Leishmania-Infected MHC Class II\textsuperscript{high} Dendritic Cells Polarize CD4\textsuperscript{+} T Cells toward a Nonprotective T-bet\textsuperscript{+} IFN-\gamma\textsuperscript{+} IL-10\textsuperscript{+} Phenotype

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Leishmania-Infected MHC Class II\textsuperscript{high} Dendritic Cells Polarize CD4\textsuperscript{+} T Cells toward a Nonprotective T-bet\textsuperscript{+} IFN-γ\textsuperscript{+} IL-10\textsuperscript{+} Phenotype

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A differential behavior among infected and bystander dendritic cells (DCs) has been explored in different infection models. We have analyzed both populations sorted on contact with visceral Leishmania infantum on a susceptible mice model evaluating the subsequent repercussions on adaptive immune response. Our results demonstrate a clear dichotomy between the immunomodulatory abilities of bystander and infected DCs. The bystander population presents increased levels of IL-12p70 and costimulatory molecules being capable to induce CD4\textsuperscript{+} T cell activation with immune protective capabilities. In contrast, infected DCs, which express lower costimulatory molecules and higher levels of IL-10, promote the development of Leishmania Ag-specific, non-protective T-bet\textsuperscript{+} IFN-γ\textsuperscript{+} IL-10\textsuperscript{+} CD4\textsuperscript{+} T cells with an effector phenotype. This specific polarization was found to be dependent on IL-12p70. Splenic infected DCs recovered from chronic infected animals are similarly capable to polarize ex vivo syngeneic naïve CD4\textsuperscript{+} T cells toward a T-bet\textsuperscript{+} IFN-γ\textsuperscript{+} IL-10\textsuperscript{+} phenotype. Further analysis revealed that only MHC class II\textsuperscript{high}-infected DCs were responsible for this polarization. The adoptive transfer of such polarized CD4\textsuperscript{+} T cells facilitates visceral leishmaniasis in BALB/c mice in a clear contrast with their counterpart generated with bystander DCs that significantly potentiate protection. Further, we demonstrated that CD4\textsuperscript{+} T cells primed by infected DCs in an IL-10 free system, thus deprived of T-bet\textsuperscript{+}IL-10\textsuperscript{+} T cells in the maintenance of infection. Overall, our results highlight novel subversion mechanisms by which nonprotective T-bet\textsuperscript{+}IFN-γ\textsuperscript{+} IL-10\textsuperscript{+} T cells are associated with chronicity and prolonged parasite persistence. *The Journal of Immunology, 2013, 191: 000–000.

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he intracellular protozoan Leishmania can produce a wide spectrum of clinical manifestations, ranging from mild cutaneous to chronic visceral pathologies, depending on the species involved. The outcome of these infections is largely dependent on host immune responses because fatalities often occur in the absence of treatment. In all cases, a protective response is associated with the development of IFN-γ\textsuperscript{+}–producing Th1 immunity, which leads to the elimination of the parasites within infected cells (1). Although IFN-γ secretion was shown to be essential for optimal parasite elimination, high levels of IFN-γ are found during active disease in visceral leishmaniasis (VL) patient’s plasma, spleen, and bone marrow, which returned to basal levels after successful treatment (2). Nevertheless, the increased IFN-γ levels were always associated with concomitant accumulation of IL-10 (2, 3), which play a dominant immunosuppressive role during VL (4, 5).

Among the different cell types that secrete IL-10 (6), recent evidences have identified CD4\textsuperscript{+}CD25\textsuperscript{+}IL-10\textsuperscript{+} Th1 cells as the critical source of IL-10 that suppresses the protective immune response against Leishmania spp. and control Toxoplasma spp. or Plasmodium–induced immunopathology (7–10). In an experimental resistant murine model of VL, a CD11c\textsuperscript{high} dendritic cell (DC)–driven expansion and/or maintenance of IL-10–producing Th1 was observed in the phase to chronicity (10, 11). These IFN-γ\textsuperscript{+}IL-10\textsuperscript{+} double-positive cells were found to arise from IFN-γ\textsuperscript{+}–producing Th1 cells and are often observed in both pathogen-
specific and nonspecific T cell clones, which argue in favor of nonspecific bystander T cell activation (9). Several mechanisms have been proposed to regulate IL-10 production on Th1 cells. Among those, TGF-β, IL-6, IL-21, as well as cytokines belonging to the IL-12 family and ICOS–ICOS ligand interaction were shown to be involved in the induction of IL-10 (12). DCs are specialized APCs that play a crucial role in driving adaptive immune responses. Intracellular pathogens are known to counteract and subvert host immune DCs and effector cell functions using distinct strategies that include interference with Ag processing/presentation, manipulation of costimulatory signals and cytokines, immunosuppression, and increased host cell viability, among others (13). Given that in any chosen model not all of the DCs are infected, one might expect a differential behavior among infected and bystander DCs. Carvalho et al. (14) have reported a functional DC dichotomy after interaction with mucocutaneous Leishmania species, where nonactivated infected DCs produced high levels of TNF-α responsible for the increased immunopathology, whereas bystander DCs exposed to soluble parasite products were activated promoting T cell responses. Considering the substantial differences between the proinflammatory response observed in mucocutaneous disease and the IL-10–driven immunopathology, whereas bystander DCs exposed to soluble parasite products were activated promoting T cell responses. Overall, we identified a cellular mechanism by which nonprotective T-bet+Ag-specific IL-10 Th1 population with effector phenotype. Moreover, we prove that only infected MHC class Ihigh (MHC IIhigh) DCs, either developed in vitro or recovered from in vivo infection, were capable to generate the Leishmania Ag-specific IL-10 Th1 population with effector phenotype. Overall, we identified a cellular mechanism by which nonprotective T-bet+IFN-γ+IL-10+ phenotype. Moreover, we prove that only infected MHC class Ihigh (MHC IIhigh) DCs, either developed in vitro or recovered from in vivo infection, were capable to generate the Leishmania Ag-specific IL-10 Th1 population with effector phenotype. Overall, we identified a cellular mechanism by which nonprotective T-bet+IFN-γ+IL-10+ phenotype. Moreover, we prove that only infected MHC class Ihigh (MHC IIhigh) DCs, either developed in vitro or recovered from in vivo infection, were capable to generate the Leishmania Ag-specific IL-10 Th1 population with effector phenotype. Overall, we identified a cellular mechanism by which nonprotective T-bet+IFN-γ+IL-10+ phenotype. Moreover, we prove that only infected MHC class Ihigh (MHC IIhigh) DCs, either developed in vitro or recovered from in vivo infection, were capable to generate the Leishmania Ag-specific IL-10 Th1 population with effector phenotype. Overall, we identified a cellular mechanism by which nonprotective T-bet+IFN-γ+IL-10+ phenotype. Moreover, we prove that only infected MHC class Ihigh (MHC IIhigh) DCs, either developed in vitro or recovered from in vivo infection, were capable to generate the Leishmania Ag-specific IL-10 Th1 population with effector phenotype. Overall, we identified a cellular mechanism by which nonprotective T-bet+IFN-γ+IL-10+ phenotype. Moreover, we prove that only infected MHC class Ihigh (MHC IIhigh) DCs, either developed in vitro or recovered from in vivo infection, were capable to generate the Leishmania Ag-specific IL-10 Th1 population with effector phenotype.

