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

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**Leishmania-Infected MHC Class II<sup>high</sup> Dendritic Cells Polarize CD4<sup>+</sup> T Cells toward a Nonprotective T-bet<sup>+</sup> IFN-γ<sup>+</sup> IL-10<sup>+</sup> 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-12p40 and costimulatory molecules being capable to induce CD4<sup>+</sup> 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<sup>-</sup>IFN-γ<sup>-</sup>IL-10<sup>+</sup> CD4<sup>+</sup> 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 naive CD4<sup>+</sup> T cells toward a T-bet<sup>-</sup>IFN-γ<sup>-</sup>IL-10<sup>+</sup> phenotype. Further analysis revealed that only MHC class II<sup>high</sup>-infected DCs were responsible for this polarization. The adoptive transfer of such polarized CD4<sup>+</sup> 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<sup>+</sup> T cells primed by infected DCs in an IL-10 free system, thus deprived of T-bet<sup>-</sup>IFN-γ<sup>-</sup> population, restore the immune response and reduce parasite load, supporting a deleterious role of IFN-γ<sup>-</sup>IL-10<sup>+</sup> T cells in the maintenance of infection. Overall, our results highlight novel subversion mechanisms by which nonprotective T-bet<sup>-</sup>IFN-γ<sup>-</sup>IL-10<sup>+</sup> T cells are associated with chronicity and prolonged parasite persistence. *The Journal of Immunology*, 2013, 191: 000–000.

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The online version of this article contains supplemental material. Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; BMM, bone marrow-derived macrophage; CD4<sup>-</sup> Byst, CD4<sup>+</sup> T cell polarized with bystander dendritic cells; CD4<sup>+</sup> DC, CD4<sup>+</sup> T cell polarized with noninfected dendritic cells; CD4 inf, CD4<sup>+</sup> T cell polarized with infected dendritic cells; DC, dendritic cell; DP, double-producer; IBMC, Instituto de Biologia Molecular e Celular; MHC II MHC class II; RPM, complete RPMI 1640; SLA, soluble Leishmania Ag; VL, visceral leishmaniasis.

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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 these, 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 immunosuppressive VL, we decided to clarify the functional role of the bystander and infected DC populations during visceral L. infantum infection on a susceptible mice model evaluating the subsequent repercussions on adaptive immune response.

Our results highlight novel subversion mechanisms used by L. infantum. We demonstrate the duality of cytokine expression postinfection, indicating that bystander DCS were prone to express IL-12p40 and IL-6, whereas infected DCS transcribe preferably postinfection, indicating that bystander DCs were prone to express CD4+ T cells toward a specific nonprotective T-bet +IFN-γ+IL-10+ Th1 phenotype. Moreover, we prove that only infected MHC class IIhigh (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- T cells are associated with chronic parasite persistence. Moreover, our data raise a potential dichotomy of IL-27/IL-12p70 in driving protective/pathogenic IFN-γ-IL-10+ Th1 double-producer (DP) responses.

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 (Direcção-Geral de Alimentação e Veterinária) guidelines. A cloned line of virulent L. infantum (MHOM/MA/67/ITMAP-263) was maintained by weekly subpassages in complete RPMI 1640 (RPMIC) supplemented with 10% heat-inactivated FBS (FCS; Lonza), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All isotype controls were obtained from BioLegend.

Abs

Abs used include: PE/Cy7 anti-mouse CD40 Ab, clone 3/23, Isotype rat IgG2a; PerCP/Cy5 anti-mouse CD80 Ab, clone 16-10A1, Isotype Armenian hamster IgG Brilliant Violet 421 anti-mouse CD86 Ab, clone GL-1, Isotype rat IgG2a; Alexa Fluor 647 anti-mouse I-A/IE Ab, clone 39-10/8, Isotype mouse (3H8SW) IgG3; allophycocyanin anti-mouse I-A/IE Ab clone M5/114.15.2, Isotype rat IgG2b; Pacific Blue anti-mouse CD41 (CD1.1, Ly-38) Ab, clone 1B1, Isotype rat IgG2b; PE/Cy7 anti-mouse CD11c Ab, clone N418, Isotype Armenian hamster IgG; allophycocyanin anti-mouse CD11c Ab, clone J558-GM-CSF. Cells were cultured at 37˚C and 5% CO2 for 3 d, after which the same amount of DC medium was added to each flask. At days 6 and 8, half of the culture supernatant was collected and centrifuged, and the cell pellet was resuspended in the same amount of fresh DC medium and put back into the original flask. At day 10, the same procedure was performed but with use of only 100 U/ml GM-CSF or 5% of GM-CSF–containing supernatant. The purity of DC preparations after 12 d of culture was always superior to 90%. Mouse naive CD4+ T cells were isolated from the spleens of naïve BALB/c or IL-10 knockout mice and purified using the CD4+CD62L+ T Cell Isolation Kit II (Miltenyi Biotec) following manufacturer’s recommendations. The purity of naive CD4+ T cell preparations was always superior to 95%.

