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Original article

Sorting of *Leishmania*-bearing dendritic cells reveals subtle parasite-induced modulation of host-cell gene expression[☆]

Hervé Lecoœur^a, Emilie de La Llave^a, José Osorio Y Fortéa^a, Sophie Goyard^a,
Hélène Kiefer-Biasizzo^b, Anne-Marie Balazuc^b, Geneviève Milon^a,
Eric Prina^{a,1}, Thierry Lang^{a,*,1}

^a Institut Pasteur, Unité d'Immunophysiologie et Parasitisme Intracellulaire, Département de Parasitologie et Mycologie, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France

^b Institut Pasteur, Plateforme de Cytométrie en Flux, 25 rue du Dr. Roux, 75724 Paris cedex 15, France

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Abstract

Once in the mouse skin, *Leishmania (L) amazonensis* amastigotes are hosted by professional mononuclear phagocytes such as dendritic cells (DCs). When monitored after parasite inoculation, the frequency of amastigote-hosting DCs is very low (<1%) in both the skin and skin-draining lymph nodes. Therefore, we designed and validated an efficient procedure to purify live amastigote-hosting DCs with the objective to facilitate quantitative and qualitative analysis of such rare cells. To this end, a *L. amazonensis* transgenic parasite expressing *DsRed2* fluorescent protein was generated and added to mouse bone marrow-derived DC cultures. Then, a high speed sorting procedure, performed in BSL-2 containment, was setup to pick out only DCs hosting live amastigotes. This study reveals, for the first time, a unique transcript pattern from sorted live amastigotes-hosting DCs that would have been undetectable in unsorted samples. It was indeed possible to highlight a significant and coordinated up-regulation of L-arginine transporter and arginase2 transcripts in *Leishmania*-hosting DCs compared to un-parasitized DCs. These results indicate that arginine catabolism for polyamine generation is dominating over L-arginine catabolism for NO generation. In conclusion, this approach provides a powerful method for further characterisation, of amastigote-hosting DCs in the skin and the skin-draining lymph nodes. © 2009 Elsevier Masson SAS. All rights reserved.

Keywords: *Leishmania*; Mouse dendritic cells; Flow-sorting; BSL-2; *DsRed2*

1. Introduction

Leishmania (L) amazonensis is a protozoan parasite that is transmitted to the dermis of its mammalian host by blood-

feeding sand flies. Laboratory mice have been shown to be relevant hosts of this *Leishmania* species for *in vivo* and *ex vivo* analyses. Once in the dermis of the mouse, the metacyclic promastigotes enter dendritic cells (DCs) [1], where they differentiate into amastigotes within the parasitophorous vacuole. The stepwise deployment of both the migratory and signalling properties of DCs hosting *Leishmania* amastigotes within the tissues is still being investigated [2–5]. Thus, it is important to characterise the features of live amastigote-harboring DCs sorted under the most rigorous conditions. Bone marrow-derived DCs cultured in the presence of GM-CSF have been shown to host *L. amazonensis* amastigotes [1,6]. However, the heterogeneity of this DC population [7,8], as well as the low frequency ($\leq 12\%$) of amastigote-loaded DCs, have prevented us from addressing questions about the range

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* Corresponding author.

E-mail addresses: herve.lecoeur@pasteur.fr (H. Lecoœur), emilie.de-la-llave@pasteur.fr (E. de La Llave), Jose.OSORIOYFORTEA@limagrain.com (J. Osorio Y Fortéa), sophie.goyard@pasteur.fr (S. Goyard), helene.kiefer-biasizzo@pasteur.fr (H. Kiefer-Biasizzo), anne-marie.balazuc@pasteur.fr (A.-M. Balazuc), genevieve.milon@pasteur.fr (G. Milon), eric.prina@pasteur.fr (E. Prina), thierry.lang@pasteur.fr (T. Lang).

¹ EP and TL contributed equally to this work.

of functions these infected DCs could display. Therefore, to overcome these limitations, we designed and validated a reliable protocol to purify live *Leishmania*-hosting DCs. The present study describes the different steps of the procedure, from the generation of transgenic *L. amazonensis* expressing the fluorescent *DsRed2* molecule (*DsRed2-LV79*) [9] to the optimal use of the FACSAria cell sorter in biosafety level 2 (BSL-2) containment [10,11].

2. Materials and methods

2.1. Mice

Female BALB/c mice and Swiss *nu/nu* mice were purchased from Charles River (Saint Germain-sur-l'Arbresle, France) and were housed following institutional guidelines of the A3 animal facility at the Institut Pasteur (Paris, France); http://webcampus.pasteur.fr/jcms/c_87141/documents.

2.2. Generation of *DsRed2*–transgenic *L. amazonensis*

The 697-bp *DsRed2* coding region was cut by BamHI/NotI from p*DsRed2* (Clontech, CA) and subsequently cloned into *Leishmania* expression vector pF4X1.HYG (Jenabioscience, Jena, Germany) previously cut by BglII/NotI. This plasmid contains the Hygromycin B marker for the selection of transgenic *Leishmania*. In this construct, the 3' and 5' UTR regions flanking the *DsRed2* and *Hyg* genes provide the required splicing and polyadenylation signals. Following linearization with SmaI, transfections were performed by electroporation in a 4 mm cuvette under the following conditions: 25 [rId7]F, 1500 V, 3.75 kV/cm [12]. Cells were incubated for 24 h in medium without the drug, and plated for selection of transgenic *Leishmania* on semi-solid medium containing 100 µg/ml of hygromycin B (Cayla, Toulouse, France). Recombination of the engineered plasmids leads to integration into the *Leishmania* rDNA locus, ensuring a permanent high level of transcription.

2.3. *L. amazonensis* metacyclic promastigote preparation and inoculation into nude mice

Wild type or *DsRed2*-transgenic *L. amazonensis* strain LV79 (WHO number MPRO/BR/72/M1841) amastigotes were isolated from Swiss nude mice inoculated 2 months before under a BSL-2 cabinet [1]. These amastigotes did not present antibodies at their surface [1,13]. Promastigotes derived from amastigotes were cultured at 26 °C in complete M199 medium [12]. Infective-stage metacyclic promastigotes were isolated from stationary phase cultures (6 day-old) on a Ficoll gradient [14]. Ten thousand metacyclic promastigotes per 10 µl of PBS were injected into the ear dermis of BALB/c mice. Lesion size was measured using a direct reading Vernier caliper (Thomas) and expressed as ear thickness.