**Materials and Methods**

**Animals and parasites**

BALB/c and IL-10 (BALB/c background)–deficient mice were obtained from Instituto de Biologia Molecular e Celular (IBMC, Porto, Portugal) animal facilities. Animal care was in accordance with institutional guidelines. All experiments were approved by and conducted in accordance with the IBMC INEB Animal Ethics Committee and the Portuguese National Authority for Animal Health (Direccio-Geral de Alimentacion e Veterinaria) guidelines. A cloned line of virulent *L. infantum* (MHOM/MA/67/ITMAP-263) was maintained by weekly subpassages in complete RPMI 1640 (RPMLc) supplemented with 10% heat-inactivated FBS (PCS; Lonza), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-Hepes (BioWhittaker, Walkersville, MD). All the experiments were realized with promastigotes at passage four as previously defined (15).

**Abs**

Abs used include: PE/Cy7 anti-mouse CD40 Ab, clone 3/23, Isotype rat IgG2b,k; PerCP/Cy5.5 anti-mouse CD80 Ab, clone 16-10A1, Isotype Armenian hamster IgG Brilliant Violet 421 anti-mouse CD86 Ab, clone GL-1, Isotype rat IgG2a,k; Alexa Fluor 647 anti-mouse I-A^+^ Ab, clone 39-10-8, Isotype mouse (C3H/HeJ) IgG3; alkalophyocyanin anti-mouse I-A^+^E Ab, clone M5/114.15.2, Isotype rat IgG2b,k; Pacific Blue anti-mouse CD1d (CD1.1, Ly-38) Ab, clone 1B1, Isotype rat IgG2b,k; PE/Cy7 anti-mouse CD11c Ab, clone N418, Isotype Armenian hamster IgG; alkalophycocyanin anti-mouse CD11c, Ab, clone N418, Isotype Armenian hamster IgG; FITC and PE/Cy7 anti-mouse IFN-γ Ab, clone XMG 1.2, Isotype IgG1, k; PE and alkalophyocyanin anti-mouse IL-10 Ab, clone JES5-16E3, Isotype rat IgG2b,k; PerCP/Cy5.5 anti-mouse TNF-α Ab, clone MP6-XT22, Isotype rat IgG1, k; alkalophyocyanin anti-mouse IL-12/IL-23 p40 Ab, clone C15.6, Isotype rat IgG1,k; PerCP/Cy5 and purified anti-mouse CD3 Ab, clone 17A2, Isotype rat IgG2b,k; LEAF purified anti-mouse TCR β chain Ab, clone H57-597, Isotype Armenian hamster IgG; PER-anti-mouse CD69 Ab, clone 1B3F1, Isotype Armenian hamster IgG; Brilliant Violet 421 anti-mouse T-bet Ab, clone 4B10, Isotype mouse IgG1, k; V500 rat anti-mouse CD4 Ab, clone RM4-5, Isotype rat IgG2a,k; Alexa Fluor 488 anti-mouse FOXP3 Flow Kit, clone MF-14; Isotype rat IgG2b,k; PerCP anti-mouse CD25 Ab, clone PC61, Isotype rat IgG1; PE anti-mouse/human CD44 Ab, clone IM7, Isotype rat IgG2b, alkalophyocyanin anti-mouse CD62L Ab, clone MEL-14, Isotype rat IgG2b,k. All Abs were purchased from BioLegend (San Diego, CA), except for CD4-V500, which was purchased from BD (San Diego, CA). All isotype controls were obtained from BioLegend.

**Generation of bone marrow DCs and naive CD4+ T cell isolation**

Bone marrow DCs were derived as described previously (16). In brief, bone marrow from femurs and tibiae of 10- to 12-wk-old mice were flushed with RPMI 1640, using syringes and 25-gauge needles. The tissue was resuspended, and bone marrow–derived DCs (BMDCs) were obtained by seeding 5 × 10⁶ bone marrow cells in 25 ml RPMI supplemented with 50 μM 2-ME (Sigma Chemical) and 200 U/ml GM-CSF (DC media: BioWhittaker, Walkersville, MD). BMDCs were harvested and resuspended in RPMI with 2% FBS after 12 d of culture, were allowed to settle, were washed two times with PBS, and labeled with 5 μM CFSE for 10 min at 37°C with occasional shaking followed by 5-min incubation at 4°C. The parasites were then washed twice and resuspended in RPMIc before proceeding to infections.

