at least for some genes, appears to be type I IFN independent. Nevertheless, the absence of a functional IFNAR in DC differentially affected the production of egg-induced, but not LPS-induced, MIP-1α (CCL3; Fig. 5, *B* and *C*).

Discussion

Coevolution has shaped host-pathogen interactions (22), resulting in a wide range of possible infection outcomes. Parasites have evolved survival strategies that include immune escape and exploitation of immune defense mechanisms (23). Schistosomes are believed to use both strategies, although at different developmental stages (24). Given the central role of DC at the host-pathogen interface (25), we compared the different transcriptional programs induced by either *S. mansoni* eggs or SLA on DC. Clustering techniques applied to 283 differentially expressed genes distinguished the two stimuli from different points of view. First, the continuous modification of DC transcriptome during the egg time-course experiment was compatible with a progressive cell differentiation process, such as maturation, whereas observations from SLA-stimulated DC samples suggested the occurrence of a stable blocking event within the first 4 h. Second, eggs modulated different amounts and subsets of genes in comparison with SLA, indicating that the two developmental stages of *S. mansoni* affected distinct

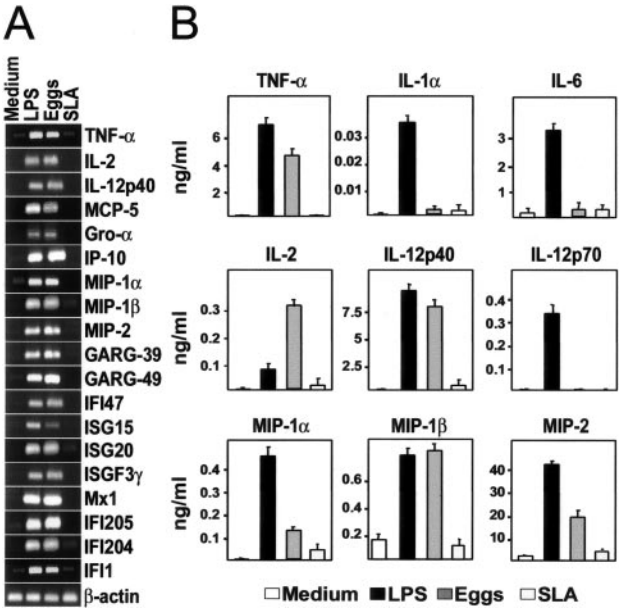

FIGURE 3. DC genes differentially regulated by LPS, eggs, or SLA validated by RT-PCR or ELISA. *A*, RNA was extracted from D1 cells at the indicated time points after stimulation and analyzed by RT-PCR for the indicated genes. Shown are the results from one representative experiment of three that gave similar results. *B*, D1 cells supernatants were harvested after 24 h of stimulation, and protein levels of selected cytokines were analyzed by ELISA. Histograms show results and SEs obtained from three independent experiments.

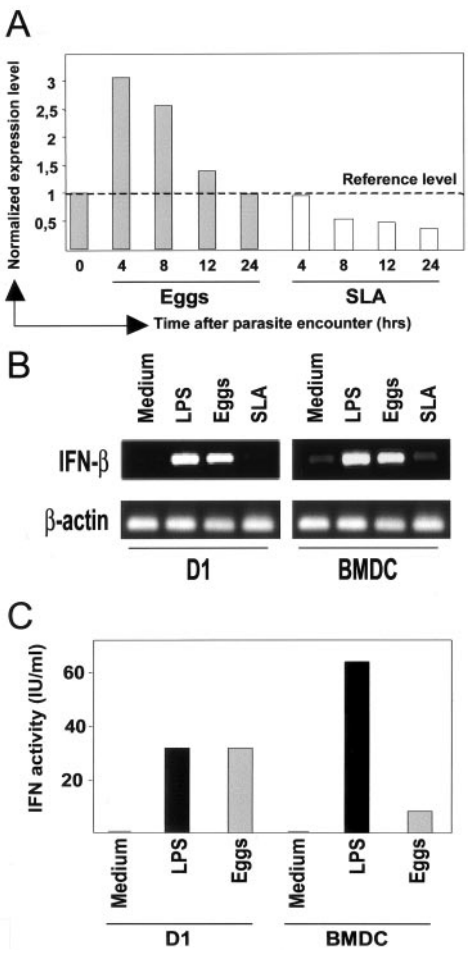

FIGURE 4. Production of type I IFN after DC-egg interaction. *A*, IFN-β normalized expression profile, as measured in the microarray experiment. The histogram shows the average of two independent experiments. *B*, Differential transcription of IFN-β gene by D1 cells and BMDC after 4-h stimulation with LPS, eggs, or SLA, analyzed by RT-PCR. The figure shows results from one representative experiment. Similar results were obtained from three independent experiments. *C*, Secretion of biologically active IFN molecules by D1 cells and BMDC 5 h after encounter with LPS or eggs. The histogram shows results obtained from one representative experiment. Identical results were obtained from two independent experiments.

intracellular pathways in DC possibly by triggering specific receptors.