Leishmania staining with CFSE

Stationary-phase promastigotes from each culture medium at a concentration of 1.2 × 106 promastigotes/ml were used for CFSE (Invitrogen) labeling. L. infantum promastigotes were washed twice 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; GammaCell 1000 Elite) parasites or 2-μm-diameter FITC-latex beads (Sigma-Aldrich) at similar coculture ratios (10 FITC-beads for 1 cell). Nonnormalized parasites were removed by gently washing after 4 h of infection, and fresh RPMIC was added to the wells. Cells were immediately recovered or maintained for 24 h. BMDCs stimulated with LPS (1 μg/ml) were used as a positive control for DC activation/maturaion. The percentage of infected BMDCs was determined by flow cytometry evaluation of CFSE+ cells in a FACSCanto 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 × 105 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 FACSCanto II cytometer. Cells were selected on the basis of forward scatter/side scatter values; BMDCs were gated on CD11c+. BMDCs were fixed with 2% PFA, washed in PBS containing 2% FCS, and permeabilized in 50 μl of 0.5% saponin, to detect intracellular cytokines. Cells were then incubated with TNF-α–PerCP/Cy5.5, IL12p40-allophycocyanin,
or isotype-matched controls (all from BioLegend) for 30 min at room temperature in the dark. The cells were then washed twice in PBS and acquired on a FACS_Canto II cytometer. All analyses were performed using FlowJo software.

**Cell sorting**

**Dendritic cells.** In some experiments, BMDCs were sorted according their CD11c+ surface expression in the case of noninfected cells and CD11c+ CFS* or CD11c+ CFS* expression in the case of bystander and infected cells, respectively. In other cases, BMDCs were sorted according their CD11c+/MHC II high or low and then infected for posterior analysis. Finally, in other experiments, BMDCs were first infected with CFS* labeled parasites and bystander or infected BMDCs were sorted according their MHC II expression (high or low) as demonstrated on gating strategy (Supplemental Fig. 1A). Six populations were sorted according to their expression on MHC II (low or high) and CFS* labeling.

**CD4+ T cells.** After coculture with the different subsets of BMDCs, CD4+ T cells were sorted after surface labeling with anti–CD3-PerCP/Cy5.5 and anti–CD4-APC, SYTOX Green Nucleic Acid Stain (Invitrogen) was 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 x 10^6) BALB/c or IL-10 knockout mice were cocultured with naive CD4+ T cells of similar origin at a responder/stimulator 10:1 cell ratio. CD4+ T activation was measured after 36 h of coculture by labeling CD4+ T cells with anti–CD69-PE, anti–CD25-FITC, anti–IFN-γ-PE, anti–IL-4-PE, and anti–IL-10-PE (BD, San Diego, CA) and following the manufacturer's recommendations. The supernatants of sorted BMDCs were collected after 24 h of culture, and the cytokine intracellular levels of CD4+ T cells were measured after PMA/Ionomycin plus brefeldin A stimulation as before.

Adoptive transfer and parasite burden

BALB/c mice were infected i.p. with 1 x 10^6 CFS* labeled L. infantum promastigotes. Mice were euthanized at 24 h or 28 d postinfection and the spleens removed. T and B lymphocytes were depleted using CD19+ and CD3+ microbeads coupled with LD columns (Miltenyi Biotec). The remaining cell suspension was labeled with anti–CD11b-PE, anti–Ly-6C-PerCP/Cy5.5, and anti–Ly-6G-Pacific Blue, and cells (in a FACSAria using the FACSDiva software) according to the surface expression CD11b+CD11c+Ly6C+ Ly6G+ and surface MHC II expression gating on infected (CFSE+CD11b+CD11c+Ly6C+Ly6G+) or bystander (CFSE- CD11b+CD11c+Ly6C+Ly6G-) splenic DCs. For all experiments, CD11b+CD11c+Ly6C+Ly6G- cells from the spleen of noninfected mice were sorted as a control. In other experiments, total splenic DCs of infected or noninfected mice were sorted following a similar approach. Noninfected, infected, or bystander splenic DCs were cocultured with naive CD4+ T cells of similar origin 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 Ag-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 (10^4) were used as APCs. These were cultured overnight with heat-killed Leishmania infantum promastigotes at a 1:10 ratio (parasite:J774; 56°C, 30 min) and irradiated. A total of 10^5 sorted CFS* labeled CD4+ T cells (CD4+ T cells polarized with infected dendritic cells [CD4 inf], CD4+ T cells polarized with bystander DCs [CD4 bystand], or CD4+ T cells polarized with noninfected DCs [CD4 CDU]), 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 (MultiAnalyses AB, Göteborg, Sweden) as the most stable for the conditions used.