**In vitro infection of DCs**

For in vitro infection, 12-d nonadherent BMDCs were seeded at 1 × 10⁶ cells/ml RPMIc in 24- or 6-well plates. After an overnight incubation period, CFSE-labeled *L. infantum* promastigotes were added to the culture at an infection ratio of 10:1 (parasites/cell). Parallel experiments were also performed using heat-killed (30 min at 56°C), fixed (10 min in glutaraldehyde), irradiated (500 Gy; Gamma-cell 1000 Elite) parasites or 2-μm-diameter FITC-labeled latex beads (Sigma-Aldrich) at similar coculture ratios (10 FITC-beads for 1 cell). Noninternalized parasites were removed by gently washing after 4 h of infection, and fresh RPMIc was added to the wells. Cells were fixed and analyzed by flow cytometry. The percentage of infected BMDCs was determined by flow cytometry evaluation of CFSE+ cells in a FACS Canto II cytometer and analyzed with FlowJo software (Tree Star, Ashland, OR) (17).

**Surface and intracellular staining of BMDCs**

For the analysis of surface stimulatory markers, 2 × 10⁶ BMDCs were incubated for 20 min with saturating concentrations of CD40-PE/Cy7, CD80-PerCP/Cy5.5, CD86- Brilliant Violet 421, MHC II–Alexa Fluor 647 CD1d–Pacific Blue, and CD11c-PE/Cy7. Mouse isotype controls were used when necessary. All of the Abs were obtained from BioLegend. After two washing steps with PBS/2% FBS, the cells were acquired by flow cytometry in a FACS Canto II cytometer. Cells were selected on the basis of forward scatter/side scatter values. BMDCs were gated on CD11c+CD4+CD62L−. All Ab prep was fixed and permeabilized in 50 μl of 0.5% saponin, to detect intracellular cytokines. Cells were then incubated with TNF-α–PerCP/Cy5.5, IL12p40–alkalophycocyanin,
BALB/c mice were infected i.p. with 1 and analyzed on a FACSCanto II cytometer. In some cases, Foxp3-PE was included in the labeling following bet–Brilliant Violet 421 and IL-10–allophycocyanin or isotype-matched mAbs. The intracellular levels of IFN-γ, TNF-α, IL-12p70, IL-6, IL-21, IL-22, IL-23, IL-27, and IL-10 on CD4+ T cells were measured after 72 h in the presence of 10 ng/ml IL-2. The CD4+ T cells intracellular cytokine levels were measured after 5 d of coculture. In some cases, IL-12p70, IFN-γ, TNF-α, IL-21, and fluorescent phages (104) were used as APCs. These were cultured overnight with heat-killed Leishmania promastigotes. Mice were euthanized at 24 h or 28 d postinfection and CD4+ T cells were measured after PMA/Ionomycin plus brefeldin A were added to exclude dead cells. All sorting experiments were conducted in a FACSAria using FACSDiva software. The purity of the separation was always confirmed by flow cytometry and was superior to 95%.

BMDCs and CD4+ T cell coculture

In vitro. Noninfected, infected, or bystander BMDCs (2 × 105) BALB/c or IL-10 knockout mice were cocultured with naive CD4+ T cells of similar origins at a responder/stimulator ratio: 10:1 cell ratio. CD4+ T activation was measured after 36 h of coculture by labeling CD4+ T cells with anti-CD69-PE, anti-CD107a–allophycocyanin, or anti-CD275 (ICOSL: BioLegend), anti-IL-27p28 (R&D Systems) or the respective isotype control (all at 10 μg/ml) were added to the cocultures. PMA/Ionomycin plus brefeldin was added during the last 16 h. CD4+ T cells were stained with anti-CD4-PerCP/Cy5.5 and anti-CD3e-FITC, and supernatants of BMDC–CD4+ T cell cocultures were collected after 72 h of culture for cytokine quantification by ELISA (IFN-γ and IL-10), using commercial sandwich immunoassay kits (BioLegend and BD) and following the manufacturer’s recommendations. The supernatants of sorted BMDCs were collected after 24 h of culture, and supernatants of BMDC–CD4+ T cell cocultures were collected after 72 h of culture for cytokine quantification by ELISA (IL-12p70 and IL-27), using commercial sandwich immunoassay kits (BioLegend and BD) and following the manufacturer’s recommendations.

Adaptive transfer and parasite burden

BALB/c mice were infected i.p. with 1 × 108 CFSE-labeled L. infantum promastigotes suspended in sterile PBS. Fifteen days after, 1 × 106 CD4+ cells were adoptively transferred by i.v. injection. Equal volume of PBS was injected in some groups as a control. Two weeks after receiving the CD4+ T cells, the animals were euthanized. The parasite burden in the spleen and liver was determined by dilution titration as described previously (19).

Cytokine ELISA

Spleen cells were cultured in the presence or absence of soluble Leishmania Ag (SLA: 25 μg/ml) at 37˚C under 5% CO2. The supernatants were collected after 72 h of culture for cytokine quantification by ELISA (IFN-γ and IL-10), using commercial sandwich immunoassay kits (BioLegend and BD, San Diego, CA) and following the manufacturer’s recommendations. The supernatants of sorted BMDCs were collected after 24 h of culture, and supernatants of BMDC–CD4+ T cell cocultures were collected after 72 h of culture for cytokine quantification by ELISA (IL-12p70 and IL-27), using commercial sandwich immunoassay kits (BioLegend and BD) and following the manufacturer’s recommendations.

