MAJOR ARTICLE

Adoptive Transfer of Dendritic Cells Pulsed With *Fasciola hepatica* Antigens and Lipopolysaccharides Confers Protection Against Fasciolosis in Mice

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Dendritic cells (DCs) can function as adjuvants able to mediate protection against different pathogens. Given that successful vaccination against *Fasciola hepatica* is mostly related to the induction of Th1 responses, we studied the potential of DCs loaded with *F. hepatica* antigens and lipopolysaccharide (LPS) (which promote DCs maturation) as a vaccine against fasciolosis in BALB/c mice. However, only a semimature phenotype was achieved when DCs were simultaneously cultured with an *F. hepatica* total extract (TE) and LPS. The activation status of TE-loaded DCs was enhanced when these cells were treated with TE 90 minutes before being stimulated with LPS (TE90 DCs). More importantly, a single vaccination of mice with TE90 DCs stimulated a systemic Th1 response and conferred protection against hepatic damage induced by *F. hepatica* infection. Thus, TE90 DCs may prove to be a useful new tool for vaccination against *F. hepatica*.

Fasciolosis, a chronic disease caused by *Fasciola hepatica*, a fluke trematodes, is becoming an emerging disease in humans, with an increasing number of cases being reported in certain regions of the world [1, 2]. During its life cycle, the parasite migrates across the peritoneal cavity and penetrates host tissues, where it is then in direct contact with immune cells. Infection with *F. hepatica* is accompanied by elevated immunoglobulin (Ig) E levels, eosinophilia, and immune responses associated with the Th2 subtype [3]. In addition, a number of works have demonstrated a relation between the development of significant levels of protection against *F. hepatica* and a specific Th1 response [4–6].

In recent years, several vaccine strategies using the adoptive transfer of antigen (Ag)–pulsed dendritic cells (DCs) have been shown to mediate protection against different types of pathogens [7]. In this way, properly conditioned ex vivo DCs mediate complete and sustained protection against murine leishmaniasis [8, 9]. However, there is still no information available about the role of DCs as a vaccination tool against *F. hepatica* infection.

During infection with *F. hepatica*, macrophages and DCs from the peritoneal cavity show a down-regulation in the expression of major histocompatibility complex (MHC) class II and costimulatory molecules, a fact associated with the impaired ability of these cells to induce an effective Th1 cell response [10]. Similarly, excretory secretory products from the parasite as well as the tegumental antigens are unable to induce classical maturation of DCs [11, 12]. Furthermore, excretory secretory products–loaded DCs exhibit a capacity to drive Th2

The Journal of Infectious Diseases

and Treg polarization of CD4+ cells from DO11.10 transgenic mice [12]. These data suggest that, because of the tolerogenic characteristic of *F. hepatica* Ags, DCs loaded with these Ags are not able to achieve a high enough status of activation to drive Th1 polarization of T cells.

Because mature DCs are efficient in promoting a Th1 response [13, 14], in the present study we demonstrated that the DCs exposed to the *F. hepatica* Ags 90 minutes before the addition of lipopolysaccharide (LPS) (TE90 DCs) achieved a fully mature phenotype. The vaccination of mice with TE90 DCs was able to induce in vivo mechanisms that led to the prevention of the hepatic damage characteristic of *F. hepatica* infection. Thus, TE90 DCs may prove to be a useful new tool in the control of *F. hepatica* infections.

METHODS

Animals and Total Extract (TE) Preparation
Six- to 8-week-old inbred female BALB/c (H-2K^d) mice were purchased from National University of Litoral (Santa Fe, Argentina). The Institutional Experimentation Animal Committee (authorization no. 15-01-44195) approved animal handling and experimental procedures. The TE of *F. hepatica* (which is a somatic extract) was obtained from mature flukes of infected bovine livers, as previously described [15], with some modifications. To remove endotoxin contamination, a sample of TE was applied to a column containing detoxi-gel endotoxin-removing gel (Pierce Biotechnology). Residual LPS present in TE, determined by Limulus amebocyte lysate test (Endosafe Times; Charles River Laboratories) was similar to that present in complete Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich), with 10% heat-inactivated endotoxin-free and fetal calf serum (Gibco; Invitrogen), 50 μmol/L 2-mercaptoethanol (Sigma-Aldrich), and 50 μg/mL gentamicin (Gibco).

DC Generation and Stimulation
The DCs were generated as previously described [13], with slight modifications. The cells were seeded at 2×10^5 cells/mL in 10 mL of complete RPMI 1640 medium supplemented with 7.5 % of supernatant from granulocyte-macrophage colony-stimulating factor–producing J558 cells (20 ng/mL final concentration in the plate). To activate the DCs, 4×10^5 cells were treated with TE (80 μg/mL), 10 μg/mL of LPS extracted from *Escherichia coli* (serotype 055:B5; Sigma-Aldrich), or LPS plus TE. Alternatively, DCs were pre-stimulated for 90 minutes with TE, followed by 16 hours of culture, with 10 μg/mL of LPS (TE90).

Fluorescence-Activated Cell-Scanning (FACS) Analysis
After the treatments, the expression of surface molecules on DCs was quantified by flow cytometry using fluorescein isothiocyanate (FITC)– or PE-conjugated Ab (CD11c, I-A^d, I-A^b, and CD40), all purchased from BD PharMingen. Samples were collected using a flow FACSCanto II (BD Bioscience), and data were analyzed using WinMDI 2.8 (Scripps Research Institute).