**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-2 p70 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 an Axioskop Z1 (Carl Zeiss, Germany) with the 63×/1.40 Plan-Apochromat objective and AxiosCam MR version 3.6 (Carl Zeiss, Germany). The filter set used included an excitation filter of 640/30 nm and an emission filter of 690/50 nm. The settings for contrast, brightness, pinhole, acquisition mode, and scanning time were maintained throughout the workflow. 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 x 10^5 bone marrow cells in 400 μM DEMEM 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. The cytokine intracellular levels of CD4+ T cells were measured after PMA/Ionomycin plus brefeldin A stimulation 10 h after.

**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
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 BMMs 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 Tnf transcripts exclusively on infected BMDCs (Fig. 1B) and a trend for Il-27p28 (although not significant) compared with uninfected and bystander (only for Il10) BDMC cells. In contrast, bystander population produces preferably infected BMDCs 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 BDMCs 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 BMMs 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 (CD25+ T cells, 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 byst 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 (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. Given the major immunosuppressive 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.

**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

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**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

FIGURE 2. Infected DCs induce *Leishmania* Ag-specific effector Th1 CD4+ T cells with IFN-γ+IL-10+ DP phenotype. Sorted CD11c+ bystander, infected, or noninfected BMDCs from BALB/c mice were cocultured with syngeneic naive CD4+ T cells. After 5 d of coculture, the IFN-γ and IL-10 intracellular levels were determined on CD4+ T cells (A). The mean ± SD from one representative experiment out of eight is shown (B). T-bet and Foxp3 expression were measured on CD4+ T cells cocultured with infected BMDCs on each of the indicated gates (C). CD44, CD62L, and CD25 surface markers were measured on the IFN-γ+IL-10+ DP CD4+ T cells (black). Isotype control is represented (gray) (D). After 5 d of coculture, the CD4+ T lymphocytes of each one of the cultures (CD4 bystander, CD4 infected, CD4 CD11c corresponding to the source of DCs that were used in the coculture, bystander, infected, or noninfected, respectively) were sorted and further cultured with APCs presenting *Leishmania* Ags. The *Leishmania*-specific proliferative capacity of each CD4+ T cell was quantified after 96 h by CFSE decay (E). The mean ± SD from one representative experiment out of five is shown (F). *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 3. IL-10 controls the activation and proliferation of CD4+ polarized by infected BMDCs. Sorted CD11c+ bystander, infected, or noninfected BMDCs from IL-10ko mice were cocultured with syngeneic naive WT CD4+ T cells. Thirty-six hours later, the activation of CD4+ T lymphocytes was measured through CD69 expression (A). The mean ± SD from one representative experiment out of five with BMDCs from BALB/c or IL-10ko origin is shown (B). Upon seventy-two hours of coculture, the proliferation of WT CD4+ T lymphocytes was measured through CFSE decay in the presence of 10 ng/ml IL-2 (C). The mean ± SD from one representative experiment out of three is shown (D). *p < 0.05, **p < 0.01, ***p < 0.001.
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 ± 6

**FIGURE 4.** IL-10 secreted from CD4+ polarized by infected BMDCs plays a crucial role in their effector functions. Naive CFSE-labeled CD4+ T (2.5 × 10^4) cells were activated with anti-CD3 (1 μg/ml) and cultured with in vitro polarized and sorted CD4 bystander, CD4 Inf, or CD4 CD11c (2.5 × 10^4) in the presence of irradiated APCs. After 5 d, the levels of IL-10 (A) and IFN-γ (B) were measured in the culture supernatant. Similarly, the levels of IFN-γ were quantified when naive CD4+ T cells (2.5 × 10^5) were cultured with in vitro polarized CD4+ T cells (1.0 × 10^5) in the presence of anti–IL-10 (10 μg/ml) or the respective isotype control (IgG1; C). The mean ± SD from one representative experiment out of three is shown. *p < 0.05, **p < 0.01.