Immunofluorescence

BMDCs were labeled with MHC II–allophycocyanin for cell sorting. After sorting, each BMDC subset was fixed in PFA 2%, and ~20,000 cells were mounted on slides with Vectashield mounting medium with DAPI (H-1200, Vector Laboratories) and imaged at room temperature using a AxioImager Z1 (Carl Zeiss, Germany) with the 63x Plan-Neofluar objective and AxioCam MR version 3.0 (Carl Zeiss, Germany). The filter set used included an excitation filter of 460/30 nm and an emission filter of 660/50 nm. The settings for contrast, brightness, pinhole, acquisition mode, and scanning time were maintained throughout the whole procedure. All images were treated using open source ImageJ (National Institutes of Health, Bethesda, MD).

Generation of bone marrow–derived macrophages and CD4+ T cell bone marrow–derived macrophage coculture

Bone marrow–derived macrophages (BMMs) were generated from the bone marrow recovered from femurs and tibiae of 10- to 12 wk-old female mice flushed with RPMI 1640, using syringes coupled with 25-gauge needles. The tissue was resuspended, and BMM were obtained by seeding 4 × 105 bone marrow cells in 400 μl DMEM supplemented with 10% of L929 supernatant as source of M-CSF for 10 d. At days 4 and 8, 10% of L929 supernatant was added to cultures. At day 9 of culture, naive CD4+ T cells were added to BMMs at a responder/stimulator 10:1 cell ratio in the presence of IL-2 (10 ng/ml) for 120 h. In some experiments, a gradient of responder/stimulator ratios was used (20:1; 10:1; 4:1; and 2:1). The intracellular levels of IFN-γ and IL-10 on CD4+ T cells were measured as depicted earlier.

TCR specificity

The AQ-specific proliferative capacity of the in vitro polarized CD4+ T cell populations was performed through Leishmania Ag presentation by APCs as previously described (18). In brief, irradiated (3000 Cgy) J774 macrophages (105) were used as APCs. These were cultured overnight with heat-killed Leishmania infantum promastigotes at a 10:1 ratio (parasite:J774; 96˚C, 30 min) and irradiated. A total of 105 sorted CFSE-labeled CD4+ T cells (CD4+ T cells polarized with infected dendritic cells [CD4 inf]), CD4+ T cells polarized with bystander DCs [CD4 byst], or CD4+ T cells polarized with noninfected DCs [CD4 CD11c], which had been polarized with the distinct BMDCs, were plated with the above and incubated in the presence of IL-2 (5 ng/ml) for 72 h at 37˚C 5% CO2. CFSE decay was measured by flow cytometry.

RNA extraction and real-time RT-PCR

Total RNA was isolated from cells with TRIzol reagent (Invitrogen, Barcelona, Spain) or RNeasy micro kit (Qiagen), according to the manufacturer’s instructions. The RNA concentration was determined by OD260 measurement using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and quality was inspected for the absence of degrading or genomic DNA contamination, using Experion RNA StdSens Chips in the Experion automated microfluidic electrophoresis system (Bio-Rad, Hercules, CA). RNA was stored in RNA Storage Solution (Ambion, Foster City, CA) at −80˚C until use. Real-time RT-PCRs were run in triplicate for each sample on a Bio-Rad MyCycler iQ5. Primers were designed using Beacon Designer software (version 7.2; PREMIER Biosoft International, Palo Alto, CA) and thoroughly tested. In brief, 200 ng total RNA was reverse-transcribed using the iScript Select cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed as described previously (17). After amplification, a threshold was set for each gene and Ct values were calculated for all samples. Gene expression changes were analyzed using the built-in iQ5 Optical system software (version 2). The results were normalized using two reference genes, HPRT-1 and GAPDH, determined with Genex software (MultiD Analyses AB, Göteborg, Sweden) as the most stable for the conditions used.

Statistical analysis

The results are presented as means ± SD, and the statistical difference between two groups was determined by the two-sided unpaired Student t test. For multiple group comparisons, the one-way ANOVA test with

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a Bonferroni multiple-comparison posttest was used. The tests were performed using GraphPad Prism (version 5.02; GraphPad Software, San Diego, CA). Statistically significant values are as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Maturation status and cytokine expression profile of bystander and L. infantum–infected BMDCs impact differently CD4 T cell activation and proliferation

We used an in vitro system where BMDCs are targeted by CFSE-labeled parasites. The infection rate was confirmed by flow cytometry analysis of CFSE+ BMDCs (Fig. 1A). Although we did not directly address promastigote internalization, we assumed the CFSE population as comprised in its majority by DCs containing intracellular parasites. In support of this assumption, we failed to detect surface-associated promastigotes in CFSE+ sorted DCs by microscopy, and similar percentages of CFSE+ DCs were detected in experiments using the crystal violet as a surface fluorescence quencher (20) (data not shown). Regarding the maturation status, the bystander BMDC population (CFSE-) expressed significantly higher levels of CD40, CD86, and MHC II molecules when compared with infected BMDCs (CFSE+) or cells not exposed to parasites. In opposition, the CD1d marker was specifically lower on the bystander population (Fig. 1A). This phenotype was BMDC specific, because bystander and Leishmania-infected BMDCs displayed similar levels of cell-surface markers (data not shown). We further evaluated the cytokine profile expressed by each one of these subpopulations by quantitative PCR. Among all tested cytokines, we detected significantly higher levels of IL10 and Tnfa transcripts exclusively on infected BMDCs (Fig. 1B) and a trend for IL-27p28 (although not significant) compared with uninfected and bystander (only for IL10) BMDC cells. In contrast, bystander population produces preferably IL12p40 and Il6 transcripts (Fig. 1B). Flow cytometry analyses confirmed that IL12p40 is preferentially expressed by bystander BMDCs (gating specifically on each population (Fig. 1B, 1C). This population produces preferably IL12p40 (although not significant) compared with uninfected and bystander, but not infected BMDCs (data not shown).

