Distinct Transcriptional Signatures of Bone Marrow-Derived C57BL/6 and DBA/2 Dendritic Leucocytes Hosting Live Leishmania amazonensis Amastigotes

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Introduction

Leishmania (L.) amazonensis perpetuates in South and Central America, its main location being the wet forests of the Amazon basin. The perpetuation of this Leishmania species relies successively on two hosts which cohabit more or less transiently within this ecosystem: blood-feeding sand flies and mammals, including wild rodents and humans. A broad spectrum of clinical manifestations, ranging from single cutaneous lesions to multiple, disfiguring sites reached by parasites emigrating from the primary inoculation site [4,5,6,7,8]. By contrast, in DBA/2 mice, at the inoculation site, the L. amazonensis population size is rapidly controlled, a process coupled to a controlled inflammatory process with limited parasite dissemination in distant tissue(s), if any [9].

Knowing that once in the dermis of the mouse, amastigotes are hosted by mononuclear phagocytes including macrophages and dendritic leukocytes (DLs) [10,11,12,13,14,15], we have addressed the following question: could the DLs harbouring live amastigotes contribute to the distinct phenotypes observed in C57BL/6 and DBA/2 mice? Since the frequency of DLs hosting live L. amazonensis amastigotes do display distinct transcriptional signatures and markers that could contribute to the distinct features observed in C57BL/6 versus DBA/2 ear pinna and in the ear pinna-DLNs during the first phase post L. amazonensis inoculation.

Conclusions/Significance:

The distinct features captured in vitro from homogenous populations of C57BL/6 and DBA/2 DLs hosting live amastigotes do offer solid resources for further comparing, in vivo, in biologically sound conditions, functions that range from leukocyte mobilization within the ear pinna, the distinct emigration from the ear pinna to the DLN of live amastigotes-hosting DLs, and their unique signalling functions to either naive or primed T lymphocytes.
Author Summary

The rapid and long term establishment of parasites such as L. amazonensis, otherwise known to strictly rely on subversion of macrophage and dendritic leucocyte (DL) lineages, is expected to reflect stepwise processes taking place in both the skin dermis where the infective form of the parasite and the skin-draining lymph node (DLN) were inoculated. Relying on mice of two distinct inbred strains—C57BL/6 and DBA/2—that rapidly and durably display distinct phenotypes at the two sites of establishment of L. amazonensis, we were curious to address the following question: could live L. amazonensis-hosting DL display unique signatures that account for the distinct phenotypes? Based on flow cytometry, genechip and real-time quantitative PCR analyses, our results did evidence that, once subverted as cells hosting live L. amazonensis, DLs from C57BL/6 or DBA/2 do display distinct profiles that could account for the i) distinct parasite load profiles, ii) as well as the distinct macroscopic features of ear pinna observed once the L. amazonensis metacyclic promastigotes completed their four day developmental program along the amastigote morphotype.

C57BL/6 and DBA/2 mice exposed or not to live L. amazonensis amastigotes.

Based on flow cytometry (FCM), genechip (Affymetrix Mouse GeneChip) and real-time quantitative PCR (RT-qPCR) analyses performed on sorted DLs hosting live DsRed2-expressing L. amazonensis transgenic amastigotes [17] many distinct features have been highlighted. DBA/2 DLs displayed transcriptional signatures and markers that could be related to the early phenotype observed in vivo, in contrast to live amastigotes-hosting C57BL/6 DLs. The data are consistent with rapid and sustained immune regulatory functions accounting for the remodeling of the DBA/2 ear as L. amazonensis protective niche. All together this study provides, for the first time, a solid base for exploring i) the inflammatory processes that maintain the amastigote population under control in DBA/2 mice and ii) the inflammatory processes coupled to extended parasite dissemination and to poor parasite population control in C57BL/6 mice.

Methods

Mice

Six week old female DBA/2, C57BL/6 and Swiss nu/nu mice were purchased from Charles River (Saint Germain-sur-l’Arbresle, France).

Ethics statement

All animals were housed in our A3 animal facilities in compliance with the guidelines of the A3 animal facilities at the Pasteur Institute which is a member of Committee 1 of the “Comité d’Ethique pour l’Experimenation Animale” (CEEA) - Ile de France - Animal housing conditions and the protocols used in the work described herein were approved by the “Direction des Transports et de la Protection du Public, Sous-Direction de la Protection Sanitaire et de l’Environnement, Police Sanitaire des Animaux under number B75-15-28 in accordance with the Ethics Charter of animal experimentation that includes appropriate procedures to minimize pain and animal suffering, TL is authorized to perform experiment on vertebrate animals (licence 75-717) issued by the Paris Department of Veterinary Services, DDSV) and is responsible for all the experiments conducted personally or under his supervision as governed by the laws and regulations relating to the protection of animals.

Preparation of L. amazonensis amastigotes and metacyclic promastigotes

DsRed2-transgenic L. amazonensis strain LV79 (WHO reference number MPRO/BR/72/M1841) amastigotes were isolated from Swiss nude mice inoculated 2 months before within a BSL-2 cabinet space as described previously [17]. These amastigotes did not present any antibodies at their surface [18]. Promastigotes derived from amastigotes were cultured at 26°C in complete DMEM medium. The metacyclic promastigote population (mammal-infected stage) was isolated from stationary phase cultures (6 day-old) on a Ficoll gradient.

Monitoring of L. amazonensis metacyclic promastigotes in ear dermis post inoculation (PI)

Ten thousand metacyclic promastigotes in 10 μl of PBS were injected into the ear dermis of C57BL/6 and DBA/2 mice. Increased ear thickness was measured using a direct reading Vernier caliper (Thomas Scientific, Swedesboro, NJ) and expressed as ear thickness.