2.4. Real-time cytofluorometric analyses of *DsRed2-LV79* amastigotes

Amastigote size was assessed with the forward scatter photodiode using the E-00 setting with a logarithmic amplification gain of 1.32. Amastigote structure was evaluated by the side scatter (SSC) photomultiplier tube, using a voltage of 363 V and a linear amplification gain adjusted to 6.88. *DsRed2* fluorescence was analysed in logarithmic scale. Real-time experiments were performed on samples containing 10⁶ parasites per ml in 3 ml of M199 medium. Basal FSC, SSC and *DsRed2* fluorescence data were acquired for 2 min, and L-leucine methyl ester (Leu-oMe) or paraformaldehyde (PFA) were added at a final concentration of 2 mM and 1%, respectively. Data were collected for the following 14 min as previously described for isolated liver mitochondria [15]. FSC and *DsRed2* fluorescence changes were analysed using the Flow Jo software (Ashland, OR).

2.5. *DsRed2-L. amazonensis* amastigote addition to DC cultures

DCs were differentiated from bone marrow cells of 6-week-old BALB/c mice according to a method adapted from Méderlé et al. [1,16]. Briefly, bone marrow cells were seeded at 2 × 10⁶ 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; Bio-Whittaker 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 [17], 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 7% CO₂. On day 6, suspended cells and loosely adherent cells were harvested using 1% EDTA (Versene) and cultured in complete IMDM supplemented with 10% of the primary culture supernatant. On day 10, cells were harvested with EDTA and distributed in hydrophobic 6-well plates (Greiner, St Marcel, France) at a concentration of 9 × 10⁵ cells/ml in 3 ml complete IMDM. On day 14, DCs were exposed to freshly isolated *DsRed2-LV79* amastigotes or to live BCG at micro-organism-DC ratios of 4:1 and 10:1, respectively. DC cultures were placed at 34 °C for 24 h.

2.6. Preparation of DC samples for cell sorting

Five minutes post the incubation in Versen-EDTA at 34 °C, DCs were carefully detached, resuspended at 4 °C in Dulbecco's PBS with 2% FCS (PBS-FCS) and transferred to a 15 ml tube (Falcon; BD Biosciences, San Jose, CA) at a concentration of 6 × 10⁶ cells/ml. Cells were centrifuged (300 g, 5 min, 4 °C) and then incubated in PBS-FCS supplemented with 10% heat-inactivated donkey serum for 5 min. Cells were incubated for 30 min in PBS-FCS containing 0.2 µg/ml of the anti-MHC class II monoclonal antibody (mAb) (M5/114) or the corresponding IgG2a isotype control mAb, both conjugated to PE-Cy5-conjugated mAb (eBioscience). After two washes, cells were resuspended at 5 × 10⁶

cells/ml in PBS containing 3% FCS and 1% J558 supernatant. Cell aggregates were dissociated on a 70 μ m filter (Falcon), and placed on ice until the cell sorting was done.

2.7. Cell sorting and biosafety guidelines

All experimental procedures were performed according to biosafety level two practises [10,11]. Biosafety guidelines can be obtained from one of us (HKB) (Plateforme de cytométrie en flux; Certification ISO 9001: 2000).

Cell sorting was performed using a FACSAria (BD Biosciences) equipped with completely sealed sample injection and sort collection chambers that operate under negative pressure. Following the M5/114 mAb staining, DCs were sorted using the BD FACSDiva software (BD Biosciences). PE-Cy5 and DsRed2 fluorescences 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. To avoid the sorting of cell doublets or cell aggregates, single cells were sequentially selected on SSC-H/SSC-W, and FSC-H/FSC-W dot plots. Infected DCs were sorted by selecting cells expressing both surface MHC Class II molecules and DsRed2. Sorting conditions included—i) sheath pressure of 70 Psi, ii) flow rate of 7 and iii) 70 μ m nozzle tip. Cells were collected at 4 °C in polypropylene tubes (BD Biosciences) previously coated with FCS (1 night at 4 °C). Sorted cells were immediately used for further studies.

2.8. Detection of 2A3-26 positive DCs by flow cytometry

After cell sorting, DCs were washed in PBS containing 1% FCS and 0.01% sodium azide, and fixed in PBS containing 1% PFA for 20 min at 4 °C. Then, DCs were washed in Perm/Wash solution from the BD cytofix/Cytoperm™ Plus Kit (BD Biosciences). Intracellular amastigotes were stained with 5 μ g/ml 2A3-26 mAb conjugated to Alexafluor 488 in Perm/Wash buffer for 30 min at 4 °C in the dark [1]. Then DCs were washed in Perm/Wash buffer and fixed with 1% PFA. Analysis was performed on the FACSAria.

2.9. Microscopic observations of sorted DCs

DsRed2⁺ MHC II⁺ DCs were centrifuged on poly-L-lysine-coated glass coverslips and incubated at 34 °C for 30 min. After thorough washing steps in PBS, cells were fixed with 4% PFA and mounted on glass slides with Hoechst 33342-containing Mowiol. Incorporation of Hoechst into DNA allows the staining of both host-cell and amastigote nuclei. Epifluorescence microscopy images -mouse MHC II molecules in red, DC and *Leishmania* nuclei in blue- were acquired on an upright Zeiss Axioplan 2 microscope monitored by the Zeiss Axiovision 4.4 software.

2.10. RNA integrity quality control

Evaluation of RNA quality was performed by optical density measurement using the Nanodrop (Kisker, <http://www.kisker-biotech.com>) and by electrophoresis on a Lab-on-a-chip product using the Agilent 2100 Bioanalyzer (Agilent, <http://www.chem.agilent.com>).