Cytokine Measurement
Cytokines were detected in culture supernatants using capture enzyme-linked immunosorbent assay (ELISA). Interferon (IFN) γ (Biosource); interleukin (IL) 10, IL-5, IL-4, and IL-12p70 (eBioscience); and tumor necrosis factor (TNF) and IL-6 (BD Pharmingen) were used as paired monoclonal antibodies in combination with recombinant cytokine standards. Assays were performed according to the manufacturer's guidelines. IL-12 (p40/p70) was detected by intracellular staining with phycoerythrin (PE)–conjugated monoclonal antibody (BD Pharmingen).

Vaccination of Mice
DCs from BALB/c mice were treated for 18 hours as described above, washed twice with phosphate-buffered saline (PBS), and injected intraperitoneally in BALB/c mice (1×10^6 cells/mice). After 7 days, spleens were removed, and suspensions of splenocytes were adjusted to 2.5×10^6 cells/mL and cultured in 96-well plates in medium alone or in the presence of 20 μg/mL of TE for 72 h. Supernatants from cultures were collected after 48 hours from IFN-γ and after 72 hours for IL-4 and IL-5 and were measured using a capture ELISA.

Vaccination of Mice and Monitoring of Liver Damage and Peritoneal and Splenic Cells Response
BALB/c mice were injected intraperitoneally once with the DCs (1×10^6 cells/mice) treated as described above. After a week, all animals were orally infected with 5 *F. hepatica* metacercariae (Baldwin Aquatics) per animal. After 2 weeks of infection, the mice were necropsied, and the livers, spleens, and peritoneal cells (PCs) were removed. Paraffin sections were cut from the livers and were stained with hematoxylin-eosin. At least 5 livers were included in each experimental group. Spleens were also removed, and splenocytes (2.5×10^6 cells/mL) were cultured in complete RPMI medium in the presence of 20 μg/mL of TE for 3 days. Supernatants were collected, and cytokines were measured by ELISA. PCs were harvested by washing the peritoneal cavity with 10 mL of PBS. Uninfected animals were used as a control group. The cells were washed and resuspended at 2.5×10^6 cells/mL and were cultured for 48 hours or 72 hours in the presence or absence of 20 μg/mL of TE. Additionally, PCs were cultured for 24 hours with LPS (10 μg/mL). Culture supernatants were collected, a capture ELISA was used for IL-12p70 detection, and the Griess reaction was used to determine nitric oxide (NO) production. To analyze the phenotype of the PC population, staining was performed of 1×10^6 cells/mL with antigen

presenting cell–labeled anti-CD3 (eBioscience) and PE-labeled anti F4/80 (Caltag Laboratories), PE-labeled anti-B220 (BD Pharmingen), and rat FITC-labeled anti Gr-1 (Ly6G/Ly6C) clon RB6-8C5, IgG2b isotype (BD Pharmingen).

Statistical Analysis

The measurement of cytokines in splenocyte supernatant was performed for 6 wells per animal with 3–5 animals being included in each group, and the data expressed as mean values ± standard deviation. The Student t test was used for all statistical comparisons; P values < .05 were considered to be statistically significant.

RESULTS

Treatment of DCs With *F. hepatica* Ags plus LPS Induces a Semimature Phenotype

As previously demonstrated with tegumental and excretory secretory *F. hepatica* Ags [11, 12], the somatic extract of *F. hepatica* (TE) was unable to promote classical DC maturation. DCs stimulated with TE increased MHC class II expression, but not CD40 expression, TNF, IL-12p40/p70 or IL-10 production, compared with unstimulated cells (control DCs) (Figure 1A). However, the simultaneous exposure of DCs to TE plus LPS (T/L) induced an increase in the percentage of cells expressing CD40 (3.2% vs 7.6%) (Figure 1A) as well as a significant increase in the TNF production and IL-12p40/p70–positive

cells, compared with control or TE-DC groups (Figure 1B). Nevertheless, in spite of T/L treatment increasing the production of the inflammatory cytokines TNF and IL-12p40/p70, the bioactive molecule necessary to drive the Th1 polarization the IL-12p70 [16] was undetectable for this treatment (Figure 2A). On measuring the cell viability using Annexin V and the 7-AAD assay, DC treated with LPS or T/L showed similar profiles for both live and apoptotic cells (Supplementary figure 1A), thus ruling out a toxic effect of TE on LPS-matured DCs.

These results demonstrate that, although the treatment with TE plus LPS was able to induce the production of TNF and IL-12p40/p70 and increase the expression CD40, it was less capable of producing the bioactive IL-12p70 necessary to induce a Th1 response.