Preparation of BMD-DLs

DLs were differentiated from bone marrow cells of DBA/2 or C57BL/6 mice according to a method described previously [18,19]. Briefly, bone marrow cells were seeded at 4×10^6 cells per 100 mm diameter bacteriological grade Petri dish (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in 10 ml of Iscove’s modified Dulbecco’s medium (IMDM; BioWhittaker Europe, Verviers, Belgium) supplemented with 10% heat-inactivated foetal calf serum (FCS; Dutscher, Brumath, France), 1.5% supernatant from the GM-CSF producing J558 cell line, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-mercaptoethanol and 2 mM glutamine. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO2. On day 6, suspended cells were recovered and further cultured in complete IMDM supplemented with 10% of the primary culture supernatant before seeding on day 10 in micro-organism-DL ratios of 5:1 and 10:1, respectively. DL cultures were placed at 34°C and sampled at 24 hours post micro-organism addition. Recovered DLs were incubated first in PBS-FCS supplemented with 10% heat-inactivated donkey serum for 15 minutes, second in PBS containing 10% FCS and 0.01% sodium azide in presence of antibodies directed against surface antigens. Extracellular staining procedures were performed with specific monoclonal antibodies (mAbs) directed against MHC class II molecules (M5/114 clone) conjugated to PE-CY5 (0.2 μg/ml) and either of the following biotinylated mAbs directed against CD86 (GL1 clone), CD80 (K-10A1 clone), CD54 (3E2 clone), CD11c (HL3) and IgG control (B81-3 clone) at 0.5 μg/ml (eBioscience, San Diego, USA). Biotinylated mAbs were revealed using 1.5 μg/ml Streptavidin-conjugated to Phycoerythrin (Molecular Probes, Cergy Pontoise, France). PE-conjugated mAb directed against CXCR4 (2B11 clone) was purchased from eBioscience. Analysis was performed on the FACS Calibur. DLs were selected on FSC-SSG parameters (to excluded debris), and on the basis of the MHC class II expression

Immuno-staining for flow cytometric analyses

On day 4 post the distribution of DLs in the 6 well plate culture, DLs were exposed or not to freshly isolated DsRed2-LV79 amastigotes or to live BCG at micro-organism-DL ratios of 5:1 and 10:1, respectively. DL cultures were placed at 34°C and sampled at 24 hours post micro-organism addition. Recovered DLs were incubated first in PBS-FCS supplemented with 10% heat-inactivated donkey serum for 15 minutes, second in PBS containing 10% FCS and 0.01% sodium azide in presence of antibodies directed against surface antigens. Extracellular staining procedures were performed with specific monoclonal antibodies (mAbs) directed against MHC class II molecules (M5/114 clone) conjugated to PE-CY5 (0.2 μg/ml) and either of the following biotinylated mAbs directed against CD86 (GL1 clone), CD80 (K-10A1 clone), CD54 (3E2 clone), CD11c (HL3) and IgG control (B81-3 clone) at 0.5 μg/ml (eBioscience, San Diego, USA). Biotinylated mAbs were revealed using 1.5 μg/ml Streptavidin-conjugated to Phycoerythrin (Molecular Probes, Cergy Pontoise, France). PE-conjugated mAb directed against CXCR4 (2B11 clone) was purchased from eBioscience. Analysis was performed on the FACS Calibur. DLs were selected on FSC-SSG parameters (to excluded debris), and on the basis of the MHC class II expression

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to discard the fraction of “contaminating” cells expressing no surface MHC class II molecules.

Intracellular staining of amastigotes was performed after fixation in PBS containing 1% paraformaldehyde (PFA) for 20 minutes at 4°C with the 2A3-26 mAb which was shown to strictly bind to the L. amazonensis amastigote [18]. DLs were washed in Perm/Wash solution from the BD Cytofix/Cytoperm™ Plus Kit (BD Bioscience) and incubated with 5 μg/ml of Alexafluor 488- conjugated 2A3-26 mAb in Perm/Wash buffer for 30 minutes at 4°C in the dark. Then DLs were washed in Perm/Wash buffer and fixed with in PBS − 1% paraformaldehyde (PFA).

**Immuno-staining for microscopic observations**

DLs were exposed or not to freshly isolated DsRed2-LV79 amastigotes at a parasite -DL ratio of 5:1. DL cultures were placed at 34°C and sampled at 5, 24, and 48 hours post parasite addition. Detached DLs were centrifuged on poly-L-lysine-coated glass coverslips and incubated at 34°C for 30 minutes. Cells were then fixed with 4% PFA for 20 minutes, permeabilised with saponin and incubated with 10 μg/ml of the amastigote-specific mAb 2A3-26-AlexaFluor 488 and 1 μg/ml of biotinylated-mAb (M5/114) directed against MHC class II molecules. The revelation was performed using 1.5 μg/ml streptavidin conjugated to Texas Red (Molecular Probes, Cergy Pontoise, France). Finally, they were mounted on glass slides with Hoechst 33342-containing Mowiol. Incorporation of Hoechst into DNA allowed the staining of both host cell and amastigote nuclei. Epifluorescence microscopy images were acquired on an upright microscope Zeiss Axioplan 2 monitored by the Zeiss Axiovision 4.4 software.

**Cell sorting in BSL2 containment, RNA extraction and integrity quality control**

DsRed2-LV79 amastigotes were added or not to cultures of C57BL/6 and DBA/2-DLs. Twenty four hours later, three samples collected from three distinct cultures of either unexposed DLs or DLs exposed to DsRed2-LV79 amastigotes were carefully sorted as previously described by Leccour et al. [20]. Briefly cells were first incubated in PBS-FCS containing 0.2 μg/ml of the anti-MHC class II mAb (M5/114) conjugated to PE-Cy5-conjugated mAb (eBioscience). After two washes, cells were resuspended at 5 x 10^6 cells/ml in PBS containing 3% FCS and 1% J558 supernatant. The cell sorting was performed using a FACSaria (BD Biosciences, San Jose, CA) equipped with completely sealed sample injection and sort collection chambers that operate under negative pressure. PE-Cy5 and DsRed2 fluoresences were collected through 695/40 and 576/26 bandpass filters respectively. FSC and SSC were displayed on a linear scale, and used to discard cell debris with the BD FACSDiva software (BD Biosciences) [17]. L. amazonensis amastigote-hosting DLs were sorted by selecting cells expressing both surface MHC Class II molecules and DsRed2 fluorescence and immediately collected for RNA extraction by using the RNeasy Plus Mini-Kit (Qiagen) as previously described [21]. However, the readout assays-Affymetrix or RT-qPCR - the RNA populations used were prepared from the same samples. The quality control (QC) and concentration of RNA were determined using the NanoDrop ND-1000 microspectrophotometer (Kisker, http://www.kisker-biotech.com) and the Agilent-2100 Bioanalyzer (Agilent, http://www.chem.agilent.com).