We then evaluated the profile of CD4+ T cells induced in those conditions. IL-4, IL-10, IL-17, and IFN-γ, initially used for discriminating Th2, Th1, Th17, and Th1 cells, respectively, were analyzed. Whereas bystander BMDCs primed CD4+ T cells to IFN-γ secretion, infected BMDCs polarize CD4+ T cells toward an IFN-γ+IL-10+ DP phenotype (the percentage of DP varied from 2.8 to 13.4% in independent experiments; Fig. 2A, 2B). Few cells were positive for IL-4 and IL-17, and we did not find any significant differences on the IL-21, IL-22, or TNF-α level among the different cocultures. The absence of contact between BMDCs and CD4+ T cells abrogate any IFN-γ and/or IL-10 production (data not shown). Interestingly, the CD4+ polarization was restricted to the DC population because DP cells were never generated when infected BMDCs were used as APCs (Supplemental Fig. 2). Studies on Leishmania spp. (7, 23, 24) and Toxoplasma gondii (9) infection classified the DP cells arising during infection as effector Th1 lymphocytes. Accordingly, the DP population generated with infected DCs expressed the highest levels of the Th1 master transcription factor T-bet, but not the classical regulatory T cell marker Foxp3 (Fig. 2C). Similarly, this population was found negative for the CD25 marker displaying an effector phenotype (CD62L−CD44−; Fig. 2D). To gain insight about the specificity of CD4+ T cells generated in vitro after 5 d of coculture, we restimulated CD4+ T cells with APCs loaded with Leishmania Ags. CD4+ inf proliferate in response to SLA, whereas those cultured with bystander cells failed to proliferate (Fig. 2E, 2F). Nevertheless, we do not discard the possibility that CD4+ cell may recognize specific Leishmania secreted products, which are poorly presented by J774 macrophages in our experimental settings regarding the initial differentiation mediated by bystander and infected DCs, and may reflect distinct Ag specificity of CD4+ T cells. Thus, although initially less potent to proliferate in the presence of infected DCs, the CD4+ inf cells acquired specific Ag response.

IL-10 controls the activation of CD4+ T cells polarized by infected BMDCs playing a crucial role in their effector functions

Considering the IL-10 inhibitory role for Ag-specific activation and proliferation of CD4+ T cells (25) and because infected BMDCs presented increased IL-10 production (Supplemental Fig. 1B), complementary experiments were performed with BMDCs recovered from IL-10 knockout mice cocultured with syngeneic BALB/c naive CD4+ T cells. Although the level of CD4+ T cell activation remained unchanged when noninfected or bystander BMDCs were used as APCs, the absence of IL-10 from infected BMDCs restored the CD4+ T activation to similar levels as bystander cocultures (Fig. 3A, 3B). Similarly, the absence of IL-10 from myeloid origin rescued the CD4+ T proliferation upon coculture with infected BMDCs to comparable levels as bystander cocul-
IL-10 secreted by infected BMDCs is responsible, at some extent, for inhibiting the activation and proliferation of Ag-specific CD4+ T cells. Cytokine quantification on coculture supernatant showed increasing quantities of IL-10 concomitantly to a decrease of IFN-γ levels when CD4 inf cells were used in comparison with CD4 byst coculture conditions (Fig. 4A, 4B). Given the major immuno-suppressive role of IL-10, we repeated the experiment in the presence of anti–IL-10 mAb. Remarkably, the blockage of IL-10 restored the IFN-γ levels in coculture with CD4 inf to similar levels of CD4 byst cocultures (Fig. 4C).

Ex vivo infected DCs polarize naive T cells toward an IFN-γ+ IL-10+ DPs CD4+ T cell phenotype

To confirm the impact of bystander and infected DCs, we infected BALB/c mice with CFSE-labeled L. infantum, and 24 h later we isolated splenic CD11c+ cells. Thereafter, we sorted infected and bystander DCs, and accessed syngeneic naive CD4+ T cell polarization upon coculture. As a control, noninfected DCs from naive

FIGURE 1. L. infantum shapes the maturation and cytokine expression profile of bystander and infected BMDCs resulting in distinct levels of T cell activation and proliferation. The surface costimulatory molecules were analyzed in bystander (blue) and infected gated populations (red) of BALB/c BMDCs infected with CFSE-labeled L. infantum. For comparison, the histograms display the same markers on noninfected BMDCs (black) (A). Four hours postinfection, CD11c+ bystander and infected BMDCs were sorted, and the transcript levels of the indicated cytokines were determined by qPCR. Values are normalized for noninfected CD11c+ sorted cells (B). The intracellular levels of IL-12p40 and TNF-α were accessed by flow cytometry 18 h postinfection (C). The graphic depicts the relative percentage of noninfected, bystander, or infected cells producing IL-12p40 and TNF-α as detected by flow cytometry (D). Sorted CD11c+ bystander, infected, or noninfected BMDCs from BALB/c mice were cocultured with syngeneic naive CD4+ T cells. Thirty-six hours later, the activation of CD4+ T lymphocytes was measured through CD69 expression (E). Seventy-two hours later, the proliferation of CD4+ T lymphocytes was measured through CFSE decay in the presence of 10 ng/ml IL-2 (F). The mean ± SD from one representative experiment out of five is shown (G). *p < 0.05, **p < 0.01, ***p < 0.001.
mice were used. The DP CD4+ T cell population was exclusively detected when CD4+ T cells were in contact with infected DCs (Fig. 5A) demonstrating that only infected splenic BMDCs are capable to polarize naive CD4+ T cell toward an DP phenotype. To ascertain the true relevance of this DP phenotype on CD4+ T cells, we evaluated their presence during different time points of infection in our in vivo susceptible model (BALB/c). In this study, the frequency of splenic DP CD4+ T cells was found to increase...
during the acute phase, reaching its peak at 28 d postinfection (0.10 ± 0.04% in naive mice; 2.40 ± 0.60% at day 28 postinfection), diminishing in percentage and total numbers during chronic phase (1.10 ± 0.25%; Fig. 5B, 5C). Thus, an ∼25-fold expansion in the number of CD4+ T cells capable of IFN-γ and IL-10 simultaneous production was found at day 28 postinfection (2.44 ± 1.02 × 10^4 in naive mice versus 6.00 ± 1.25 × 10^5 at day 28 postinfection). Importantly, at day 84 postinfection, ∼81.1 ±
4.1 Leishmania Ag-specific IL-10−–producing cells and 32.2 ± 13.4 IFN-γ+ were characterized as DPs.