RNA Integrity Number (RIN) scores were monitored for each sample providing an objective and standardised measure of RNA quality on a scale of 1–10 (the value 10 corresponding to the highest quality) [18,19].

2.11. Transcriptional analyses by RTqPCR and Genechip analysis

Total RNAs were extracted from biological triplicate DC cultures. For RTqPCR analyses, RNAs were reverse transcribed as previously described [20]. A SYBR Green-based real-time PCR assay (QuantiTect SYBR Green Kit, Qiagen) for quantitation of several 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 input into qBase, a flexible and open source program for qPCR data management and analysis [21]. For normalisation calculations, 8 candidate control genes were tested (*cycA*, *pbgd*, *h6pd*, *hprt*, *tbp*, *l19*, *gapdh*, *rp11e*) with geNorm [22] and Normfinder programs [23]. *gapdh* and *rp11e* were selected as the most stable reference genes.

GeneChip hybridisations were performed following the Affymetrix two-cycle labelling protocol. QC assessment of Affymetrix recommended QC metrics was done using AffyGCQC program [24]. Affymetrix MIAME-compliant data have been made available through Gene Expression Omnibus databases (www.ncbi.nlm.nih.gov/projects/geo/, accession: GSE16644). Data processing, background correction, normalisation and signal quantification were carried out using GC-Robust Multiarray Analysis (GC-RMA) algorithm. Local pooled error tests [25] were performed to identify significant differences in gene expression between parasite-free and sorted parasite-harboring DCs. Benjamini–Hochberg multiple-test correction [26] was applied to control for the number of false positives (False Discovery Rate or FDR). We used GC-RMA algorithm, LPE tests and Benjamini–Hochberg multiple-test correction as implemented in Partek[®] Genomics Suite[™] (Partek, St Louis, Missouri).

2.12. Statistical analyses

Two-sided Student's paired t-tests were used to compare data from flow cytometry experiments and gene expression studies performed on sorted samples ($6 < n < 13$). A nonparametric Mann-Whitney bilateral U test was used for gene expression comparisons on unsorted samples ($n = 5$).

3. Results

3.1. Principal features of DsRed2-expressing *L. amazonensis* parasites

Methods for sorting *L. amazonensis*-hosting DCs require the detection of intracellular amastigotes. Consequently, we

generated transgenic LV79 *L. amazonensis* parasites that stably express the fluorescent molecule *DsRed2* (*DsRed2-LV79*). These parasites displayed characteristics similar to wild type parasites (WT-LV79) such as the flow cytometric properties forward scatter-FSC and side scatter-SSC (morphological features) of both the promastigote developmental stage *in vitro* (data not shown) and the amastigote developmental stage (recovered from footpads of *L. amazonensis*-hosting nude mice) (Fig. 1A). The lesion onset and features were similar following the inoculation of the *L. amazonensis-DsRed2* and WT-LV79-parasites to BALB/c mice (Fig. 1B).

The orange fluorescence of *DsRed2-LV79* amastigotes was detected both by epifluorescence microscopy and by flow cytometry (FC) with a 488 nm excitation wavelength (Fig. 1C). A key advantage of these fluorescent amastigotes was that neither cellular fixation nor permeabilisation was needed to efficiently detect them within the parasitophorous vacuole, allowing for the sorting of live DCs hosting live amastigotes. Indeed, the use of *DsRed2* fluorescence revealed only live amastigotes, since amastigotes killed by the leishmanicidal drug Leu-oMe [27] rapidly lost *DsRed2* fluorescence (Fig. 1D, E).