**FIGURE 5.** Ex vivo infected DCs induce the polarization of CD4+ T cells with IFN-γ IL-10+ DP phenotype. BALB/c mice were infected i.p. with 1 × 10^8 CFSE-labeled L. infantum promastigotes. Twenty-four hours later, the spleens were removed and the bystander and infected DCs sorted. As a control, DCs were sorted from the spleen of noninfected mice. Each DC population was cocultured with syngeneic naive CD4+ T cells for 5 d, and the IFN-γ and IL-10 intracellular levels were determined by flow cytometry (A). The percentage (B) and total number (C) of splenic CD4+ T cells with Leishmania Ag-specific IFN-γ IL-10+ DP phenotype were quantified in noninfected mice (naive) or after 14, 28, or 84 d of infection (d14, d28, or d84, respectively). The spleens of noninfected (naive) or d28 infected mice (infected) were recovered and the CD11c+ MHC IIhigh DC population purified by cell sorting. Each DC population was cocultured with syngeneic naive CD4+ T cells for 5 d at the displayed conditions, and the IFN-γ and IL-10 intracellular levels were determined by flow cytometry (D). Noninfected, infected, or bystander BMDCs sorted according their MHC II expression (high or low) were cocultured with naive CD4+ T cells. After 5 d of coculture, the IFN-γ and IL-10 intracellular levels were determined on CD4+ T cells (E).
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 naive cells. We isolated total splenic DCS defined as CD11c⁺ MHC II⁺ from naive 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⁺, 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⁺–infected BMDCs, whereas MHC II⁺⁺ 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⁺⁺–infected DCS have a direct impact on naive T cell polarization toward the development of *Leishmania* Ag-specific effector CD25− Tbet⁺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 rLL-6, rLL-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 × 10⁷ viable CD4⁺ T cells polarized with bystander (CD4 byst) or infected

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**FIGURE 6.** Critical role for IL-12p70 in the development of IFN-γ⁺ IL-10⁺ DP CD4⁺ T cells. Sorted CD11c⁺ bystander, infected, or noninfected BMDCs from BALB/c mice were cocultured with syngeneic naive BALB/c CD4⁺ T cells in the presence or absence of rIL-12p70 (10 ng/ml), anti-IL-12p40, or the correspondent isotype control (10 μg/ml), and the intracellular levels of IFN-γ and IL-10 on CD4⁺ T cells measured according to Cytometric assay. The mean ± SD from one representative experiment out of four is shown. *p < 0.05, **p < 0.01, ***p < 0.001 in comparison with isotype control or mock treated (None) (B). The levels of IL-12p70 and IL-27 were quantified on the supernatant of sorted CD11c⁺ bystander, infected, or noninfected BMDCs cultured in the absence or CD4⁺ T cells or presence (+CD4⁺ T cells) of naive CD4⁺ T cells (C). The mean ± SD from one representative experiment out of three is shown. *p < 0.05, **p < 0.01, ***p < 0.001.
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 adoptively transferred with CD4 inf (containing the T-bet+IFN-γ+IL-10+ population) displayed a preferential 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

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**FIGURE 7.** Adoptive transfer of T-bet+IFN-γ+IL-10+ T cells is associated with incapacity to mount an effective immune response. Sorted bystander, infected, or noninfected BMDCs from BALB/c mice were cocultured with syngeneic naïve BALB/c CD4+ T cells (A). After 5 d of coculture, CD4+ T lymphocytes of each one of the cultures (CD4 byst, CD4 Inf, CD4 CD11c corresponding to the source of DCs that were used in the coculture, bystander, infected, or noninfected, respectively) were sorted and injected i.v. in previously infected BALB/c mice. Two weeks later, the spleens and livers were recovered and parasite loads determined in all groups. The levels of IFN-γ and IL-10 were measured by ELISA in the supernatant of splenocytes cultured in the presence or absence of SLAs for 3 d (B). A similar experiment as shown in (A) was performed with BMDC and CD4+ T cells from IL-10ko origin (C). Each value represents the mean ± SD from at least eight mice per group (*p < 0.05, **p < 0.01, ***p < 0.001).
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 environment. 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). 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.

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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 reexposure 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 blockage of IL-12p70 abrogated the generation of such population. In symtomatic 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 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 IIhigh dermal DC immigrants and epidermal Langerhans cells (43). In addition, MHC IIhigh 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 IIhigh 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 IIhigh BMDCs showed significant increased levels of II10 and Tfα, whereas infected MHC IIlow did not modify the expression of any of the tested cytokines. Finally, the profile of bystander MHC IIhigh revealed increased II12p40 and IIf6 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 BMDCs revealing opposite roles on T cell activation and polarization. First, IL-10 secreted from MHC IIhigh 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-10ko 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-cell IFN-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+ CD4+ T populations are induced in the context of *Leishmania* infection could represent a new strategic therapeutic target.