We then assessed whether splenic DCS from chronically infected mice were capable of generating in vitro DP CD4 T cells from naïve cells. We isolated total splenic DCS defined as CD11c+ MHC II\text{high} from naïve or 28 d postinfection mice and accessed syngeneic CD4 T cell polarization upon coculture. Again, the DP CD4+ T cell population was exclusively detected when CD4+ T cells were in contact with DCs recovered from infected mice in a dose-dependent manner (Fig. 5D). Because we were unable to discriminate between bystander and infected spleen DCs using CFSE-labeled parasites after 28 d of infection, the cocultures were performed with increased quantities of DCs. This was a necessary step because at chronic phase, the percentage of actually infected DCs should be reduced.

Because splenic DCS are in their majority MHC II\text{high}, we performed in vitro complementary experiments to evaluate the impact of surface MHC II in CD4+ polarization. The DP CD4+ T cells were found only when these were polarized by MHC II\text{high}–infected BMDCs, whereas MHC II\text{low} polarizes only IFN-γ+ single-producer CD4+ T cells (Fig. 5E), thus demonstrating the different impact of high and low MHC II–infected BMDCs on CD4+ T cell polarization. As expected, bystander or noninfected cells were always unable to generate DP CD4+ T cells independently of their MHC II intensity. Altogether, our data demonstrate that MHC II\text{high}–infected DCS have a direct impact on naive T cell polarization toward the development of Leishmania Ag-specific effector CD25− T-bet+IFN-γ+IL-10+ T cells.

Critical role for IL-12p70 in the development of IFN-γ+IL-10+ DP CD4+ T cells

Several cytokines have been proposed to induce DP CD4+ T cells. Therefore, we performed complementary experiments to explore which cytokines could be involved in the polarization of DP cells by infected DCs. As expected, the addition of IL-12p70 increased the secretion of high levels of IFN-γ (Fig. 6A, 6B). In infected Ag-presenting BMDCs, IL-12p70 increased the percentage of DP CD4+ T cells. In contrary, the addition of IL-12p70 had no impact on DP-producing cells from bystander cells. By blocking IL-12p70 activity, we were able to impede the emergence of DP CD4+ T cells (Fig. 6A, 6B). As expected, the addition of IL-12 potentially increased the expression of single IFN-γ producers, whereas its neutralization by the use of a mAb, but not with mAb isotype control, abrogated IFN-γ production. Among all other cytokine cocktails tested, we have found no differences in the percentage of DP CD4+ T cells induced by infected BMDCs in the presence of rIL-6, rIL-21, rTNF-α, or rTGF-β, or after ICOS–ICOS ligand blockage (Supplemental Fig. 3A, 3B). IL-27 added at the beginning of the culture did not impact on DP but abrogated the production of IL-10 single-producer cells (Supplemental Fig. 3C, 3D). Moreover, the blockage of IL-27 by a specific Ab did not alter the polarization of CD4+ T cells cocultured with infected BMDCs toward a DP phenotype (Supplemental Fig. 3A, 3B). We then quantified by ELISA the presence of endogenous IL-12p70 and IL-27 in the coculture experiments. Indeed, a significant increase of IL-12p70 levels was found only on cocultures involving the infected BMDCs (Fig. 6C), whereas IL-27 levels were increased at equivalent levels on both bystander and infected BMDCs/CD4+ T cell cocultures (Fig. 6C). These data suggest the main role of IL-12p70 in triggering the DP CD4+ populations.

CD4+ T cells primed by infected DCs but deprived of T-bet+ IFN-γ+IL-10+ DP population restore the immune response and reduce parasite load

The functional in vivo significance of the CD4+ T lymphocyte populations derived from in vitro cultures with infected or bystander BMDCs was then evaluated by adoptive transfer in BALB/c mice. Groups of eight age-matched BALB/c mice at the peak of the acute phase of infection were i.v. injected with 1 × 106 viable BALB/c CD4+ T cells. In contrary, the addition of IL-12p70 had no impact on DP-producing cells from bystander cells. By blocking IL-12p70 activity, we were able to impede the emergence of DP CD4+ T cells (Fig. 6A, 6B). As expected, the addition of IL-12 potentially increased the expression of single IFN-γ producers, whereas its neutralization by the use of a mAb, but not with mAb isotype control, abrogated IFN-γ production. Among all other cytokine cocktails tested, we have found no differences in the percentage of DP CD4+ T cells induced by infected BMDCs in the presence of rIL-6, rIL-21, rTNF-α, or rTGF-β, or after ICOS–ICOS ligand blockage (Supplemental Fig. 3A, 3B). IL-27 added at the beginning of the culture did not impact on DP but abrogated the production of IL-10 single-producer cells (Supplemental Fig. 3C, 3D). Moreover, the blockage of IL-27 by a specific Ab did not alter the polarization of CD4+ T cells cocultured with infected BMDCs toward a DP phenotype (Supplemental Fig. 3A, 3B). We then quantified by ELISA the presence of endogenous IL-12p70 and IL-27 in the coculture experiments. Indeed, a significant increase of IL-12p70 levels was found only on cocultures involving the infected BMDCs (Fig. 6C), whereas IL-27 levels were increased at equivalent levels on both bystander and infected BMDCs/CD4+ T cell cocultures (Fig. 6C). These data suggest the main role of IL-12p70 in triggering the DP CD4+ populations.

