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Lassa Virus Infection of Human Dendritic Cells and Macrophages Is Productive but Fails to Activate Cells

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Lassa fever is a hemorrhagic fever caused by Lassa virus (LV), an old-world Arenavirus. Little is known about the immune responses that occur during the disease, but protection seems to be linked to the induction of cellular responses specific for viral glycoproteins. Conversely, severe Lassa fever may be associated with immunosuppression. We studied the infection of human dendritic cells (DC) and macrophages (MP) by LV. Both these cell types are susceptible to LV infection. Viral nucleoprotein was detected in DC and MP, and high and moderate viral titers were obtained with culture supernatants of DC and MP, respectively. LV did not induce apoptosis in DC and MP. These cells were not activated by LV infection. No change was observed in the expression of surface molecules involved in activation, costimulation, adhesion, and Ag presentation following LV infection, or in the functional properties of DC. Inflammatory cytokine production was not detected at the mRNA or protein level after LV infection of DC and MP. Thus, MP, and particularly DC, are crucial targets for LV and are probably involved in the early replication of LV from the initial site of infection. The lack of activation and maturation of cells following infection may be associated with the immunosuppression observed in severe LV infection.

Materials and Methods

Virus

LV (AV strain (21)) was cultured in the Vero E6 cell line at 37°C. Viruses from the serum of a patient were subjected to four passages on Vero E6 cells. The cell-free supernatant, with a viral titer of 10^6 Focus-forming units (FFU)/ml was then used as the infectious virus stock. This supernatant was also irradiated (5 × 10^4 rad) and used as inactivated LV (22). We checked that cell lines and viruses were not contaminated with mycoplasmas. All experiments with infectious viruses were conducted in Biosafety Level 4 facilities.

Virus titration

Vero E6 cells were incubated for 1 h at 37°C with several dilutions of cell-free supernatants and 1.6% carboxy-methyl-cellulose (BDH Laboratory Supplies, Poole, U.K.) in DMEM (Life Technologies, Cergy-Pontoise, France) was then added. Infectious foci were detected after 5 days of culture, by incubation with a mixture of mAbs against LV NP (mAbs 52-158-3, 52-54-6, and 52-189-13 (23), generously provided by Dr P. Jahrling, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD) followed by HRP-conjugated goat polyclonal anti-mouse IgG (Sigma-Aldrich, Saint Quentin Fallavier, France) and di-amino-benzidine. Results are expressed as FFU per million cells.

Obtention of DC and MP

Fresh human peripheral blood was obtained from the Etablissement Français du Sang (Lyon, France). Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque (Pharmacia). Uppsala, Sweden was then supplemented by centrifugation through a 50% Percoll gradient (Pharmacia) for 20 min at 400 × g. The low-density fraction from the interface was recovered, washed twice in RPMI 1640-Glutamax I (Life Technologies) supplemented with 10 mM HEPES (Life Technologies), 1% penicillin-streptomycin (Life Technologies), 1% nonessential amino acids (Life Technologies), and 10% FCS (Life Technologies) (C-RPMI), and incubated for 15 min in C-RPMI supplemented with 5% human AB serum (Etablissement Français du Sang). Monocytes were further purified by immunomagnetic depletion (Dynabeads; Dynal, Oslo, Norway) with a mixture of mAbs against C3D (SPV-T3b; Dynal), CD14 (AB1; Dynal) and CD56 (C218; Immunotech, Marseille, France). DC were generated by culturing monocytes at a density of 10^6 cells/ml in C-RPMI supplemented with 1000 U/ml recombinant human (rh) GM-CSF (Peprotech, London, U.K.) and 500 U/ml rhLIF (Peprotech). We replaced 40% of the culture medium every 2 days and replenished the supply of cytokines. The cells were harvested after 7 days: 70–90% were CD1a-expressing immature DC. MP were obtained by culturing monocytes in C-RPMI supplemented with 10 U/ml mCM-CSF (Peprotech) for 7 days and replacing the medium and cytokines every 2 days. Mature DC were obtained by adding 500 ng/ml LPS (Sigma-Aldrich) to cell cultures 24 h before infection.