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**Disclosures**

We have no financial conflicts of interest.

**References**


receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. J. Immunol. 183: 797–801.
S1. Maturation status and survival of bystander and *L. infantum* infected BDMCs. The percentage of infected cells in relation to their surface MHCII expression was performed accordingly to the depicted gating strategy (A). The percentage of infected MHCII$^{\text{low}}$ or high is given by the percentage of the respective infected population over the corresponding bystander cells (B). BMDCs were sorted according to their surface MHCII expression (high or low) and infected with CFSE-labeled parasites. After 24 hours, the MHCII intensity on bystander and infected cells as well as the percentage of infection on MHCII$^{\text{low}}$ and MHCII$^{\text{high}}$ populations were determined by flow cytometry (C). Sorted MHCII (high or low) BMDCs were infected with CFSE-labeled *L. infantum* or cultured with irradiated CFSE-parasites or FITC-Beads and their uptake analyzed by flow cytometry. The percentage of uptake in each case is shown (D). Representative immunofluorescence pictures are depicted for each gated population (a, b, c and d) according the gate strategy shown on figure S2C. The scale bar represent 10 μm (E). Sorted MHCII$^{\text{high}}$ or low bystander, infected or non-infected BMDCs from Balb/c mice were co-cultured with syngeneic naïve Balb/c CD4$^+$ T cells. Thirty-six hours later, the activation of CD4$^+$ T lymphocytes were measured through CD69 expression (F). RNA levels of the indicated cytokines were determined by qPCR for MHCII$^{\text{high}}$ (G) or MHCII$^{\text{low}}$ (H) infected and bystander BMDCs sorted as depicted on Fig. S1A. Values are normalized for non-infected cells MHCII$^{\text{high}}$ or low, respectively (A-B). Sorted MHCII$^{\text{high}}$ bystander, infected or non-infected BMDCs from BALB/c mice were co-cultured with syngeneic naïve BALB/c CD4$^+$ T cells. Seventy-two hours later the proliferation of CD4$^+$ T lymphocytes were measured through CFSE decay in the presence of 10 ng/ml of IL-2 (I). The mean ± S.D. from one representative experiment out of three is shown (J). Each value represents the mean ± S.D. from at least four independent experiments. (*p<0.05; ** p<0.01; *** p<0.001).
S2. Infected bone marrow-derived macrophages (BMM) are unable to induce Th1 CD4⁺ T cells with IFN-γ⁺IL-10⁺ double producer phenotype. Sorted bystander, infected or non-infected BMM from BALB/c mice were co-cultured with naïve BALB/c CD4⁺ T cells. After 5 days of co-culture, the IFN-γ and IL-10 intracellular levels were determined on CD4⁺ T cells. The results are from a representative experiment of three carried out independently.
S3. The addition or blockage of distinct anti or pro-inflammatory cytokines does not modifies the IFN-γ⁺ IL-10⁺ double producer phenotype induced by infected DCs. Sorted bystander, infected or non-infected BMDCs from BALB/c mice were co-cultured with naïve BALB/c CD4⁺ T cells in the presence of distinct cytokines (IL-6, IL-21, TNF-α, TGF-β; added at a final concentration of 10 ng/ml) or in the presence of mAb against CD275 (ICOSL), IL-27p28 or the correspondent isotype controls (all mAb were added at a final concentration of 10 μg/ml). The IFN-γ and IL-10 intracellular levels were determined on CD4⁺ T cells after 5 days of co-culture (A). The mean ± S.D. from one representative experiment out of three is shown (B). Similarly, sorted bystander, infected or non-infected BMDCs from BALB/c mice were co-cultured with naïve BALB/c CD4⁺ T cells in the presence of IL-27 or IL-27 plus IL-12p70 (all cytokines added at a final concentration of 10 ng/ml). The IFN-γ and IL-10 intracellular levels were determined on CD4⁺ T cells after 5 days of co-culture (C). The mean ± S.D. from one representative experiment out of three is shown (D) (*p<0.05).
S4. Profile of naïve CD4+ T cells from IL-10 knockout mice co-cultured with bystander, infected or non-infected BMDCs from IL-10 knockout mice. Sorted bystander, infected or non-infected BMDCs from IL-10ko mice were co-cultured with syngeneic naïve IL-10ko CD4+ T cells. After 5 days of co-culture, the IFN-γ and IL-10 intracellular levels were determined on CD4+ T cells (A). The mean ± S.D. from one representative experiment out of three is shown (B) (*p<0.05).