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BMDCs (CD4 inf). As controls, we transferred equal numbers of CD4 CD11c or treated the mice with an equivalent volume of PBS (Fig. 7A). Subsequent assessments of parasite burden in the spleen and liver of all groups of mice were made at 15 d after adoptive transfer. Remarkably, the transfer of CD4+ T cells primed by bystander DCs induced a significant decrease in liver and spleen parasite burden (1.54 and 1.31 log, respectively) when compared with mice that received PBS or CD4 CD11c (Fig. 7A). More importantly, this protection was lost when CD4+ T cells were polarized with infected DCs. Indeed, the transfer of a CD4+ population containing T-bet+IFN-γ+IL-10+ T cells not only failed to induce any protection in the liver (6.24 ± 0.16 for CD4 inf against 5.84 ± 0.23 and 5.56 ± 0.22 for PBS and CD4 CD11c, respectively), but it could even increase splenic parasite burden (6.71 ± 0.15 for CD4 inf against 5.91 ± 0.46 and 5.72 ± 0.22 for PBS and CD4 CD11c, respectively; Fig. 7A). The levels of IL-10 and IFN-γ produced by splenocytes after stimulation with SLA (25 μg/ml) demonstrated an increase in *Leishmania*-specific IFN-γ secretion in the group receiving CD4 byst cells, whereas spleen cells from mice that were adaptively transferred with CD4 inf (containing the T-bet+IFN-γ+IL-10+ population) displayed a preference for secretion of IL-10 (Fig. 7B). This led to a disequilibrium of the IFN-γ/IL-10 ratio, a known indicator of the infection outcome (19), contributing to an impaired effective immune response and prolonged parasite persistence.

To ensure that the observed effect was related to IL-10 from lymphoid origin, we performed the same assay in the context of an IL-10–free system therefore absent of T-bet+IFN-γ+IL-10+ T cells. IL-10ko CD4+ T cells were polarized with noninfected, bystander, or infected IL-10ko BMDCs. No significant differences were found on the levels of IFN-γ produced by the polarized CD4+ T cells (Supplemental Fig. 4A, 4B). Once more, the adoptive transfer of CD4+ T cells polarized with bystander BMDCs induced a significant decrease in liver and spleen parasite burden (1.45 and 1.40 log, respectively; Fig. 7C). Importantly, CD4+ T cells primed by infected DCs in an IL-10–free system reduced significantly the parasite burden in both the liver and spleens of infected mice. These results clearly demonstrated the critical role of T-bet+IFN-γ+IL-10+ cells in the maintenance of infection.

Discussion

DCs are specialized APCs that play a crucial role in driving adaptive immune responses. However, most of the analyses performed on DC functions upon protozoan infections are generally performed without discriminating between the infected and the bystander (exposed but noninfected) populations. Exploring the CFSE-labeled *L. infantum*-BMDCs model allowed us to underline the existence of two major distinct DC subsets with opposite roles for T cell activation and polarization. Hence when facing a matured DC, *Leishmania* induces the secretion of myeloid IL-10 that limits T cell activation and proliferation. On the other side, the bystander population, which contacted with the parasite or with parasite-secreted products, presents increased transcription levels of inflammatory cytokines being capable to induce CD4+ T cell activation and proliferation with immune protective capabilities. Our results demonstrated that in vitro or ex vivo only infected DCs...
induced the polarization of naive CD4+ T cells toward an IFN-γ+ IL-10+ DP phenotype. This phenotype of DP cells corresponds phenotypically and functionally to the IL-10–producing Th1 identified in cutaneous or VL lesions (7, 11, 23, 26). Most importantly, we demonstrate that the adoptive transfer of DP cells favors disease progression and highlights the importance of IL-10 as a major factor inhibiting parasite elimination. In contrast, the adoptive transfer of CD4+ T cells polarized in the presence of bystander DCs displayed a striking phenotype with reduced splenic burden and enhanced IFN-γ production. Therefore, our results contrast with other infectious models such as malaria or *Toxoplasma*, where the generation of DP cells is protective (8, 27).

The general consensus for an effective response toward all forms of leishmaniasis is the preferential development of Th1–mediated immune response. Nevertheless, patients with active VL disease present high levels of IFN-γ and IL-12p70 that are concomitantly detected with elevated IL-10 production (2, 23, 28). Among its cellular sources, IL-10 of myeloid origin has been long associated with disease progression (29–31). In this study, we demonstrate that the secreted IL-10 from infected DCs impaired to some extent the development of an adaptive response by decreasing the activation and proliferation of Ag-specific CD4+ T cells. More importantly, recent data indicated that IL-10–producing Th1 cells, which are activated early in a strong inflammatory setting, are the critical mediators of immune suppression in a chronic cutaneous or VL (7, 23). The detailed characterization of this population indicated that the DP cells are 'T-bet', Foxp3+, and CD25+ while maintaining an effector phenotype as previously described (CD62L–, CD44+) (7, 9, 24). Moreover, the impact of the adoptive transfer of IFN-γ+IL-10+ DP cells on disease progression highlights the importance of lymphoid IL-10 as a major factor inhibiting parasite elimination because the adoptive transfer of T cells deficient for IL-10 cultured in the same conditions do not exacerbate disease progression.