Infection of DC and MP by LV

Cell pellets were resuspended in cell-free supernatants containing infectious or inactivated LV, or with Vero E6 supernatant for mock infection. Cells were incubated for 1 h at 37°C with gentle shaking. They were then thoroughly washed in C-RPMI, and cultured at a density of 10^6 cells/ml in C-RPMI supplemented with the appropriate cytokines at 37°C. Medium and cytokines were renewed every 2 days. In some cases, DC and MP were activated 2 h after infection by adding 250 ng/ml soluble rCD40 ligand (sCD40L) and 1 μg/ml enhancer (Alexis, Carlsbad, CA) for DC and 500 ng/ml LPS for MP.

Immunofluorescence

DC and MP were harvested 48 h after infection and centrifuged onto coated slides (Shandon, Cheshire, U.K.) in a CytoSPIN 3 cytospin centrifuge (Shandon). Cells were fixed with 3% paraformaldehyde (PFA) in PBS at 10 min at room temperature (RT) and were incubated in 5% AB+ human serum for 15 min. CD1a− or CD14− specific mAbs (BD PharMingen, San Diego, CA) diluted 1/20 in PBS were then added and the cells were incubated for 1 h at RT. They were then incubated with rhodamine-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA). Cells were permeabilized by incubation with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 5 min. A mixture of two FITC-conjugated mAbs specific for LV NP (52-54-6 and 52-189-13) was then added and the cells incubated for 1 h at RT. Slides were mounted in FluorPrep mounting medium (Dako, Trappes, France), and images were captured with Qfluor software (Leica, Cambridge, U.K.).

Detection of mRNA by RT-PCR

Total RNA was isolated from 5 × 10^6 cells, using the RNAeasy kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. Contaminating genomic DNA was digested with DNase I (Qiagen) during the extraction procedure. First-strand cDNA was synthesized from 200 ng of RNA, using Superscript II reverse transcriptase (Life Technologies) and oligo(dT)15 (Life Technologies); 10% of the products of this reaction were used as a template for qualitative and real-time PCR. We checked for the absence of genomic DNA amplification for each gene by adding RNA directly to the amplification mixture without reverse transcription. The following primers were used for qualitative PCR (annealing temperatures are indicated for each primer pair): β-actin, 5'-CAGGCACACGCGCGT GAT-3' and 5'-GCCACGGAGTTCAGCAG-3'; IL-6, 5'-AGTT GCCCTTCTCGTG-3' and 5'-ATTGGCGAAAGCCCTCA-3'; 55°C; IL-12p35, 5'-GAGAAGGGTGCTGATTG-3' and 5'-CCCTCCACGTC AACCTGACC-3'; 60°C. DNA was amplified by PCR with TaqDNA polymerase (Roche, Mannheim, Germany) for 40 cycles (cytokines) or 35 cycles (actin). PCR products were separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining (data not shown). For real-time PCR, predeveloped primers and TaqMan probes for β-actin, TNF-α, IL-1β, IL-10, IL-12p35, TGFβ, IFN-γ, CCR5, CCR6, CCR7, macrophage inflammatory protein (MIP)-1α, MIP-1β, and IL-8 were used (Applied Biosystems, Courtaboeuf, France). DNA was amplified using TaqMan Universal Master Mix (Applied Biosystems) on an ABI PRISM 7000 real-time thermocycler (Applied Biosystems), according to the manufacturer’s instructions. DNA fragments encoding cytokines were amplified in duplex with β-actin and relative mRNA levels for each sample were calculated as follows: Δ cycle threshold (Ct) = Ct gene X – Ct β-actin. Ratio (mRNA in infected cells/mRNA in mock-infected cells) = 2^(-ΔCtinfected − ΔCtmock).