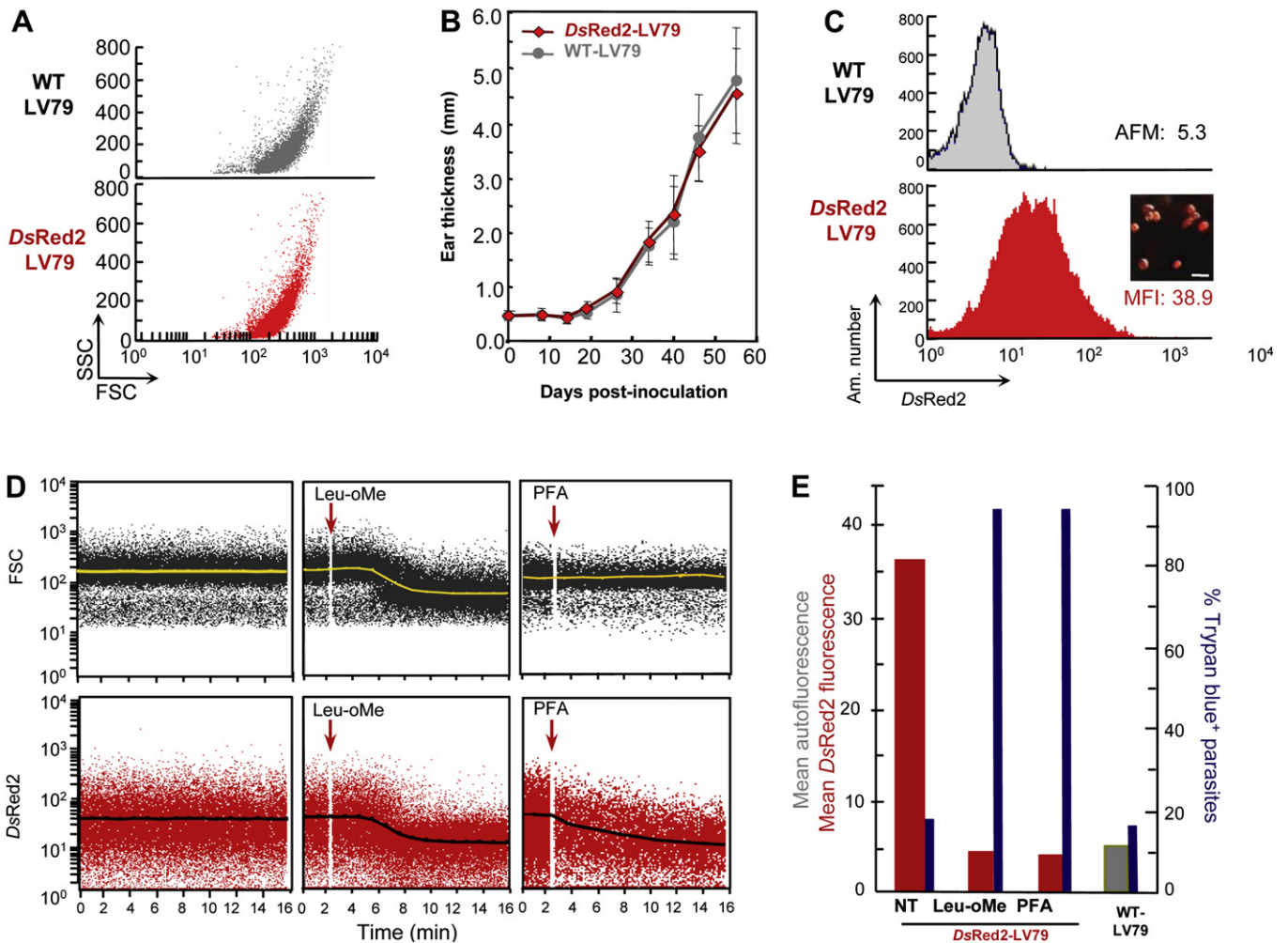


Fig. 1. Characteristics of *L. amazonensis* amastigotes expressing *DsRed2*. *DsRed2*-transgenic (*DsRed2-LV79*) and Wild type (WT-LV79) amastigotes were isolated from lesions of infected Swiss nude mice and further analysed by flow cytometry and epifluorescence microscopy. Analyses were performed without the addition of fixatives. (A) Flow cytometric analyses of forward (FSC) and side scatter (SSC) of *ex vivo* amastigotes post purification. (B) Ear clinical features (thickness) in BALB/c mice inoculated with WT-LV79 or *DsRed2-LV79* amastigotes. Metacyclic WT- (grey line) or *DsRed2-LV79* (red line) parasites (10,000) were inoculated into the ear dermis of 7 BALB/c mice ($n = 14$ ears per group). Ear thickness was monitored every week for 8 weeks. Medians and standard deviations (SD) are indicated. (C) Detection of *DsRed2* fluorescence from *DsRed2-LV79* amastigotes. WT-LV79 autofluorescence (grey histograms) and *DsRed2-LV79* orange fluorescence (red histogram) were analysed by cytofluorimetry. The mean of autofluorescence (AFM) and fluorescence intensity (MFI) are indicated. The *DsRed2* fluorescence of transgenic amastigotes was also detectable by epifluorescence microscopy (insert). Scale bar, 1 μ m. (D, E) Impact of Leu-oMe or PFA exposure on *DsRed2* fluorescence emission by isolated amastigotes (D) Real-time FC analyses of FSC (upper panels, black dots) and *DsRed2* fluorescence (lower panels, red dots) of amastigotes exposed to 2 mM Leu-oMe or 1% PFA. The addition of Leu-oMe or PFA is indicated by a red arrow. Lines correspond to real-time median values of FSC (upper panel) and *DsRed2* fluorescence (lower panels). The leishmanicidal effect of Leu-oMe was correlated with a rapid loss of *DsRed2* fluorescence. (E) Impact of a 24 h exposure to Leu-oMe or PFA. The MFI of transgenic (red bars) and WT (grey bars; mean autofluorescence) amastigotes was measured by FC analysis and parasite death was assessed by trypan blue staining (blue histograms).

3.2. Setting up a reliable *in vitro* DC culture for further characterisation of amastigote-hosting DCs

DCs were derived *in vitro* from bone marrow progenitors of BALB/c mice [1]. In these cultures more than 95% of cells harboured the phenotype of myeloid DCs, and co-expressed CD11c, CD11a, CD11b and MHC class II molecules [1]. Only a minor fraction (<5%) of the cultured cells did not express any MHC class II molecules (Fig. 2A) and did not display the morphology of DCs. DCs were cultured for 24 h in the absence or in the presence of freshly isolated *DsRed2*-LV79 amastigotes, this *Leishmania* developmental stage being efficiently phagocytosed by DCs [1]. DCs hosting *DsRed2*-LV79 amastigotes were easily detected via *DsRed2* fluorescence by both epifluorescence microscopy and flow cytometry (Fig. 2A, B, C). The percentage of

infected cells and the mean *DsRed2* fluorescence, both determined by flow cytometry analyses, varied with the parasite:DC ratio (Fig. 2A, B). First, we addressed the questions of the correlation between the percentages of the living *DsRed2*⁺ DCs and the intracellular presence of amastigotes, the latter being determined with the 2A3-26 mAb, which has been shown to bind to the amastigote stage of *L. amazonensis* [1]. To this end, DCs were cultured with different amastigote:DC ratios (ranging from 0.5–8) for 24 h, fixed and permeabilised. Then, intracellular amastigotes were immunostained with the 2A3-26 mAb. The percentages of *DsRed2*-LV79-hosting DCs were highly correlated to the percentages of 2A3-26 positive DCs ($r^2 = 0.9736$, $p < 0.001$) (Fig. 2B). Consequently, FC analysis of *DsRed2* fluorescence was a reliable approach to detect live *DsRed2*-LV79 amastigote-hosting DCs.