Consistent with DC maturation process, the egg stage, but not the larval stage, of *S. mansoni* sustains the maximization of Ag presentation efficiency in DC by inducing the up-regulation of H-2M, which plays a crucial role in the peptide loading of MHC class II molecules (26) and of the costimulatory molecules CD40 and ICAM-1. Cathepsins D and L, which are believed to remove invariant chain from its complex with MHC class II molecules (27), are down-regulated by SLA, but are not modulated by eggs, suggesting a reduction in the Ag processing capacity exerted by the larval stage on DC.

During their maturation process DC undergo profound cytoskeletal rearrangements that are critical for the formation of an immunological synapse in their interaction with T cells (28). In this study we observed that the actin binding protein, advillin; the intermediate filament binding protein, plectin 1; and the small monomeric GTPase, Arhu, homologous to Cdc42, were either specifically enhanced by eggs or specifically silenced by SLA, indicating a more impaired cytoskeletal rearrangement ability of SLA-treated DC compared with egg-treated DC.

One of the key features of inflammation is the chemokine-mediated leukocyte recruitment to the site of infection (29). Indeed, DC stimulated with the egg stage, but not with SLA, expressed and secreted a large number of proinflammatory cytokines, such as TNF-α, and chemokines, such as IP-10 (CXCL10), monocyte chemoattractant protein-5 (CCL12), MIP-1α (CCL3), MIP-1β (CCL4), MIP-1γ (CCL9), and MIP-2 (CXCL2), that are known to collectively attract granulocytes, immature DC, NK cells, and activated T cells (29). In accordance with the previously described Th2-type immune response induced in vivo in the course of helminth infection (30), DC produced no IL-12p70 after parasite encounter. Moreover, *S. mansoni* eggs, but not SLA, induced the production of high amounts of IL-2, which could be important for DC-mediated activation of NK cells (31) or NKT cells (32) as well as for priming naive T cells (33).

Recent studies in both human and mouse models have shown that type I IFN are able to adjuvate the maturation of DC both in vitro and in vivo (34, 35). The results presented in this work demonstrate an inducible production of IFN-β by mouse myeloid DC in response to helminth eggs, but not to SLA. We have observed that the DC-derived IFN-β molecule efficiently triggered the IFNAR expressed on DC, thus providing an autocrine and/or paracrine stimulation mechanism. Upon triggering, IFNAR is known to mediate the phosphorylation of STAT1, hence causing its translocation into the nucleus and its assembly to the multiprotein transcription factor ISGF3 (36). ISGF3 is known to bind the IFN-stimulated response element present in the promoter region of many IFN-inducible genes (37), including ISG15, IP-10, and IFN regulatory factor-7, all of which have been identified in the present study as being specifically induced by eggs, but not by SLA. Indeed, we have shown that the type I IFN-mediated autocrine/paracrine stimulation mechanism is necessary not only for the expression of classical IFN response genes, but also for the production by