**GeneChip hybridization and data analysis**

Two hundred ng of total RNA per sample were processed, labelled and hybridized to Affymetrix Mouse Gene ST 1.0 arrays, following Affymetrix Protocol (http://www.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf).

Three Biological replicates per condition were run. Following hybridization, the arrays were stained and scanned at 532 nm using an Affymetrix GeneChip Scanner 3000 which generates individual CEL files for each array. Gene-level expression values were derived from the CEL file probe-level hybridization intensities using the model-based Robust Multichip Average algorithm (RMA) [22]. RMA performs normalization, background correction and data summarization. An analysis is performed using the LPE test [23] to identify significant differences in gene expression between parasite-free and parasite-harbouring DLs, and a p-value threshold of p<0.05 is used as the criterion for significant differential expression. The estimated false discovery rate (FDR) was calculated using the Benjamini and Hochberg approach [24] in order to correct for multiple comparisons. A total of 1,340 probe-sets showing significant differential expression were input into Ingenuity Pathway Analysis software v3.5.1 (http://www.ingenuity.com), to perform a biological interaction network analysis. The symbols of the modulated genes are specified in the text (fold change [FC] values between brackets), while their full names are given in additional file 1. MIAME-compliant data are available through GEO database http://www.ncbi.nlm.nih.gov/geo/ accession GSE.

**Validation of microarray analyses by RT-qPCR**

Total RNAs from DLs cultures were reverse-transcribed to first strand cDNA using random hexamers (Roche Diagnostics) and Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Life Technologies). A SYBR Green-based real-time PCR assay (Quantı́Tect SYBR Green Kit, Qiagen) for relative quantification of mouse target genes was performed on a 384-well plate LightCycler 480 system (Roche Diagnostics). Crossing Point values (Cp) were determined by the second derivative maximum method of the LightCycler 480 Basic Software. Raw Cp values were used as input for qBase, a flexible and open source program for qPCR data management and analysis [25]. Relative expression for 8 transcripts (cd2, e177, ccl19, cost, ccl2, cxcr4, ad274, tsp5f4) were calculated for sorted LV79-hosting DLs using sorted DLs from Leishmania unexposed cultures as calibrators. For normalization calculations, candidate control genes were tested (pgk1, HPd, ldha, nono, g6pd, icps, tthp, lyt1, gdpd, gmpdh, ychaz and ywhaz) with the geNorm [26] and Normfinder programs [27]. Tthp and nono were selected as the most stable reference genes for the C57Bl/6 DLs. RpIIf and tthp were selected for the DBA/2 DLs.

**Transcriptional analysis in tissues by RT-qPCR**

At day 4 and 7 post the inoculation of 10^4 metacyclic promastigotes, three mice were sacrificed, the abundance of some transcripts being determined by real time RT-qPCR. Control, naive mice were analyzed in parallel. Whole ear pinnas and ears-DLN were removed and fragmented using the Precelys 24 System [21]. Total RNAs were extracted and processed for RT-qPCR as described above. Ldhb and nono were selected as the most stable reference genes for the C57Bl/6 and DBA/2 ears. tthp and nono were selected for the as the most stable reference genes for C57Bl/6 DLNs while ychaz and nono were selected for the DBA/2-DLNs.

**Leishmania quantification in ears and ears-DLN**

The experimental procedure for quantifying Leishmania in tissues was done as previously described by de La Llave et al [21]. Briefly, serial 10-fold dilutions of parasites (10^9 to 10^0) were added to either ears or car-DLN recovered from C57Bl/6 or DBA/2 naive...
mice. Total RNAs were extracted and processed for RT-qPCR as described above. The primers for Leishmania gene target (ssrRNA) to quantify the number of parasites were F- CCATGTCG-GATTTTGAT and R- CGAAGCCTAGCCTAGAG [28]. A linear regression for each standard curve was determined; number of parasites against the relative expression of ssrRNA values.

**Statistical analyses**

Two-sided Student’s paired t-tests were used to compare FCM experiments (4<n<6). A Mann-Whitney test was used to compare ear thickness measurements and number of parasites.

**Results and Discussion**

*L. amazonensis* amastigotes set up in distinct dermis niche post inoculation in C57BL/6 and DBA/2 mice

C57BL/6 and DBA/2 mice were given into the ear pinna dermis a low number (10^4) of *L. amazonensis* (LV79 strain) metacyclic promastigotes. The monitoring of ear macroscopic features up to 100 days post inoculation (PI) has evidenced mouse inbred strain-specific features (Figure 1). C57BL/6 mice did not display any significant inflammatory signs during the early phase (ranging from day 0 to day 22 PI, phase 1), whereas they later display sustained inflammatory signs (after 22 days, phase 2; figures 1A, 1B). During the early phase, only a few parasites can be quantified in the ear pinna, the ear pinna-DLN displaying lower figures 1A, 1B). During the early phase, only a few parasites can be displayed any significant inflammatory signs during the early phase features up to 100 days post inoculation (PI) has evidenced mouse inbred strain-specific features (Figure 1). C57BL/6 mice did not display any significant inflammatory signs during the early phase (ranging from day 0 to day 22 PI, phase 1), whereas they later display sustained inflammatory signs (after 22 days, phase 2; figures 1A, 1B). During the early phase, only a few parasites can be quantified in the ear pinna, the ear pinna-DLN displaying lower number of parasites (10^4 parasites/DLN; figure 1C). In contrast, in DBA/2 mouse ear pinna, a mild inflammatory process was observed immediately post the inoculation whereas a rapid increase of the amastigote population size was noted in both the ears and ears-DLN. The second phase was delineated by the persistence of inflammatory process (Figure 1) coupled to the control of parasite load in the ear pinna and ear-DLN (data not shown).