Treatment of DCs With TE Before Being Stimulated With LPS Results in a Fully Mature Phenotype

Given that a semimature phenotype was achieved when DCs were simultaneously treated with T/L, we decided to test different times of TE addition to a DC culture before LPS stimulation to increase their maturation status. Treatment of DCs with TE 90 minutes before being stimulated with LPS (TE90 DC) significantly enhanced the levels of IL-12p70, TNF, and IL-6, compared with those released by T/L-treated DC (Figure 2). However, similar levels of IL-10 production and CD40 expression were found with both treatments (Figure 2
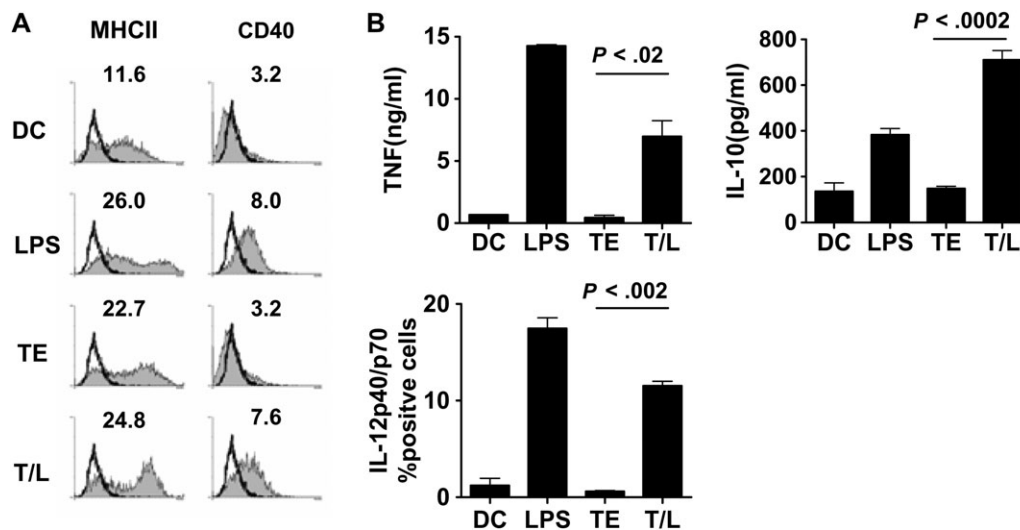

Figure 1. Total extract (TE) plus lipopolysaccharide (LPS) treatment induced a semimature phenotype in dendritic cells (DCs). DCs were treated for 18 hours with medium alone (DC), LPS, TE, or a combination of TE plus LPS (T/L). *A*, cell surface expression of major histocompatibility complex (MHC) class II and CD40 is shown by the filled histograms, with cells stained with control antibodies (Abs) being indicated by the empty histograms. Values represent the percentage of positive cells. A fluorescence-activated cell-scanning analysis was performed by gating cells on the basis of CD11c⁺ cells. *B*, tumor necrosis factor (TNF) and interleukin (IL) 10 concentrations were measured in culture supernatants by enzyme-linked immunosorbent assay, with data shown as mean values ± standard deviation of triplicate wells, and the values are representative of 3 cultures per group. Intracellular expression of IL-12 (p40/p70) on DCs, stimulated as described above and incubated with Golgi Stop for the last 4 hours of culture. Cells were washed, stained for CD11c, fixed, permeabilized, and stained with anti-IL-12(p40/p70) Ab as described in Materials and Methods. Values are given as the percentage of total CD11c⁺ IL-12–producing cells. A graphical representation showing the average results of single determinations of 2 independent experiments is presented.
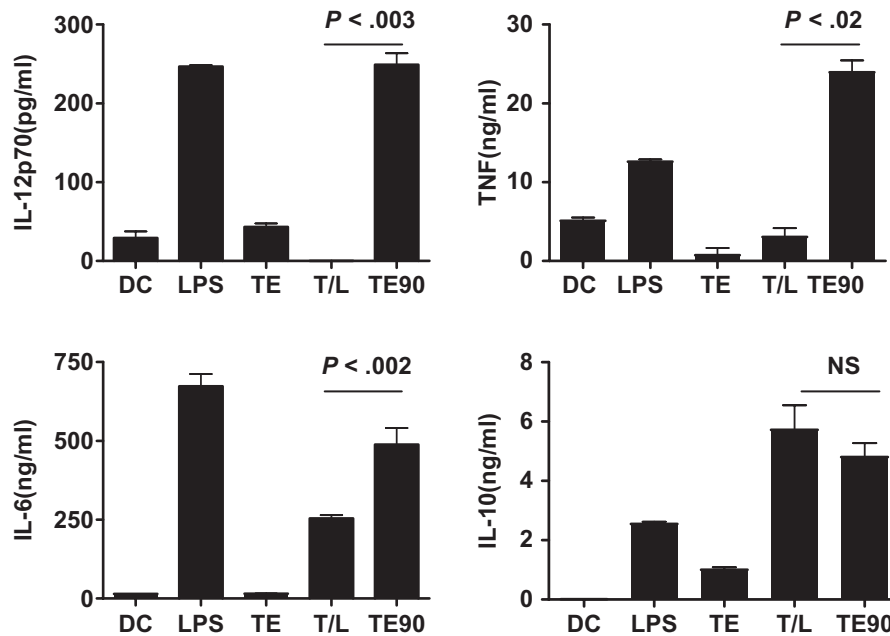
Figure 2. A delayed administration of lipopolysaccharide (LPS) to total extract (TE)–treated dendritic cells (DCs) increased the activation status of these cells. DCs were cultured with medium (DC), LPS, TE, or TE plus LPS (T/L) or were stimulated for 90 minutes with TE followed by 16 hours of culture with LPS (TE90). The supernatants of all cultures were collected at 18 hours, and the interleukin (IL) 12p70, tumor necrosis factor (TNF), IL-6, and IL-10 concentrations were measured in culture supernatants by enzyme-linked immunosorbent assay. Data are means ± standard deviation of quadruplicate wells per group and are representative of 3 independent experiments.