Our data also demonstrated the dynamics of DP CD4+ T cells during acute and chronic infection. A clear increase of this population was observed upon infection, reaching its peak in the transition to chronic phase (day 28). Although the observed contraction phase of DP cells is in late chronic phase, their relative abundance among single IFN-γ+ or IL-10+–producing cells is sustained at high levels. This suggests a modification of the splenic microenvironment toward a more parasite-permissive cytokine enrichment. Importantly, we demonstrated that splenic DCs recovered at day 28 postinfection were very efficient in the induction of DP cells. Thus, our data demonstrate a previously proposed association between the frequency of IFN-γ+IL-10+ DP CD4+ T cells and susceptibility in leishmaniasis (7, 11, 32). A recent study underlined, in a resistant model (C57BL/6 mice) of visceral *L. donovani* infection, also the role of splenic CD11c+DCs in the development of IL-10–producing Th1 cells and disease progression (10). Nevertheless, the differential behavior among infected and bystander DCs and the cytokines involved in CD4+ T cell polarization were not explored.

It is well-known that the balance of IL-10 and IL-12 is central in determining T cell activation (33). Our results indicated that infected DCs control the level of activation of naive T cells through their capacity to secrete IL-10 because once this cytokine is blocked, the amount of IFN-γ–producing CD4+ T cells induced is increased to levels similar to the ones induced by bystander or noninfected DCs. However, we found that these DP cells acquire a striking capacity to proliferate secondary to a re-exposure to *Leishmania* Ag presentation. Most interestingly, we demonstrated that IL-12p70 play a role in the polarization of IFN-γ+IL-10+ DP CD4+ T in our experimental settings. Thus, the blocking of IL-12p70 abrogated the generation of such population. In symptomatic VL, patients present significantly higher levels of IL-12 than asymptomatic or healthy individuals in the same endemic area (28, 34). Although infected DCs did not up-regulate IL-12p40 or IL-12p35 mRNA transcription upon *L. infantum* infection, we detect higher levels of IL-12 in the supernatants coculture. This apparent paradox may be explained by the requirement of DC–T cell surface interactions for the secretion of large amounts of IL-12p70 (35). In addition, membrane-associated IL-12p70 stores were shown to be released by human and murine DCs after in vitro or in vivo contact with visceral *Leishmania* species (36). Overall, our data demonstrate that IL-12 is needed to prime CD4+ T cells toward a DP phenotype.

Other stimuli such as IL-27 (8, 37), IL-21 (38), TGF-β (39), ICOS, and the transcription factor c-maf (40) have also been shown to drive IL-10 expression. IL-27 was shown in different infectious models to be crucial in the development of IFN-γ+IL-10+ DP CD4+ T cells (8, 41, 42). More recently, a correlative presence of IL-27–producing splenic DCs and DP CD4+ T polarization in vivo was shown, although a direct link was not proved (10). In our DC–*L. infantum* experimental model, we failed to confirm this link. Not only did IL-27 fail to induce DP cells, but we did not detect any increment on the transcription or the secretion levels of IL-27p28, EB13, or IL-27 upon infection. Instead, in this study, we found that the addition of IL-27 inhibits the expression of IL-10.

An ongoing debate discusses the DC subtypes responsible for the induction of protective immunity against *Leishmania* infection. *Leishmania* parasites in different steps of the infection process will interact with DCs presenting distinct maturation degrees. In the course to visceralization, *Leishmania* parasites interact with both MHC II+ dermal DC immigrants and epidermal Langerhans cells (43). In addition, MHC II+ dermal monocyte-derived DCs (44) or even resident MHC IIlow lymphoid tissue DCs (45) were suggested to play a preponderant role in the development of T cell immunity against pathogens. Once in the spleen, *Leishmania* will contact with DCs expressing high levels of cell-surface MHC II molecules (10, 46). In this article, we demonstrated that only infected MHC II+DCs, both of in vitro or ex vivo origin, were capable of inducing the polarization of CD4+ T cells toward a DP phenotype. Moreover, only infected MHC II+ BMDCs showed significant increased levels of IFN-γ and *Tnfα*, whereas infected MHC IIlow did not modify the expression of any of the tested cytokines. Finally, the profile of bystander MHC II+ revealed increased *Il12p40* and *Il16* transcripts, which was not surprising because the majority of bystander cells displayed a matured phenotype. Although high levels of MHC II surface expression on DCs can be viewed as essential for Ag presentation and DP polarization, our results revealed a profound difference in cytokine expression, which probably represents the main determinant in DP polarization that we observed. Thus, MHC IIlow did not express IL-10. Altogether, our results highlight a new level of complexity in *Leishmania*–DCs interaction and suggest that future studies should dissect DC immune responses in view of MHC II expression.

Our results highlight novel subversion mechanisms used by *L. infantum* parasites. We demonstrate a clear dichotomy between bystander and infected BDMCs revealing opposite roles on T cell activation and polarization. First, IL-10 secreted from MHC II+DCs is capable to restrain to a certain amount the activation and proliferation of CD4+ T cells. Second, our work identified for the first time, to our knowledge, an infectious model where IL-12p70–driven IFN-γ+IL-10+ DP CD4+ Th1 cells play a critical role in the maintenance of protozoan infection, underlying the role of lym-
phoid IL-10 as judged by the adoptive transfer of IL-10kn CD4+ T cells contrasting the beneficial role of this subset (IFN-γ/IL-10+ DP cells) in T. gondii or Plasmodium spp. infections. Our observation that IL-12p70 favors nonprotective T-betIFN-γ/IL-10+ T cells without any involvement of IL-27 unravel a potential dichotomy of IL-27/IL12p70 in driving protective/pathogenic IFN-γ/IL-10+ DP Th1 responses. Thus, the identification of the mechanisms by which IFN-γ/IL-10+ DP CD4+ T populations are induced in the context of Leishmania infection could represent a new strategic therapeutic target.

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Disclosures
The authors have no financial conflicts of interest.

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