We reasoned that early distinct DLs-dependent immune processes- promoting either rapid or slow remodeling of the dermis as amastigote-protective niches- could account for the distinct features displayed, over time, by the *L. amazonensis* amastigotes-hosting ear pinna of the C57BL/6 and DBA/2 mice. Being aware that, whatever the tissues, the DL frequency is very low, we considered biologically sound to start the comparative analysis with GM-CSF-dependent C57BL/6 or DBA/2 cultured DLs, once they were hosting, or not, live *L. amazonensis* amastigotes. Briefly, C57BL/6 and DBA/2 bone marrow cell suspensions were exposed or not to live DsRed2 *L. amazonensis* amastigotes and carefully sorted from otherwise heterogeneous cultures. The immunolabelling of surface MHC class II molecules was evidenced common transcripts of C57BL/6 and DBA/2 DLs such whatever the DL genetic background (Figure 3C). We first evidenced common transcripts of C57BL/6 and DBA/2 DLs such as *arg1* (17%), monoamine oxidase A (*maoa*) and spermidine/spermine N1-acetyltransferase 1 (*sat1*) were upregulated (Figure 3B). These analyses being set up, we found biologically relevant to highlight some components of the early immune shaping of C57BL/6 versus DBA/2 ear pinna as unique skin sites where the

A reliable *in vitro* model for comparing features of C57BL/6 and DBA/2 DLs harbouring *L. amazonensis* amastigotes

We used a carefully designed *in vitro* model [20] based on cultures of mouse BMD-DLs in which more than 97% of cells expressed CD11c, CD11a and CD11b (data not shown). When the presence/absence of surface MHC class II molecules was monitored on whole cell cultures by fluorescence microscopy and FCM, three phenotypically distinct cell subsets were evidenced (Figures S1A–C). The population of cells that did not express surface and intracellular MHC class II molecules were considered as “Contaminating” Cells (CC). The two other cell populations partition between i) a majority of cells displaying a moderate surface MHC class II amount (MHC II<sub>low</sub>; bona fide mature DLs) and ii) a minority of cells expressing very high levels of MHC II molecules (MHC II<sub>high</sub>; bona fide mature DLs). DsRed2 *L. amazonensis/LV79* amastigotes were put in contact with BMD-DLs (MOI of 5/1) and analysed 3, 24 or 48 hours later (Figure 2). Intracellular amastigotes (2A3-*26*) detected by immunofluorescence microscopy analysis were evidenced in all BMD-DL subsets with much higher number of amastigotes in CC (data not shown). Low percentages of DLs hosting 2A3-*26* parasites were also documented by FCM analyses at 24 hours post amastigote addition (23.0%+/−12.6 and 26.0%+/−8.1 of 2A3-*26* cells in C57BL/6 and DBA/2 BMD-DLs, respectively, for n = 9 experiments). Interestingly, while the percentage of DLs housing amastigotes did not change from 5 hours to 24 hours (Figure 2A), the number of intracellular amastigotes did slowly expand whatever the mouse genotype (Figure 2B) over the otherwise limited temporal window we did focus on. *L. amazonensis* amastigote-hosting DLs were sorted by selecting cells expressing both surface MHC Class II molecules and DsRed2 fluorescence (see below).

Distinct signatures displayed by sorted C57BL/6 and DBA/2 DLs housing live *L. amazonensis* amastigotes

Gene expression profiles of DLs hosting or not live amastigotes. We next performed a genome-wide transcriptional analysis by comparing the gene expression profiles of control DLs unexposed to *Leishmania* amastigotes and live amastigote-hosting DLs from both mouse strains. To provide a robust comparative analysis and allow generating a detailed picture of the distinct features of C57BL/6 and DBA/2 DLs, a special attention was given to specifically gated or sorted cells expressing class II molecules (Figures S1A, S1C; subsets MHC II<sup>low</sup> and MHC II<sup>high</sup>). Despite their low frequency, MHC positive DLs hosting live parasites were easily sorted by using the detection of fluorescent DsRed2 protein expressed in transgenic *L. amazonensis* amastigotes [17] (Figure 3A). Affymetrix analyses performed on total RNAs revealed that sorted DLs hosting DsRed2 LV79 amastigotes displayed discrete transcriptional modifications compared to control BMD-DLs, both in term of modulated gene numbers and of modulation magnitude. Out of 28,853 mouse genes, 858 and 932 were captured with a differential expression at the 5% significance level in C57BL/6 and DBA/2 DLs, respectively (Figure 3B). Of these, 450 were common between the two mouse strains (Figure 3B). A similar fold-change distribution was observed whatever the DL genetic background (Figure 3C). We first evidenced common transcripts of C57BL/6 and DBA/2 DLs such as those involved in arginine metabolism which could be play an important role in the early parasite multiplication within DLs from both strains (Figure S2). Indeed, transcripts for key enzymes involved in pathway of arginine metabolism such as Arginase-1 (*argl*), monoamine oxidase A (*maoa*) and spermidine/spermine N1-acetyltransferase 1 (*sat1*) were upregulated (Figure S2). These modulations were also confirmed by the gold standard approach of RT-qPCR (data not shown). Consequently, *L. amazonensis* amastigotes probably sustain a metabolic flux that promotes the biosynthesis of polyamines to facilitate the intracellular amastigote multiplication which was evidenced as soon as 24 hours post-addition of amastigotes (Figure 2B).

These analyses being set up, we found biologically relevant to focus on distinct transcriptional signatures. Such a choice allows highlighting some components of the early immune shaping of C57BL/6 versus DBA/2 ear pinna as unique skin sites where the
amastigote population size increase is respectively slow or rapid. Curiously, while a delayed amastigote growth profile is coupled to a clinically silent process in the C57BL/6 ear pinna, the rapid amastigote growth onset in the DBA/2 mouse ear pinna is coupled to a mild inflammatory process.

**Distinct transcriptional signatures of C57BL/6 and DBA/2 DLs hosting live amastigotes.** We focused our attention on two different—though temporally linked families—of transcriptional signatures that were unique to each mouse: i) transcripts related to DL adhesion and de-adhesion, the expected outputs being distinct local live amastigote-hosting DL dissemination within the dermis and distinct emigration of live amastigote-hosting DL from the ear to the ear-DLN and ii) transcripts linked to the interactions between DLs and non DL leucocytes, a special attention being given to T lymphocytes which are known to be the main partners of DLs in the LNs draining any peripheral upstream tissues.