and data not shown, respectively). Because it has been demonstrated that the delayed administration of LPS to DC cultures induces a high secretion of IL-12 but minimal IL-10 secretion [17], we wondered whether the high status of activation achieved in TE90 DC was only attributable to the simple delay in the addition of LPS. Moreover, proteins from TE involved in the inhibition of LPS-induced DC maturation may be cleaved by proteases contained in TE [18]. To investigate these hypotheses, we cultured TE or TE plus LPS in medium for 90 minutes before adding these stimuli to DC cultures (TE$_{DC90}$ and T/L$_{DC90}$, respectively) (Supplementary figure 1B). After 18 hours of culture, T/L$_{DC90}$ produced significantly lower levels of proinflammatory cytokines than did the DCs treated with LPS from the beginning of the culture, suggesting that neither a simple delay in the addition of LPS nor a loss of suppressor activity of TE had happened during the TE90 treatment.

Taken together, our data show that the treatment of DCs with TE prior to Toll-like receptor ligand addition generated a fully mature phenotype in these cells, with even higher levels of some proinflammatory cytokines occurring than those produced by the treatment of DCs with the Toll-like receptor ligand alone.

Vaccination With TE90 DCs Induces a TE-Specific Th1 Response in Mice

To analyze the ability of TE90 DCs to prime an *F. hepatica* T cell response in vivo, BALB/c mice were vaccinated with these cells.

Seven days later, the splenocytes were restimulated with TE, and the secretion of Th1- and Th2-type cytokines was analyzed. Cultures of splenocytes from immature (DC), TE, or T/L DC recipient mice were used as controls. As shown in Figure 3, vaccination with TE90 DCs was able to induce a systemic TE-specific Th1 response, with a high level of IFN-γ and minimal IL-5 after TE restimulation (Figure 3). As expected, TE DC vaccination induced a systemic TE-specific Th2 response (low IFN-γ and high IL-5 levels) (Figure 3). Therefore, the activation status achieved by TE90-treated DCs seems to be crucial to produce a bias toward a specific Th1 response in vivo.

TE90 DC Vaccination Protects Against *F. hepatica* Infection–Induced Hepatic Damage

During fasciolosis, it has been demonstrated that the major effector mechanisms, which occur in the peritoneal cavity, reduce the number of flukes able to reach the liver. For this reason, the evaluation of hepatic damage is a measure of the intensity of the killing mechanisms that take place in the peritoneum and is helpful in assessing the protection induced by vaccines against an *F. hepatica* challenge [19–21]. Therefore, we determined whether TE90 DCs could act as a cellular vaccine against *F. hepatica* infection by measuring the prevention of hepatic damage in vaccinated mice. BALB/c mice were vaccinated with untreated DC or with LPS-, TE-, T/L-, or TE90-treated DCs or with PBS as a control and were then infected as described in Methods, with the livers being taken for histopathological
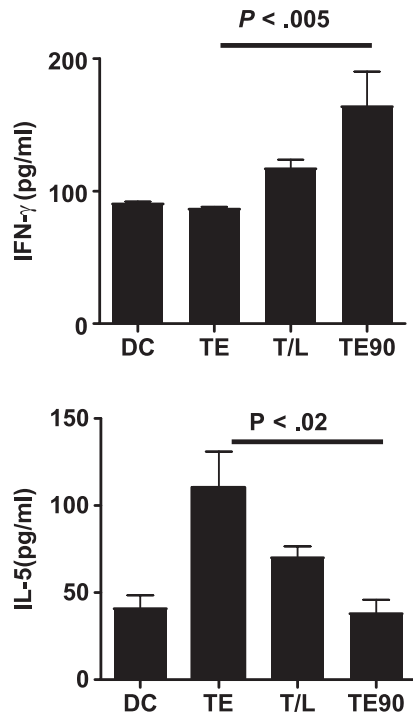
Figure 3. A single injection of dendritic cells (DCs) stimulated for 90 minutes with total extract (TE) followed by 16 hours of culture with LPS (TE90 DCs) induced an increase in interferon (IFN) γ production by splenocytes of injected mice. BALB/c mice were vaccinated with 1×10^6 DCs treated with medium (DC), TE, or TE plus lipopolysaccharide (LPS) for 18 hours or with TE90 DCs. After 7 days, mice were sacrificed, and single-cell suspensions of the spleens were cultured in the presence of TE (20 μg/mL). Supernatants were collected at 72 hours of culture, and the levels of IFN-γ and interleukin (IL) 5 were determined by enzyme-linked immunosorbent assay. Data represent the mean values $\pm$ standard deviation of triplicate wells of splenocytes cultures from 4–5 mice per group and are representative of 3 experiments with similar results. P represents a significant difference between TE- and TE90-treated DC-recipient mice.