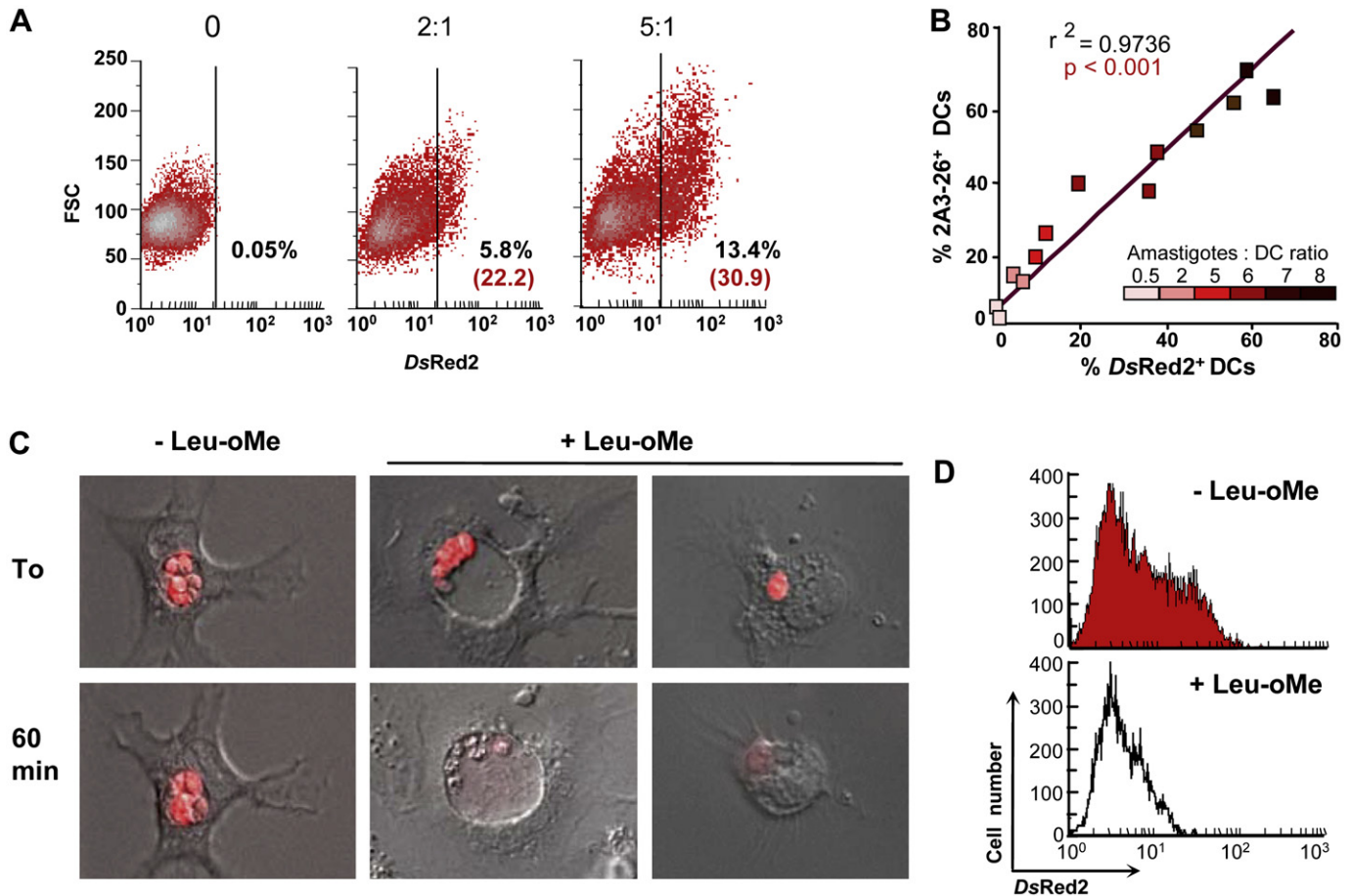


Fig. 2. Detection of live *DsRed2*-LV79 amastigotes in DCs. (A) Flow cytometric analysis of *DsRed2*-LV79 fluorescence and FSC from unfixed DC samples. DCs were cultured for 24 h in the presence of *DsRed2*-LV79 amastigotes at different parasite to DC ratios. Analysis was performed in live samples. The MFI (red text) and the percentages (black text) of *DsRed2*-LV79-loaded DCs and amastigote-free DCs are indicated. Note the FSC increase in amastigote-loaded DCs. (B) Correlation between the flow cytometric quantitation of *DsRed2*⁺ and 2A3-26⁺ DCs. DCs were incubated at different ratios (0.5–8) parasites per DC as indicated with the colour code) for 24 h. The quantitation of infected DCs was based on the expression of *DsRed2* or on 2A3-26-AF fluorescence. The coefficient of correlation and the p value are indicated. (C, D) Relationship between amastigote viability and *DsRed2* fluorescence *in cellula*. *DsRed2*-LV79 amastigotes were added to DC cultures (ratio of 5:1). Twenty-four hours later, Leu-oMe (2 mM) was added or not to the *DsRed2*-LV79-loaded cultures for 60 min. Epifluorescence microscopy (c) and flow cytometry analyses (D) were performed. Leu-oMe-induced parasite death led to the release of *DsRed2* into the parasitophorous vacuole or into the cytoplasm. This process was associated with a loss of *DsRed2* fluorescence that was detected by both FC and epifluorescence microscopy approaches.

Secondly, we optimised the sorting procedure by setting up *in vitro* DC cultures with DCs hosting from one to four amastigotes. This intracellular amastigote number is similar to that observed in CD11c⁺ DCs collected from the lymph nodes of BALB/c mice 10 weeks after the inoculation of a low number of parasites into the ear dermis (Lang *et al.*, unpublished data).

The “multiplicity of infection” of five was chosen for two reasons: i) the percentage of parasitized DCs is higher than with the 2:1 ratio (see Fig. 2A) ii) the percentage of *DsRed2*⁺ cells reached 11.3% ± 2.5% (one of eight representative experiments is displayed in Fig. 2B) and iii) within this 11.3% of live DCs hosting parasites more than 80% of DCs harboured one to four amastigotes (Supplementary Fig. 1).

Lastly, we wanted to ensure that only DCs hosting live amastigotes would be sorted. Thus, *DsRed2* LV79-hosting DC cultures were exposed to the leishmanicidal drug Leu-oMe [27]. Not only there was a rapid diffusion of the fluorescence out of the amastigotes within parasitophorous vacuole, but there was also complete fluorescence extinction 60 min after the drug addition (Fig. 2C and D). These results indicated that *DsRed2* fluorescence emission from *Leishmania*-infected DCs was only related to live amastigotes.