ELISA detection of cytokines in supernatants

Supernatants from cultures of DC and MP were harvested, centrifuged, and stored at −80°C. Commercial ELISA kits were used for TNF-α, IL-10, IL-1β (CLB, Amsterdam, The Netherlands), IL-12 (Cytimmune Sciences, College Park, MA), IL-8, MIP-1α, MIP-1β (R&D Systems, Abingdon, U.K.) detection, according to the manufacturer’s instructions.

Flow cytometry

MP were harvested in PBS containing 0.5 mM EDTA at various times after infection, incubated for 15 min in PBS-5% AB+ human serum, and stained for 30 min at 4°C with the following mAbs: FITC-conjugated anti-human CD14 (RM052), FITC-conjugated anti-HLA-DR (B3;12.2), PE-conjugated anti-CD1a (BL6), PE-conjugated anti-CD14, PE-cyanin 5 (PC5)-conjugated anti-ILT3 (ZM3.8) (Immunotech, Marseille, France), FITC-conjugated anti-CD86 (FUN-1), PE-conjugated anti-CD80 (L307.4), PE-conjugated anti-CD83 (HB1Se), CyChrome-conjugated anti-HLA-ABC (G46-2.6), CyChrome-conjugated anti-CD95 (DX2), CyChrome-conjugated anti-CD45 (HA58) (BD Pharmingen). Isotypic controls were performed by staining cells with FITC-conjugated (Pharm FITC-congala, Sweden) (MOPC 21), PE-conjugated IgG2a (G155-178), and CyChrome-conjugated IgG1 (MOPC-21) (BD Pharmingen). The purity of monocyte populations was checked by staining cells with FITC-conjugated anti-CD8 (B9.11), PE-conjugated anti-CD19 (J4.119), and PCS-conjugated anti-CD3 (UCH1) (Immunotech) Abs. Cells were washed in 2.5% FCS in PBS, and resuspended in 10% PFA in PBS, LV NP was detected by intracellular staining as follows. Cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), according to the manufacturer’s instructions. Cells were incubated for 30 min at 4°C with the mixture of two FITC-conjugated anti-LV NP mAbs described above or with FITC-conjugated mouse IgG1 (MOPC-21). They were then washed twice and resuspended in 3% PFA in PBS. Double staining for Annexin V FITC binding and 7-aminoactinomycin D (7AAD) (BD Pharmingen) was performed according to the manufacturer’s instructions, to assess cell viability. Absolute counts were obtained with FlowCount-calibrated fluorospheres (Beckman Coulter, Villepinte, France), used according to the manufacturer’s instructions. Flow cytometry was performed in an EPICS-XL four-color cytometer (Beckman Coulter), with EXPO 32 ADC software (Beckman Coulter).

Internalization of dextran-FITC

DC were incubated for 1 h at 37°C (or at 4°C for control) with 20 μg/ml dextran-FITC (Sigma-Aldrich) in C-RPMI. Cells were harvested in cold PBS supplemented with 0.5 mM EDTA, washed in PBS, resuspended in 3% PFA in PBS, and analyzed by flow cytometry.
Chemotaxis assay

Cell migration was performed in 24-well Transwell cell culture chamber (Costar, Corning, NY). DC were harvested 72 h after mock or LV infection, and $10^5$ cells in 100 µl of C-RPMI were added to each transwell insert. Six hundred microliters of serum-free medium containing 100 ng/ml rh MIP-1α, MIP-3α, or MIP-3β (Peprotech) were added to the lower compartment. In other experiments, immature DC migration assays toward supernatants of mock and LV-infected DC harvested 3 days after infection were performed. After 2 h of incubation at 37°C, the cells that migrated through the 8.0-µm pore polycarbonate membranes in the lower compartment were collected and counted on a flow cytometer (EPICS-XL; Beckman Coulter) with FlowCount-calibrated fluorospheres (Beckman Coulter). The lower compartment of control chambers contained medium alone, while the absence of chemokinesis was demonstrated by the absence of cell migration when both compartments contained chemokines. Each assay was performed in duplicate and two independent experiments have been done.

Statistical analysis

Student’s t test was used to analyze differences between datasets for the assays. Statistical data were obtained using Excel software (Microsoft, Redmond, WA).