Figure 4. Vaccination with dendritic cells (DCs) stimulated for 90 minutes with total extract (TE) followed by 16 hours of culture with LPS (TE90 DCs) prevented hepatic damage in *Fasciola hepatica*–infected mice. BALB/c mice were vaccinated with DCs treated with medium alone (DC), lipopolysaccharide (LPS), TE, or TE plus LPS (T/L) for 18 hours or with TE90 DCs or vehicle phosphate-buffered saline (PBS) as a control. Seven days after vaccination, all animals were orally infected. Then, 14 days later, the animals were sacrificed, and the livers were removed, fixed with 10% of phormaldehyde in PBS, and stained with hematoxylin-eosin. Liver sections from untreated BALB/c mice were included as a normal control (−). Thin arrows show inflammatory infiltrates, thick arrows indicate young flukes, and asterisks denote fibrotic areas. Original magnification, 400×. The results are representative of at least 5 animals per group, and the experiment was repeated twice.

analysis. Although the liver parenchyma of control uninfected animals showed a normal histological pattern, livers from infected mice treated with the vehicle alone (PBS) displayed a wide range of hepatic lesions and inflammatory changes (Figure 4). The infected mice presented migratory tunnels containing young flukes of different sizes and cellular debris surrounded by slight or abundant leukocyte infiltrates (Figure 4). In addition, livers from both untreated and LPS-treated DC recipients showed extensive signals of damage, including widespread areas of hemorrhage and the presence of abundant immune cell infiltrates that replaced wide areas of hepatic parenchyma (Figure 4). In a similar way, mice receptors of TE- or T/L-treated DCs revealed severe hepatic injury, demonstrated by abundant inflammatory infiltrates and the presence of small flukes in the TE group (Figure 4) and fibrosis in the T/L group (Figure 4). In contrast, the livers of mice vaccinated with TE90-treated DCs showed a more preserved microarchitecture, with
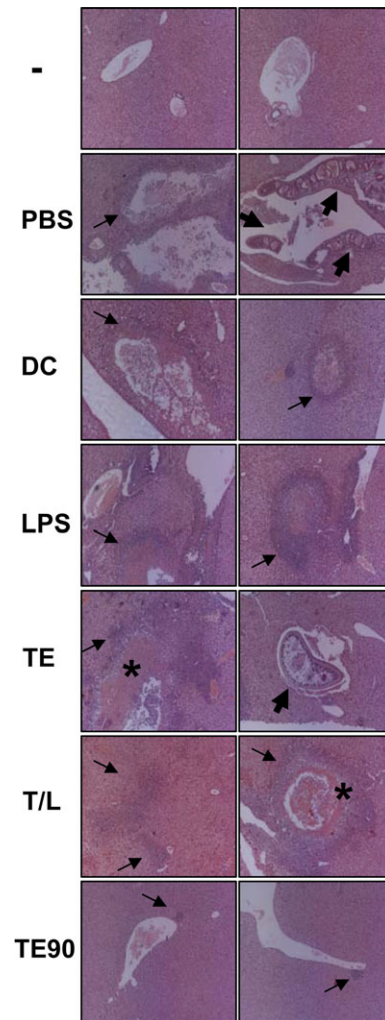
only minor perivascular infiltrate foci being observed (Figure 4). Results showing the histopathological analysis of the livers are summarized in Table 1.

Splenocytes from mice that had been vaccinated with differentially treated DCs were restimulated with TE, and the secreted cytokines were analyzed. Splenocytes from mice injected with TE90 DC produced a significant increase in IFN-γ levels, compared with all other treatments, and a decrease in

Table 1. Histopathological Lesions of Livers From Mice Vaccinated With Differentially Activated Dendritic Cells (DCs)

Treatment	NEC	HF	T	INFF	GR
PBS	+++	+	+++	++++	+++
DC	+++	++	+++	+++	++
LPS	++++	+	++	+++	+++
TE	+++	+	++	++++	++
T/L	+++	++	++	+++	+++
TE90	+	−	−	+	+

Abbreviations: GR, granulomas; HF, hemorrhagic foci; INFF, inflammatory foci; LPS, lipopolysaccharide; NEC, necrosis; PBS, phosphate-buffered saline; T, tracts; TE, total extract; TE90, DCs stimulated for 90 minutes with TE followed by 16 hours of culture with LPS; T/L, TE plus LPS; −, none; +, mild; ++, moderate; +++, severe; ++++, very severe.

IL-10 production (Figure 5). In contrast, splenocytes from mice injected with TE DC produced large amounts of IL-4 and IL-5 (Figure 5). These results suggest that TE90 DCs were able to induce in vivo mechanisms that led to the prevention of hepatic damage characteristic of *F. hepatica* infection. In addition, vaccination with TE90 DCs was able to stimulate a systemic primary Th1 response, which was maintained during *F. hepatica* infection.

Vaccination of Mice With TE90-Treated DC Induced the Recruitment of Macrophages With a Decrease in Eosinophils in the Peritoneal Cavity of Infected Mice

Next, we evaluated the cellular recruitment and the immune response in PCs of vaccinated and infected mice. Mice were injected intraperitoneally once with differentially treated DCs and then infected with the parasite. At 14 days after infection, PCs were analyzed by cytometry and gated into 3 regions (R1, R2, and R3; Figure 6A). Among the different treatments, vaccination with TE90 DCs induced a substantial increase in the percentage of F4/80^{high}Gr1$^-$ cells (defined as macrophages) [22] of the total live peritoneal cells in R1 (Figure 6A), whereas in R2 this treatment induced a reduction in the percentage of F4/80^{int}Gr1$^+$ cells (defined as eosinophils) [22] (Figure 6A). In contrast, the injection of TE-treated DCs induced the lowest percentage of macrophages and the highest percentage of eosinophils, compared with all the other treatments (Figure 6A). Cells in R3 were of types B (B220+) and T (CD3+) lymphocytes, with no changes being observed in these populations for the differential DC treatments (data not shown). Next, PCs were stimulated with TE for 48 h, and the production of IFN-γ and IL-4 was analyzed. The PCs from TE90 DC recipient mice showed the highest ratio of IFN-γ/IL-4 (Figure 6B). PCs from TE DC vaccinated mice showed the lowest ratio of IFN-γ/IL-4 but the highest IL-5 production (Figure 6B), which correlated with the presence of eosinophils detected by cytospin (Figure 6C).