3.3. High speed sorting of rare live *DsRed2* amastigote-hosting DCs in BSL-2 containment

The distribution of MHC class II molecules in DC cultures exposed to amastigotes was very similar to those observed in DCs left unexposed to the amastigotes (Fig. 3A1 and A2). The immunolabelling of surface MHC II molecules allowed us to exclude the low fraction of amastigote-hosting cells that did not express surface MHC II. The biparametric dot plot (Fig. 3A) was used to define the sorting gate on MHC II⁺ *DsRed2*⁺ cells. The sorting was performed in samples exhibiting moderate (9%) or low percentages (0.5%, Fig. 3A) of MHC II⁺ *DsRed2*⁺ cells. The risk of laboratory personnel exposure to aerosols during the cell sorting was minimised by the application of adequate BSL-2 measures (see “methods”). The Aerosol Management System added to the FACSaria cell sorter allowed for the vacuuming of aerosols into a special ultra-low penetrating air filter, and to generate an additional negative pressure in the sort collection chamber. The biosafety level 2 containment was performed by totally covering the cell sorter and the operator with a specialised biological safety cabinet (EP 20-16 hood), providing a localised negative pressure (see methods).

Due to the relative fragility of DCs, different experimental sorting conditions were tested, including variations on the pressure level (20 or 70 psi), the nozzle size (100 or 70 µm) and the flow rate (2 or 7) (Supplementary Fig. 2). Cell sorting procedures resulted in a moderate loss of viability in both control and infected cultures. The “70 µm/70 psi/flow rate of 7” combination resulted in a slight loss of viability, but permitted a higher rate of sorting, and thus shortened the sorting procedure and limited potential associated artefacts like DC activation/maturation. Consequently, this condition

was applied for every cell sorting for infected DCs. The purification of sorted *DsRed2*⁺ DCs was first analysed by measuring parasite-specific RNAs in sorted and unsorted infected DC cultures (Supplementary Fig. 3). Firstly, all these transcripts were enriched in sorted samples. Secondly, these conditions enabled us to obtain pure sorted cells regardless of the initial percentage of amastigotes-hosting DCs present in the culture (9% or 0.5%) (Fig. 3A), as demonstrated by FC and microscopy analysis, and to select leucocytes that harboured at least two amastigotes (Fig. 3B). After every cell sorting, sequential decontaminating procedures were applied according to the conclusions drawn from Supplementary Fig. 4.

3.4. Transcriptional signatures of live sorted *L. amazonensis*-hosting DCs

Since the preservation of RNA integrity was essential for our downstream investigations, electrophoresis on Agilent Lab-on-chips was applied to total RNA extracted from sorted and unsorted infected DC cultures. For all samples, RNA integrity numbers (RIN) were greater than 8 (Fig. 3C), and no differences could be detected between sorted and unsorted samples. These data revealed that RNA from sorted cells displayed the desired quality for Real-Time quantitative Polymerase Chain Reaction (RTqPCR) or Affymetrix studies.

We then verified that the cell sorting procedure on DC cultures exposed or not (control DCs) to amastigotes did not result in any DC maturation. To do so, as described under “Methods”, a sort of the whole cell culture was performed according to FSC/SSC parameters and compared to unsorted samples. The cell sorting procedure did not modify the abundance of transcripts known to be modulated in DC activation, such as those coding for the chemokine-receptor 7 (CCR7), the membrane glycoprotein CD200, the DC-specific ICAM3-Grabbing Nonintegrin/CD209, the co-signalling molecules CD80, B7-H1, SLAMF1 and the chemokine-receptor 4 (CXCR4) (two-sided Student’s paired t-test *p*-value > 1% with 6 < *n* < 13; not shown). Then, the impact of loading with *L. amazonensis* was determined by measuring the abundance of RNA transcripts encoding CCR7, CD200, CD209, plus the Arg2 (arginase type II) and the arginase transporter Slc7a2 (Fig. 4A, B). As a positive control for the optimal induction of DC activation, cultures were exposed to live BCG for 24 h [1] (Fig. 4A). In unsorted cultures exposed to BCG, a strong and significant increase in transcript abundance was observed for the whole cell culture when compared to control DCs (*p* < 0.0001). In DCs exposed to *DsRed2*-LV79 amastigotes, no statistically significant differences in transcript abundance were observed when compared to control DCs (Fig. 4A). In contrast, the sorting of MHC class II⁺ *DsRed2*⁺ DCs by flow cytometry revealed a significant modulation of transcripts that would not have been detected without the sorting procedure (Fig. 4B1). Indeed, a positive modulation was detected for CCR7 (*p*-value < 0.01), CD200