**Signatures that could contribute to distinct leucocyte mobilization within the ear pinna.** The mild inflammation displayed from day 4 onward post metacyclic promastigotes in DBA/2 ears is likely assessing complex processes. Affymetrix-based analyses revealed complex patterns of transcriptional modulations of pro-inflammatory chemokines and chemokine receptors that can promote the recruitment and/or persistence of different leucocyte lineages in the dermis, contributing to the rapid amastigote establishment and expansion (Figure S3). These transcript modulations in DBA/2 DLs included i) cxcl4 (+2.88) for the maintenance of senescent neutrophils and the recruitment of monocytes [29], ii) ccl2 (+3.38; RT-qPCR: +22.2), ccl3 (+4.58), ccl4 (+2.58), ccl7 (+2.57) and ccl19 (+1.16; RT-qPCR: +6.8) [30] for the recruitment of NK lymphocytes, and iii) ccl2 (+3.38) [31,32] for the recruitment of monocytes/macrophages [31,32]. By contrast, we evidenced a weak modulation of these transcripts in C57BL/6 DLs which could be related to the absence of inflammatory process during the early phase in mice.

These variations may be associated to modulation of transcripts which encode for molecules involved in the invasive properties of DLs hosting live amastigotes within the skin. The dermal extracellular matrix (ECM) features are among the contributors to the DL invasiveness. Different metallo-proteinases known to act on the dermis ECM proteins could locally remodel the ECM facilitating DL migration through the remodelled ECM. Thus, among the transcript data sets, a particular attention was given to

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**Figure 1. Distinct ear pinna features and amastigote population size values in C57BL6 and DBA/2 mice.** Ten thousand LV79 metacyclic promastigotes were inoculated into the ear dermis of C57BL/6 (n=41) and DBA/2 (n=65) mice. A–C) Ear thickness was monitored post LV79 inoculation (black dots) or not (white dots) in ears up to 80 days for C57BL/6 mice and up to 100 days for DBA/2 mice. Results are expressed as means and standard deviations. * indicates significant p values between inoculated and un-exposed/control ears. Phase 1 (day 0 to day 22 PI) and phase 2 (post day 22) are depicted. B, C) Mean delta of ear thickness and parasite load in C57BL/6 and DBA/2 mice are displayed over the phase 1 (day 4 to day 22). (B) – The delta of ear thickness was obtained from inoculated ear thickness measures of all mice from day 4 to day 22 minus ear thickness values of unexposed/control mice. (C) Mean amastigote load quantification in ears and ears-DLNs of C57BL/6 and DBA/2 mice by RT-qPCR (n = 12 mice) over the phase 1. Means and SDs are shown. * p<0.002.

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transcripts coding the Matrix Metallo Proteinases/MMPs. In DBA/2 DLs hosting live amastigotes, transcripts encoding for MMP12 (+1.45) and MMP2 (+2.53) were specifically increased. MMP12 has potent ECM remodelling properties due to its specific elastolytic activity, but may also participate to the inflammatory process through the activation of TNF [33]. Moreover, MMP12 presents potent direct pro-inflammatory properties including the ability to induce neutrophil influx, cytokine and chemokine production [34]. Finally MMP2 was also shown to activate the IL-1 precursor to the active form [35,36]. Altogether, these DBA/2-specific transcriptional modifications could favour a local but mild inflammation that could be critical for the further development of an effective immunological control of Leishmania amastigote population size. By contrast, in C57BL/6 DL-hosting live amastigotes, despite the up regulation of ccl2 (+1.90; RT-qPCR +6.34) and ccl3 transcripts (+3.61), the concomitant down modulation of transcripts coding for inflammatory cytokine receptors IL-1R2 (-1.94), II-1RII (-2.38) and IL2Rα (-1.99) and the up regulation of IL1r1 (+1.69) may account for the absence of clinically detectable inflammatory process during the phase 1.

Distinct transcriptional signatures that could contribute to a distinct emigration from the ear pinna to the DLN of live amastigotes-hosting DLs. The modulation of transcripts encoding for so called “classical maturation markers” such as CD54 and CD80 and MHC class II molecules was not observed in DBA/2 and C57BL/6 DLs post contact with living amastigotes. This state of “immature-like phenotype” of DLs has been confirmed by the absence of surface expression modulation of these classical maturation markers in live amastigotes-hosting DLs. This apparent lack of maturation process strongly contrasted with the “full maturation” of DLs exposed to live BCG [Figures 4A, 4B]. Of note, the absence of modulation of these markers has also been evidenced in the 2A3-26 (amastigote loaded) versus 2A3-26 (amastigote-free) DLs present in the same culture (Figures 4C, 4D).

However, we noticed transcriptional signatures of alternative DL maturation pathways: for instance, in DBA/2 DLs hosting live amastigotes, we evidenced a strong increase in transcripts coding for CXCR4 molecules (+2.47; RT-qPCR+9.05) which may drive the “maturing” DLs first towards the lymphatic vascular bed and then into T cell areas within the lymph nodes (supporting data S5). This observation was confirmed by RT-qPCR (Figure 5A) and importantly also at the protein level by FACS analysis (Figure 5B).

In addition, as already mentioned, ccl9 (+1.38; RT-qPCR 1.34) transcripts was observed in DBA/2 DLs that could account DL sensitization to CXCR4-dependent migration from the skin to DLNs [37]. Interestingly, an up-regulation of ccr4 transcripts was also evidenced in DBA/2 ears and ear-DLNs sampled at day 4 and 7 post L. amazonensis inoculation (Figure S3; figure 5C). Moreover an up regulation of ccl17 (+1.88; RT-qPCR 2.13) were observed in DBA/2 DLs: the higher abundance of ccl19 transcripts probably result from the increased CCL2 production (+3.38; RT-qPCR +2.2) and CCL2 ligation to its receptor (CCR2) [39]. Moreover, in addition to the up-regulation of ccr4 transcripts (+2.53) [40] we evidenced the up regulation of transcripts encoding for CXCL14 (+2.97) a potent chemo-attractant and activator of DLs which might be involved in DL homing in vivo [41,42]. We also evidenced other transcripts such as cdc42ep (+1.62) coding for the CDC42EP2 which acts downstream of the Rho GTPase CDC42 to induce pseudopodia formation and play a decisive role in DL migration in vivo through adequate coordination of both cortical and non-cortical cytoskeletal flow [43]. Additionally we noted an increase of transcripts encoding for the GTPase activating protein ArhGAP10 (+2.15) which functions as GTPase-activating protein for Cdc42 and F-actin dynamics at the level of Golgi apparatus [44]. All together these results seem to indicate a modulation of components that...
sequentially elicit key changes in dynamics and in the architecture of cells, allowing coupled migration and maturation of DBA/2 live amastigotes-hosting DLs.