We then evaluated whether PC from TE90 DC vaccinated and infected mice were able to response to a microbial stimulus such

as LPS. Thus, PCs were stimulated with LPS, and the NO and IL-12p70 production were determined. Although PCs from LPS DC, T/L DC, or TE90 DC recipient mice had the same levels of NO production, PC from TE90 DC recipient mice produced the highest level of IL-12p70 (Figure 6D). Thus, these data highlight the fact that TE90 DCs are capable of changing the cellular type recruited in the peritoneal cavity of *F. hepatica*–infected mice and of increasing the peritoneal cell's ability to respond to inflammatory stimuli, such as LPS.

DISCUSSION

This study describes a novel strategy to induce a highly effective immunity against an extracellular pathogen using fully mature DC treated with *F. hepatica* Ags before the addition of LPS. We have demonstrated that the prevention of hepatic damage by the vaccination with a single dose of TE90 DC before *F. hepatica* infection is correlated with the development of a specific and systemic Th1 response.

Furthermore, vaccination with TE90 DCs induced the recruitment of activated macrophages in the peritoneal cavity of infected mice, which is an important site for the immunological killing of migrating parasites [23], and this might suggest the involvement of these cells in effector mechanisms against this parasite.

PCs from mice injected with DCs stimulated with LPS, T/L, or TE90 DCs all secreted similar amounts of NO, although only TE90 DC were capable of inducing protection in the vaccinated mice. In addition to the increased amount of NO and IL-12p70 production that occurred upon transfer of TE90 DCs, recipient mice demonstrated the highest ratio of IFN-γ/IL-4 in PCs, which reflects the predominance of Th1 rather than Th2 cytokines. Overall, this may contribute to an inflammatory environment at a migratory site of the parasite, and a reduced number of flukes able to reach the liver might explain the reduced pathology observed. Furthermore, TE90 DC vaccination decreased the recruitment of eosinophils into the peritoneal cavity of injected mice and also the ability of PCs to secrete Th2 cytokines.

Although a protective role has been assigned to eosinophils against helminth parasites [24], an increased percentage of this population in PCs from mice injected with TE-treated DCs did not correlate with the prevention of hepatic damage. Recent evidence shows that excretory-secretory products from the parasite induce early apoptosis in vitro and in vivo, with eosinophils having the typical characteristics of apoptotic cells observed during experimental infection with the parasite [25]. However, we cannot rule out the involvement of eosinophils in effector mechanisms against the parasite, because the cellular activation profile of the peritonel cells in TE90 DC receptor mice was different from that observed in mice that had only been infected.
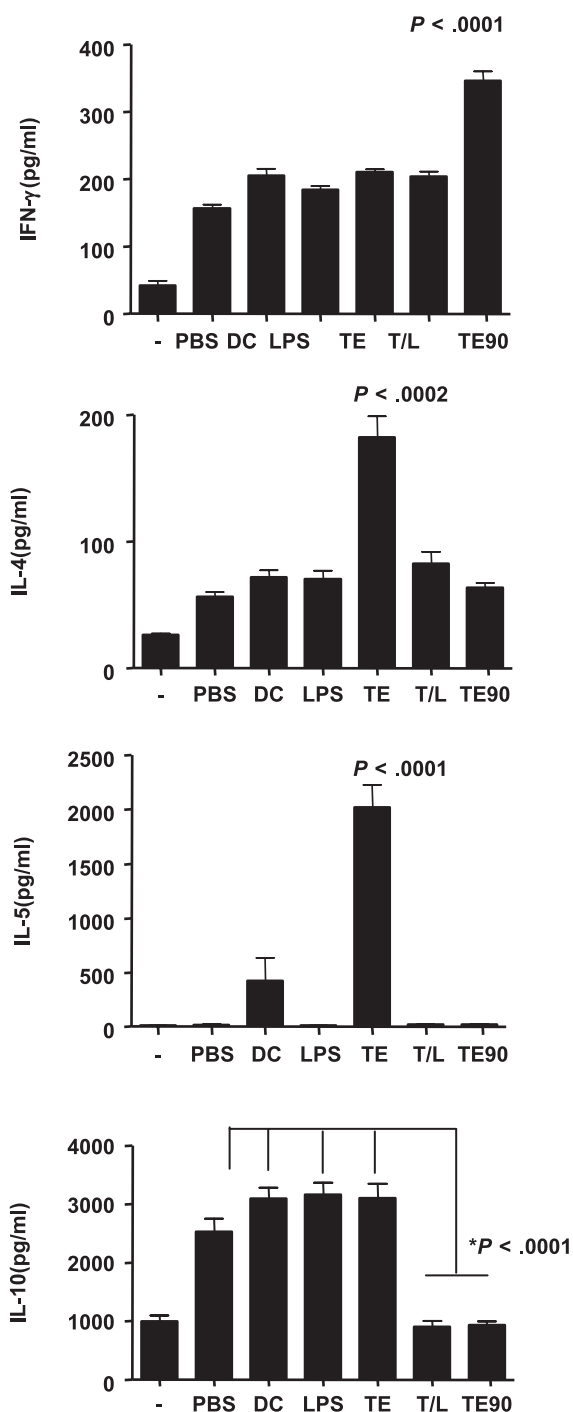
Figure 5. Vaccination with dendritic cells (DCs) stimulated for 90 minutes with total extract (TE) followed by 16 hours of culture with LPS (TE90 DCs) induced a Th1 response in splenocytes of infected mice. BALB/c mice were vaccinated with 1×10^6 DCs treated with medium (DC), lipopolysaccharide (LPS), or TE plus LPS for 18 hours or with TE90 DCs or vehicle phosphate-buffered saline (PBS) as a control. After 7 days, mice were infected with the parasite. Then, 14 days later, the mice were killed, and single-cell suspensions of the spleens were cultured in the presence of TE (20 μg/mL). Splenocytes from untreated BALB/c mice were included as normal controls (−). Supernatants were collected at 72 hours of culture, and the levels of interferon (IFN) γ, interleukin (IL) 10, IL-4, and IL-5 were