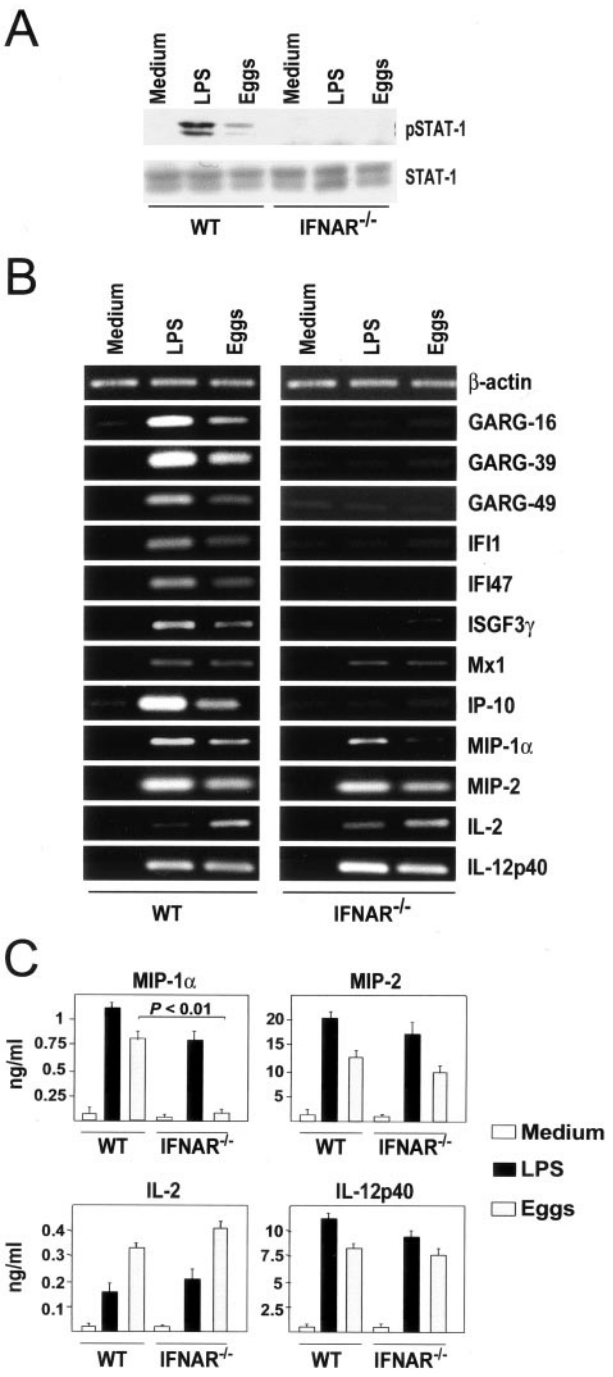

FIGURE 5. Type I IFN autocrine/paracrine stimulation of DC induced by *S. mansoni* eggs. *A*, STAT1 phosphorylation in BMDC derived from wild-type or IFNAR$^{-/-}$ mice, analyzed by Western blotting of protein extracts harvested 4 h after LPS or egg stimulation. Shown are results obtained from one representative experiment of three that gave similar results. *B*, RT-PCR analysis of selected cytokines and IFN-responsive genes in IFNAR-competent or -deficient BMDC after encounter with LPS or eggs. Results are from one representative experiment of three that had similar results. *C*, Secretion of selected cytokines by IFNAR$^{+/+}$ or IFNAR$^{-/-}$ BMDC in response to LPS or egg stimulation. The histogram shows results and error bars from three independent experiments.

mouse DC of key inflammatory chemokines, such as IP-10 and MIP-1α. Synthesis of IP-10 during *Mycobacterium tuberculosis* infection in humans has been recently shown to be mainly dependent on *M. tuberculosis*-induced production of type I IFN by infected DC, leading to a selective recruitment of NK cells and activated T cells (38). Moreover, recent in vivo studies have demonstrated that type I IFN, induced during viral infections in mice, can promote NK cell and macrophage trafficking via a mechanism that requires MIP-1α expression (39). Taken together, our data indicate myeloid DC as one possible mediator of type I IFN signaling as well as one plausible source of IP-10 and MIP-1α production also in response to helminth infections.

The comparative gene expression analysis reported in this study revealed two different DC global transcriptional modifications induced by either *Schistosoma* eggs or SLA, consistent with the different responses induced in vivo by these two parasite stages. It is noteworthy that the general effects described in this report exerted by live parasites on DC are not mimicked by their corresponding Ag extracts (not shown), suggesting that physical contact, rather that soluble factors, are involved in these phenomena. Taken as a whole, our observations have provided new molecular insights into the host-parasite interaction established in the course of schistosomiasis leading to the identification of a type I IFN-dependent mechanism by which DC may amplify inflammatory reactions in response to helminth infection. This pathway was known to be triggered by LPS during endotoxic shock (40) as well as by various infectious agents (41) that enhance antimicrobial immunity, suggesting usage of shared receptors or convergence of downstream signal transduction pathways. An important point that needs to be addressed in the future is to know whether the DC gene expression profiles induced by parasites described in this report impact on the DC-mediated immune response in vivo. Finally, identification of DC receptors involved in recognition of helminth eggs would be of interest. Possible candidates include members of the Toll-like receptor family (42–44), some of which are known to be able to induce transcription of IFN-β (45), or other pathogen recognition receptors (46).

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