By contrast, we evidenced a modulation of small number of transcripts in C57BL/6 BMD-DLs. Thus, \textit{ccr2} transcript was downregulated (21.68; RT-qPCR: 22.08), without any change in \textit{ccl19} transcript abundance, suggesting that no maturation could be initiated in those live amastigote-hosting DLs. We also noted a significant down modulation of transcripts encoding DC-SIGN (\textit{cd209c}; 22.21), a lectin otherwise known to be involved in DL-neutrophil cross-talks, that could prevent the Mac-1 and CEACAM1-displaying neutrophil-dependant DL maturation [45]. Moreover, DC-SIGN is also involved in the specific migratory processes of DLs, through the binding to ICAM-2 which is abundantly expressed by blood and lymphatic vascular bed [46].

Thus, all together these in vitro generated data highlight transcriptional signatures that may contribute to the distinct features observed in C57BL/6 versus DBA/2 ear pinna and in the ear pinna-DLNs during the first phase post \textit{L. amazonensis} inoculation.

**Distinct transcriptional signatures that could contribute to unique communication with T lymphocytes.** DLs represent the most potent source of co-signaling molecules active on naive as well as on T natural regulatory T lymphocytes (Treg). In peripheral tissues, once they have sensed microbial or endogenously derived agonists, DLs generally migrate to the T cell area in DLNs. The distinct values of live amastigote population size observed in the ear pinna of C57BL/6 and DBA/2 mice from days 4 to 22 might reflect distinct priming and or effector/regulatory functions of CD3 T lymphocytes. Indeed, while a pronounced up regulation of transcripts encoding for CD4 and CD8\textbeta at day 4 PI (Table 1) was detected in the amastigote-hosting DBA/2 ears, no modulation of these transcripts was detected in amastigote-hosting C57BL/6 ears. We further considered the above features by focusing on any signalling molecules-encoding transcripts otherwise known to act on naive T lymphocytes that cross the HEV of the ear- DLN and migrate through the secondary lymphoid organ matrix. IL-7 (+1.69) and IL-7R\alpha (+1.58)-encoding transcripts were found positively modulated in DBA/2 DLs. Since DBA/2 DL survival could also be favoured by...
CXCR4 engagement (+2.47) [47], they could support, through IL-7 and MHC Class II co-expression, the proliferation of both i) CD4+ T lymphocytes [48,49,50,51] and ii) CD8+ T lymphocytes [52]. Interestingly, in ears from DBA/2 mice, natural Treg transcripts were detected at day 4 PI (Table 1; foxp3+6.7+/24.8), a feature that could reflect communication with live amastigotes-hosting DLs: indeed not only the cd200r3 transcript encoding for a receptor shown to be expressed on regulatory DLs was not down regulated [53] but transcripts encoding for chemokines otherwise known to recruit Tregs (ccl17+1.88; RT-qPCR +1.34) were up-regulated. Altogether, in DBA/2 ears, from day 4 to day 22, the parasite load increase could assess the unique communication between regulatory DL and regulatory T lymphocytes. By contrast in ears of C57BL/6 mice, not only no up regulation of foxp3 transcripts was evidenced at day 4 PI but alternatively two interesting modulations were documented in BMD-DLs i) the down regulation of cd200r3 transcripts (-2.21) and ii) the up regulation of transcripts encoding for TNFsf4 (OX40L; +1.66; RT-qPCR 7.34), a factor shown to inhibit the induction of iTReg cells [54]. The down modulation of de-sign transcripts (cd209c; -2.21) could also result in abortive DL-T cell interactions, the binding of DL DC-SIGN to lymphocyte ICAM-3 being impeded [55]. Together with the up-modulation of tnfrsf23 transcripts (+1.94) and down modulation of icosl (cd275; -1.56) transcripts, the DC-SIGN regulation could prevent T lymphocytes to be properly activated, thus limiting their proliferation and the developmental process completion to end-stage lymphocytes displaying either effector or regulatory functions [56,57].

In conclusion, the more or less rapid and long term establishment of parasites such as L. amazonensis amastigotes, otherwise known to strictly rely on subversion of macrophage and dendritic leucocyte lineages, is expected to reflect stepwise and complex processes taking place in both i) the skin dermis where were inoculated their flagellated ascendants-the metacyclic promastigotes- ii) and the skin-DLN. Relying on mice of two distinct inbred strains- C57BL/6 and DBA/2- that rapidly
and durably display distinct phenotypes at the two sites of establishment of *L. amazonensis* amastigotes, we were curious to address the following question: could live *L. amazonensis* amastigotes-hosting DL display unique signatures which account for the distinct phenotypes?

Whatever the mouse genotype origin of the BMD-DLs, the *L. amazonensis* amastigotes establish themselves similarly a phenotype that reflects similar subversion of the DL arginine-dependent pathway. Thus, comparing the other transcriptional profiles as that reflects similar subversion of the DL arginine-dependent pathway. Thus, comparing the other transcriptional profiles as well as the expression of some key proteins allowed highlighting a promising set of transcripts that could account for a more rapid onset of the amastigote population expansion in the C57BL/6 than in the DBA/2 mouse ear pinna post the intra-dermal inoculation of 10^5 *L. amazonensis*. Our results did evidence that once subverted as cells hosting live *L. amazonensis* amastigotes, DLs from C57BL/6 or DBA/2 differently expressed transcripts involved in recruitment of other non-dendritic leukocytes as well as in the remodelling of the dermis extracellular matrix (Figure 6).