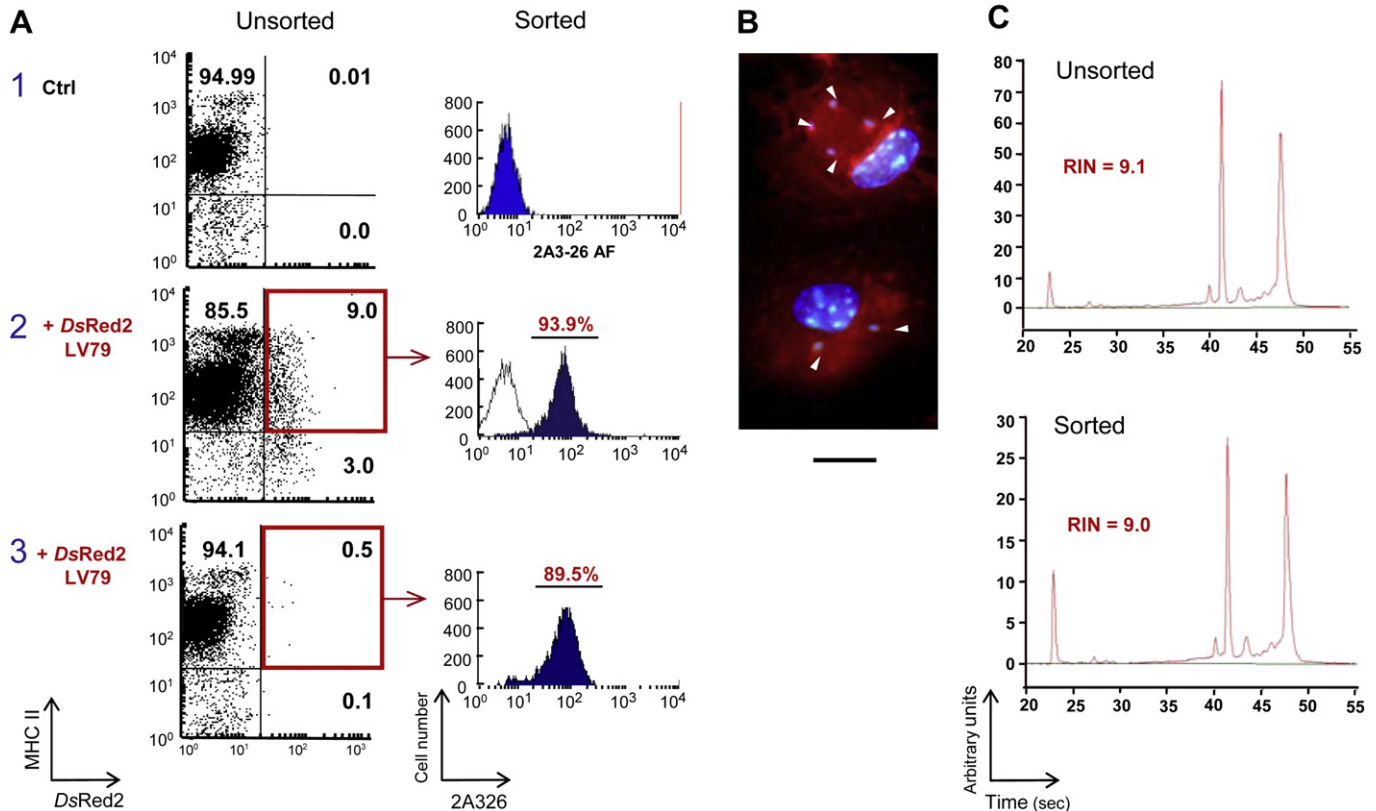


Fig. 3. Protocol for high speed sorting of live *DsRed2-LV79* amastigote-hosting DCs (A) Biparametric *DsRed2-LV79/MHC II PE-CY5* dot plots of representative control (Ctrl; panel 1) and amastigote-loaded DC cultures (*DsRed2*; panels 2 and 3). In panel 2, amastigotes were added to DC cultures at a ratio of 5 amastigotes per DC for 24 h. In panel 3, by 24 h post inoculation, DCs were detached and diluted with uninfected cells to obtain 0.5% infected DCs. For each panel, the percentages of single and double positive cells are indicated. The gating strategy was performed by selecting *DsRed2/MHC II* double positive DCs (red gate). Sorted DCs were fixed/permeabilised and labelled with the 2A3-26 mAb (black histogram) or with control mAb (white histogram), and analysed by flow cytometry. The percentage of 2A3-26⁺ cells, *i.e.*, harbouring intracellular amastigotes is indicated. (B) Epifluorescence microscopy analysis of sorted DCs. Sorted DCs were distributed on coverslips and fixed. DC and parasite nuclei were stained with Hoechst 33342 (blue spots indicated by arrows). The red fluorescence (MHC II PE-Cy5) reveals the surface immunostaining of MHC class II performed before the sorting. Scale bar, 10 μ m. (C) Evaluation of RNA quality RNA profiles from unsorted or sorted amastigote-loaded DCs were assessed by electrophoresis on Agilent Lab-on-Chips. RIN values are indicated.

($p < 0.05$), *Slc7a2* ($p < 0.1$), and *Arg2* ($p < 0.05$), (two-sided Student's paired t-test with $6 < n < 9$). In addition, a negative modulation was observed for *CD209* ($p < 0.01$). Therefore, a genome-wide transcriptional analysis was performed by comparing the gene expression profiles of control DCs and amastigote-hosting DCs using the Affymetrix technology. Among the 45,101 probe-sets of the Mouse430_2 GeneChip, 613 (1.4 %) were captured with a differential expression at the 5% significance level (Fig. 4B2). Comparable fold change values were obtained between the Affymetrix technology and the RTqPCR method. Among these probe-sets, 470 represented genes with a known function. Compared analysis pinpointed the modulation of various genes involved in the L-arginine and polyamine pathways (Fig. 4B3) that would not have been detected in unsorted cells. Indeed, modulation of transcripts encoding the arginine transporter *Slc7a2* and *Arg2* were revealed (mean FC +5.63 and + 4.2) in sorted *Leishmania*-hosted DCs. This analysis also revealed the weak modulation of genes involved in the polyamine pathway such as *Sat1* (spermidine/spermine N1-acetyltransferase 1; FC + 2.16).

4. Discussion

In the present study we describe a reliable and safe method for sorting live DCs hosting *L. amazonensis*. Taking into consideration both scientific issues, including the rarity of live amastigotes-harbouring DCs in the mouse tissues hosting *Leishmania*, and ethical issues, such as avoiding the sacrifice of numerous mice, we designed the amastigote-hosting DC sorting procedure with model DC populations generated and mastered *in vitro*. To this end, we describe a high speed sorting procedure performed in a biosafety level 2 containment [10,11], that allows for the purification of rare amastigote-hosting DCs and accurate monitoring of DC features. The use of *L. amazonensis* transgenic parasites expressing the *DsRed2* fluorescent protein, which revealed only live amastigotes, allowed us to sort live *DsRed2* MHC class II positive DCs that represented only 0.5 % of total cells, a percentage close to the one found in tissues of *Leishmania*-infected mice [28]. A major advantage of this strategy is the ability to sort live cells, the DC death being always a rare outcome. This strategy also allows for the isolation of a high quality RNA from the sorted cells, which