Despite histological analysis of infected livers having demonstrated the presence of flukes, thereby confirming the efficiency of infection, spleen cells from infected mice (PBS group) produced low levels of IFN-γ and IL-4 in response to *F. hepatica* Ags, in contrast with the Th2-polarized response reported by other authors [10, 26]. These differences, however, could be explained by the time when the spleens were collected, because 2 weeks after infection was probably not long enough to induce a Th2 bias. Nevertheless, Th2 polarization was in fact achieved in TE-treated DC-receptor mice, but this did not correlate with a reduction in hepatic damage, in agreement with previous reports showing the involvement of the Th1 response in protective mechanisms against the parasite, and suggesting that the well-described Th2 cytokine expression following *F. hepatica* infection [27, 28] may benefit the parasite more than the host. Moreover, TE90 treatment was efficient to inhibit IL-10 production by splenocytes after TE restimulation, which is a cytokine that has been described to increase during infection [10]. Thus, this treatment promotes a Th1 response without the presence of Th2 or IL-10.

Different Ags from *F. hepatica,* such as TE, tegumental Ag (Teg) [11], and excretory-secretory products [12], are not capable of providing signals to DC to induce maturation. However, they are able to inhibit DC maturation initiated by TLR signals. In the present study, the inhibitory effect exerted by TE on LPS-activated DC was reverted when the treatment was performed prior to LPS stimulation (TE90). Our data are supported by a previous work that showed a modulatory effect of LPS on DCs, where a delayed addition of LPS to DC cultures promoted the secretion of an IL-12p70 response [17]. However, the activation status achieved in TE90 DCs cannot be attributable only to this fact, because the simultaneous addition of TE plus LPS after 90 minutes to DC cultures (T/L$_{DC90}$) did not enhance DC maturation. Moreover, based on these observations, it is possible to assume that the addition of any *F. hepatica* Ag previous to the exposure of LPS to DC culture could induce an increased amount of IL-12p70. However, data from Hamilton et al [11] showed that preincubation of Teg Ags before LPS was added to DCs did not improve the IL-12p70 production, which suggests that the modulation of DC maturation depends on the time of the exposure to the stimulation signal as well as on the composition of the Ags added to the cultures.

The differences between our data and those published by Hamilton et al [11] may be based on the type of Ags used in