The early invasiveness-related phenotype displayed by DBA/2 BMD-DLs could account for the early onset of the amastigote population expansion in the ear pinna. Moreover, the up regulation of the genes encoding chemokines and chemokine receptors suggested that DBA/2 BMD-DLs would be more responsive to chemotactic gradients and thus amenable to enter into afferent lymphatics.

| Table 1. Monitoring of transcript abundance in the C57BL/6 and DBA/2 ear pinnas collected at day 4 and 15 post inoculation/pi of *L. amazonensis*. |
|---|---|---|---|---|
| FC in transcripts | C57BL/6 | DBA/2 |
| ear pinna | Day 4 | Day 15 | Day 4 | Day 15 |
| cd4 | 1.18 ± 0.57 | 0.71 ± 0.49 | 4.7 ± 2.2 | 0.66 ± 0.07 |
| cd8ji | 0.34 ± 0.6 | 1.02 ± 0.56 | 18.8 ± 2.6 | 4.64 ± 2.2 |
| foxp3 | 1.24 ± 0.33 | 0.88 ± 0.13 | 6.7 ± 4.8 | 0.35 ± 0.31 |
| cxcr4 | 0.79 ± 0.12 | 0.43 ± 0.42 | 3.13 ± 2.2 | 0.55 ± 0.11 |

Ten thousand metacyclic promastigotes of *L. amazonensis* were inoculated into ear pinna dermis of C57BL/6 and DBA/2 mice. At days 7 and 15 post-inoculation, the ear pinnas were removed and processed for RNA isolation. Transcript abundance was determined by real-time RT-qPCR. Results are indicated as Fold Changes (FC) unexposed ears being used for calibrator for the calculation of relative expressions. One representative out of 3 independent experiments with three ears that were individually processed the means and SD being displayed.
Though we did/do not ignore the limitations of in vitro generated DLs, we were expecting that once, sorted on the basis of their status of phagocytic leukocytes hosting live *L. amazonensis* amastigotes, and depending from their mouse genotype origin, these DLs will allow extracting unique signatures that dictate the balance between pro-inflammatory and counter-inflammatory agonists. The latter would operate, within the skin and the skin-DLN, to both i) prevent progressive and irreversible skin damages and ii) promote the rapid skin remodelling as a niche where *L. amazonensis* amastigotes will reach the developmental stage that allows their persistence. Whatever the DL subsets either present or recruited in the dermis, such a rapid reprogramming of *L. amazonensis* amastigotes-hosting DLs is expected to operate: indeed the live amastigotes-derived agonists detected by DL sensors are likely the agonists that dominate over all the other endogenously agonists that allow delineating the distinct lineage subsets that populate the dermis. Thus despite the limitations highlighted above, once any discrete sub-phenotypes have been delineated with quantitative parameters, high content transcriptional profiles emerging from in vitro generated populations of DL hosting live *L. amazonensis* amastigotes become solid resources for further biologically sound in vivo investigations.

**Figure 6. The distinct transcriptional signatures of BMD-DLs housing live LV79 amastigotes.** Authors’ interpretation of key distinct early events is depicted. Are highlighted five immune processes including DL property to recruit leucocytes and initiate inflammatory processes DL invasiveness (1, 2) and migration (3, 4) DL property to communicate with T lymphocytes (5).
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IIlow immature-like DL/iDL. The lower panel shows a DL and the one in medium panel is representative of a MHC class IiDLs, i.e. cells that do express MHC class II molecules at low or high level. (B) upper panel corresponds to a MHC class II high semi-mature-like in vivo red gate in panel (A). Median and standard deviations are shown (n = 5 independent experiments).

References

1. Barral A, Petersen EA, Sacks DL, Neva FA (1983) Late metastatic Leishmaniasis in the mouse. A model for mucocutaneous disease. American Journal of Tropical Medicine & Hygiene 32: 277–285.
2. Murray HW, Berman JD, Davies CR, Saravia NG (2005) Advances in leishmaniasis. Lancet 366: 1561–1577.
3. Silveira FT, Lainson R, Shaw JJ, De Souza AA, Ishikawa EA, et al. (1991) Cutaneous leishmaniasis due to Leishmania (Leishmania) amazonensis in Amazonian Brazil, and the significance of a negative Montenegro skin-test in human infections. Trans R Soc Trop Med Hyg 85: 735–738.
4. Afonso LC, Scott P (1995) Immune responses associated with susceptibility of C57BL/6 mice to Leishmania amazonensis. Infect Immun 61: 2952–2959.
5. Courret N, Lang T, Milton G, Antoine JC (2003) Intradermal inoculations of low doses of Leishmania major and Leishmania amazonensis metacyclic promasti- gotes induce different immunoparasitic processes and status of protection in BALB/c mice. Int J Parasitol 33: 1373–1383.
6. Jones DE, Buxbaum LU, Scott P (2000) IL-4-independent inhibition of IL-12 responsiveness during Leishmania amazonensis infection. J Immunol 165: 364–372.
7. Soong L, Chang CH, Sun J, Lougley BJ, Jr., Ruddle NH, et al. (1997) Role of CD4+ T cell in pathogenesis associated with Leishmania amazonensis infection. J Immunol 158: 5374–5383.
8. Soong L, Xu JC, Greaves IS, Kima P, Sun J, et al. (1996) Disruption of CD40-CD40 ligand interactions results in an enhanced susceptibility to Leishmania amazonensis infection. Immunity 4: 263–273.
9. Reed SG, Andrade ZA, Roters SB, Inverso JA, Sadigursky M (1986) Leishmania mexicana amazonensis infections in ‘resistant’ mice following removal of the draining lymph node. Clin Exp Immunol 64: 8–13.
10. Antoine JC, Prina E, Courret N, Lang T (2004) Leishmania spp.: on the interactions they establish with antigen-presenting cells of their mammalian hosts. Adv Parasitol 58: 1–68.
11. Baldwin T, Henri S, Curtis J, O’Keeffe M, Vremec D, et al. (2004) Dendritic cell polyamine pathways in LV79-housing DLs. Total RNAs were collected from the analyses of either LV79-hosting or control DLs are indicated for C57BL/6 (black text) and DBA/2 mice (grey text).

Author Contributions

Conceived and designed the experiments: EG HL GM EP TL. Performed the experiments: EG HL GS TL. Analyzed the data: EG HL GS TL. Contributed reagents/materials/analysis tools: GS JYC. Wrote the paper: EG HL GM EP TL.
29. Dreul TF, Senior RM, Chaud D, Griffin GL, Heinrikson RL, et al. (1981) Platelet factor 4 is chemotactic for neutrophils and monocytes. Proc Natl Acad Sci U S A 78: 4584–4587.