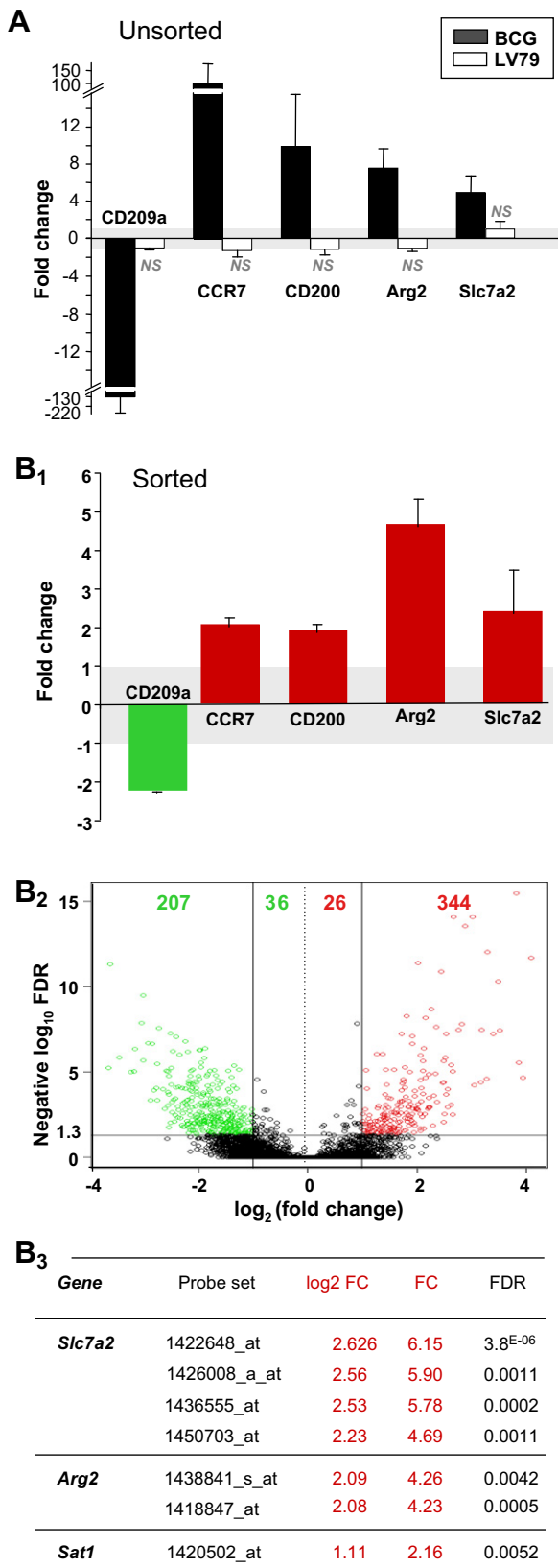


Fig. 4. Differential transcript analysis of sorted MHC II positive DCs harbouring or not *DsRed2-LV79* (A) Transcript analysis of unsorted DC cultures *DsRed2-LV79* (5 per DC) or BCG (10 per DC) were added to DCs for 24 h. RNA extraction, reverse transcription and RTqPCR were performed

can then be used for different purposes, including gene expression analyses with real-time PCR and oligonucleotide microarray hybridisations. This new approach revealed for the first time a diverse expression pattern in transcripts from sorted live amastigote-hosting DCs that would have been undetectable in unsorted samples. As a relevant biological feature of the amastigote-hosting DCs, we identified a significant up-regulation of transcripts coding for molecules involved in L-arginine metabolism, which is known to be induced as either a source of NO or as a source of polyamines [29,30]. The significant and coordinated up-regulation of an L-arginine transporter, arginase2, and spermidine/spermine N1-acetyltransferase 1/Sat1 [31], indicates that arginine catabolism for the polyamine generation is dominating over L-arginine catabolism for NO generation. It would be interesting to follow the modulation of these metabolic pathways at different time points post *L. amazonensis* intradermal inoculation to mice. Indeed, within the dermis loaded with intracellular amastigotes, DCs could display phenotypes ranging from NO to polyamine synthesis. The recent study performed in C57BL/6 mice suggests that the presence of iNOS-producing inflammatory DCs in the *Leishmania major*-loaded footpads was closely associated with the parasite load reduction phase, the latter assessing the dominance of a type 1 environment in the *L. major*-loaded footpad [32].

The technique developed herein for *in vitro* DC samples will enable many new applications by taking advantages of polychromatic flow cytometry analyses. Indeed, it would allow for the specific sorting of DC subsets according to the simultaneous expression of surface molecules (CD11c, CD80, CD86, chemokine-receptors, anti-apoptotic signalling receptors...). In addition, the settings and sorting conditions that we defined here constitute useful parameters for the further sorting of amastigote-hosting DCs from mouse biological samples such as the ears and the ear-draining lymph nodes.

on unsorted DC cultures. Displayed data are the mean fold changes for Arg2, CCR7, CD200, CD209 and Slc7a2 transcripts between DCs in contact with BCG or amastigotes and control DCs ($n = 5$ experiments). For all target genes under study there is a statistically significant difference between control DCs and DCs with BCG ($p < 0.001$; nonparametric Mann-Whitney bilateral U test) but not between control DCs and DCs in contact with *Leishmania* amastigotes (NS = non significant). (B) Transcript analysis of sorted DCs harbouring *DsRed2-LV79*. Analyses were performed by RTqPCR (b1) and Genechip analysis (Affymetrix) (b2, b3). (B1) Bar charts of a representative experiment showing the fold change values of 5 transcripts between *DsRed2*-housing sorted DCs (*DsRed2*/MHC II positive cells) and sorted control DCs (MHC II positive cells). (B2) Volcano plot representation of the differential gene expression between sorted *L. amazonensis* housing-mouse DCs and non parasitised DCs. Modulated probe-sets with False Discovery Rate (FDR) < 0.05 are above the horizontal line. The number of modulated probe-sets is indicated. A total of 613 probe-sets show significant differential expression. Up-regulated probe-sets with a fold change > 2 [\log_2 (fold change) > 1] are on the right side of the right vertical line, while down-regulated probe-sets (207) with a fold change < -2 are on the left side of the left vertical line. A total of 62 probe-sets showed differential expression with a fold change between -2 and $+2$. (B3) List of DC transcripts related to L-arginine metabolism and the polyamine pathway regulated in sorted infected DCs.

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Appendix. Supplementary material

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.micinf.2009.09.014](https://doi.org/10.1016/j.micinf.2009.09.014).

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