determined by enzyme-linked immunosorbent assay. Data represent the mean values ± standard deviation of 6 wells of splenocyte cultures from 4–5 mice per group and is representative of 3 experiments with similar results. *P* represents a significantly different value between TE90-treated DC-recipient mice and the rest of the groups. *P* means a significantly different value between TE90- or T/L-treated DC-recipient mice and the rest of the groups.
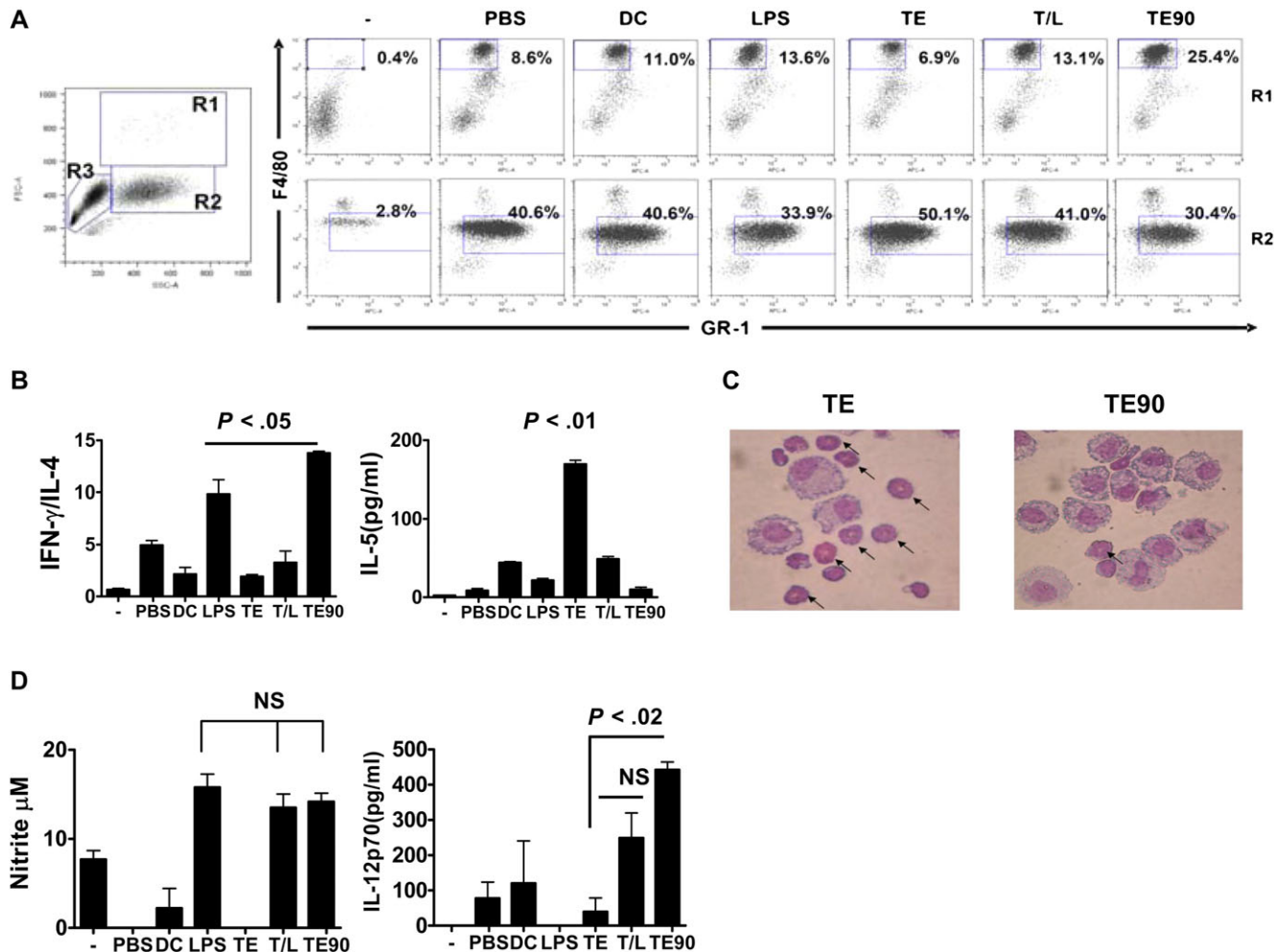
Figure 6. Activated macrophages are recruited to the peritoneal cavity of mice vaccinated with dendritic cells (DCs) stimulated for 90 minutes with total extract (TE) followed by 16 hours of culture with LPS (TE90 DCs) and infected with the parasite. BALB/c mice were vaccinated with DCs treated with medium alone (DC), lipopolysaccharide (LPS), TE, or TE plus LPS (T/L) for 18 hours or with TE90 DCs or phosphate-buffered saline (PBS) as control. Seven days after vaccination, all animals were infected with the parasite. Then, 7 days later, the animals were killed, and peritoneal cells (PCs) were isolated from *Fasciola hepatica*–infected and untreated control mice by peritoneal lavage. *A*, cells were stained with antibodies (Abs) against the cell surface marker as indicated, and fluorescence-activated cell-scanning analysis was performed. Dot plots represent the regions according to forward versus side scatter, and the cells were gated into 3 regions (R1, R2, R3; *left panel*). The number of F4/80^{high}Gr1$^-$ cells in R1 and F4/80^{low}GR1$^+$ in R2 divided by the total number of live cells is shown as a percentage. Data are representative of 2 experiments with similar results. *B*, PCs were cultured for 48 hours with TE (20 μg/mL) for interferon (IFN) γ detection or 72 hours for interleukin (IL) 4 or IL-5. The culture supernatants were collected, and cytokines were detected by enzyme-linked immunosorbent assay (ELISA). The graph represents the ratio between IFN-γ versus IL-4 concentrations or IL-5 production. PCs were collected from 4 to 5 mice and pooled, with the values representing the mean value ± standard deviation of 6 wells from the pool of PCs and data being representative of 2 experiments. *C*, representative field for PCs stained with May Grunwald Giemsa from TE- or TE90-treated DC-recipient mice, with the arrows showing eosinophils. Original magnification, 400×. *D*, PCs were cultured for 24 hours with LPS (10 μg/mL) for IL-12p70 and nitrite detection. The culture supernatants were collected, and cytokines were detected by ELISA, with 100 μL of supernatant being mixed with 200 μL Griess reactive to quantify the nitrites. Each experiment was done in triplicate and repeated twice.

the experiments. Although TE is a complex mixture of Ags that have shown cross-reactivity with proteins present in excretory-secretory products or Teg Ags [29], some other proteins appear to be exclusive to this preparation [30], which could have a global effect on DC activation different from that observed with excretory-secretory products or Teg Ags.

In conclusion, it can be clearly seen that DC-pulsed ex vivo with parasite Ags and LPS achieved a maturation status that enabled these cells to confer resistance to infection with

trematode helminth by the priming of a systemic Th1 response, which probably contributed to the killing of the parasite in the peritoneal cavity or in the early stages of migration through the liver.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit

Notes

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