30. Muller K, van Zandbergen G, Hansen B, Laufo H, Jahnke N, et al. (2001) Chemokines, natural killer cells and granulocytes in the early course of Leishmania major infection in mice. Med Microbiol Immunol 190: 73–76.

31. Gu L, Rutledge B, Fierro I, Ernst C, Grewal I, et al. (1997) In vivo properties of monocyte chemoattractant protein-1. J Leukoc Biol 62: 577–580.

32. Serbina NV, Jia T, Hohl TM, Parner EG (2006) Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol 24: 421–452.

33. Churg A, Wang RD, Tai H, Wang X, Xie C, et al. (2004) Tumor necrosis factor-alpha drives 70% of cigarette smoke-induced emphysema in the mouse. Am J Respir Crit Care Med 170: 492–498.

34. Nenan S, Boichot E, Lagente V, Bertrand CP (2005) Macrophage elastase (MMP-12): a pro-inflammatory mediator? Mem Inst Oswaldo Cruz 100 Suppl 1: 167–172.

35. Schoube B, Mach F, Libby P (1998) Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. J Immunol 161: 3340–3346.

36. Yu WH, Weisner JF, Jr. (2000) Heparan sulfate proteoglycans as extracellular docking molecules for matrixins (matrix metalloproteinase 7). J Biol Chem 275: 4183–4191.

37. Stute S, Quast T, Gerbikzi T, Savinco T, Novak N, et al. (2010) Requirement of CCL17 for CCR7- and CXCR4-dependent migration of cutaneous dendritic cells. Proc Natl Acad Sci U S A 107: 8736–8741.

38. Sallusto F, Palermo B, Lenig D, Miettinen M, Matikainen S, et al. (1999) Distinct patterns and kinetics of chemokine production regulate dendritic cell function. Eur J Immunol 29: 1617–1625.

39. Jimenez F, Quinones MP, Martinez HG, Estrada CA, Clark K, et al. (2010) CCR2 plays a critical role in dendritic cell maturation: possible role of CCL2 and NF-kappa B. J Immunol 184: 5571–5581.

40. Ratzinger G, Stoitzner P, Ebner S, Lutz MB, Layton GT, et al. (2002) Matrix metalloproteinases 9 and 2 are necessary for the migration of Langerhans cells. FEBS Lett 579: 6159–6168.

41. Yu WH, Weisner JF, Jr. (1998) Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. J Immunol 161: 3340–3346.

42. Shurin GV, Ferris RL, Tourkova I, Perez L, Lokshin A, et al. (2005) Loss of new chemokine CXCL14 in tumor tissue is associated with low infiltration by dendritic cells (DC), while restoration of human CXCL14 expression in tumor cells causes attraction of DC both in vitro and in vivo. J Immunol 174: 5490–5498.

43. Lammermann T, Renkawitz J, Wu X, Hirsch K, Roakebusch C, et al. (2009) CCL12-dependent leading edge coordination is essential for interstitial dendritic cell migration. Blood 113: 5703–5710.

44. Dubois T, Chavrier P (2005) [ARHGAP10, a novel RhoGAP at the cross-road between ARF1 and Cdc42 pathways, regulates Arp2/3 complex and actin dynamics on Golgi membranes]. Med Sci (Paris) 21: 692–694.

45. van Gisbergen KP, Ludwig IS, Geijtenbeek TB, van Kooij Y (2005) Interactions of DC-SIGN with Mac-1 and CEACAM1 regulate contact between dendritic cells and neutrophils. FEBS Lett 579: 6159–6168.

46. Geijtenbeek TB, Krooshoop DJ, Bleis DA, van Vliet SJ, van Duijnhoven GC, et al. (2000) DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. Nat Immunol 1: 333–337.

47. Kabashima K, Sugita K, Shiraishi N, Tamamura H, Fujiy N, et al. (2007) CXCR4 engagement promotes dendritic cell survival and maturation. Biochem Biophys Res Commun 361: 1012–1016.

48. Guimond M, Veenvstra RG, Grinuler DJ, Zhang H, Cai Y, et al. (2009) Interleukin 7 signaling in dendritic cells regulates the homeostatic proliferation and niche size of CD4+ T cells. Nat Immunol 10: 149–157.

49. Kondrack RM, Harbertson J, Tan JT, McBreen ME, Surih CD, et al. (2003) Interleukin 7 regulates the survival and generation of memory CD4+ T cells. J Exp Med 198: 1797–1806.

50. Seddon B, Tomlinson P, Zamorska R (2003) Interleukin 7 and T cell receptor signals regulate homeostasis of CD4+ memory cells. Nat Immunol 4: 600–606.

51. Tan JT, Duddl E, LeRoy E, Murray R, Sprent J, et al. (2001) IL-7 is critical for homeostatic proliferation and survival of naive T cells. Proc Natl Acad Sci U S A 98: 8732–8737.

52. Schum KS, Karpf WC, Jameson SG, Lefrancois L (2000) Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol 1: 426–432.

53. Sato K, Ezumi K, Fukuya T, Fujita S, Sato Y, et al. (2009) Naturally occurring regulatory dendritic cells regulate murine cutaneous chronic graft-versus-host disease. Blood 113: 4780–4789.

54. Ishii N, Takahashi T, Soroho S, Sugamura K (2010) OX40-OX40 ligand interaction in T-cell-mediated immunity and immunopathology. Adv Immunol 105: 63–98.

55. Geijtenbeek TB, Toesremo R, van Vliet SJ, van Duijnhoven GC, Adema GJ, et al. (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 100: 575–585.

56. Kim J, La S, Kim BS, Kwon BS, Kwon B (2003) Newly identified [correction of identified] members of the TNF receptor superfamily (mTNFRH1 and mTNFRH2) inhibit T-cell proliferation. Exp Mol Med 35: 154–159.

57. Nurieva RI, Mai XM, Forbush K, Bevan MJ, Dong C (2003) B7h is required for T cell activation, differentiation, and effector function. Proc Natl Acad Sci U S A 100: 14163–14168.