Results

DC and MP are susceptible to LV infection

Immature DC and MP were infected with LV (strain AV (21)) at a multiplicity of infection (MOI) of 2 FFU/cell. Cells were harvested after 2 days, centrifuged onto coated slides, and double-stained with a mixture of two NP-specific mAbs and a phenotype-specific mAb (CD1a or CD14). NP was detected in 80–95% of MP (Fig. 1, A and C), and CD14 was detected in the membrane (Fig. 1B). DC were also susceptible to LV infection, as shown by the detection of NP in 60–90% of DC, which otherwise express CD1a (Fig. 1, E and F). NP was also demonstrated to be present in DC and MP by flow cytometry. Intracellular NP production was detected as early as 1 day after infection, reaching a peak at day 3 in both DC and MP (Fig. 2, A and B), and declining thereafter (data not shown).

Immature DC produce more LV particles than MP

Viral particles were titrated in supernatants of DC and MP infected at various MOI. LV infection is productive in both DC and MP, particularly with an MOI of 0.2 (Fig. 3A). With MOI of 2 and 10, virus production in supernatants was lower for DC, and not significant for MP, given the large number of residual viral particles found 1 h after infection. The production of viral particles was observed in supernatants of DC and MP as soon as 24 h after infection. Virus production peaked at 72 h in DC and 48 h in MP, and was still efficient at 7 days for DC (MOI = 0.2). More viral particles were produced in DC than in MP (at least 30 times more), with the level of production almost reaching that in Vero E6 cells (Fig. 3A). We assessed the susceptibility of mature DC to LV infection by activating DC with LPS 24 h before infection. LV infection of mature DC was also productive, and infection followed a similar time course to that in immature DC (data not shown). We compared viral particle production three days after infection in mature and immature DC (Fig. 3B). Mature DC produced significantly fewer viral particles than their immature counterparts ($p < 0.05$). Thus, mature DC are susceptible to LV infection, but to a lesser extent than immature DC.

LV infection does not induce apoptosis of DC and MP

We investigated whether apoptosis was associated with LV infection of DC and MP by staining cells with annexin V and 7AAD various numbers of days after infection. The percentage of apoptotic cells was similar in mock- and LV-infected DC, whether or not they were activated with sCD40L 2 h after infection. (Fig. 4A)

![FIGURE 1. Detection of viral NP by immunofluorescence in DC and MP. MP (A–C) and DC (D–F) were harvested 2 days after LV infection (MOI = 2; B, C, E, and F) or mock infection (A and D). Cells were stained with irrelevant mouse IgG plus TRITC-conjugated goat anti-mouse (GAM) and FITC-conjugated mixed mAbs directed against NP (A); mouse anti-CD14 plus GAM IgG and FITC-conjugated anti-NP Ab (B); FITC-conjugated anti-NP Ab (C); mouse anti-CD1a plus GAM IgG and FITC-conjugated anti-NP mAb (D–F). Original magnifications: ×400 (A and B), ×630 (C–F). The data are from one experiment representative of four.](http://www.jimmunol.org/)

![FIGURE 2. Detection of viral NP by flow cytometry in DC and MP. Intracellular viral NP was detected by direct immunofluorescence using FITC-conjugated mixed mAbs directed against NP on day 1 mock-infected DC and MP, day 1 LV-infected DC and MP, and day 3 LV-infected DC and MP (A and B). An FITC-conjugated mouse IgG2a was used as an isotypic control (not shown). The data are representative of four independent experiments.)](http://www.jimmunol.org/)
flow cytometry, but no difference was observed between mock- and LV-infected DC and MP (data not shown). Thus, LV infection does not reduce the viability of DC and MP.

DC and MP are not activated following LV infection

The expression of several molecules involved in cell activation, costimulation, or Ag presentation at the surface of DC and MP was analyzed during the course of LV infection. DC expressed CD1a, whereas CD14 was detected only on the surface of MP (Fig. 5A). The expression of CD86, CD80, CD40, CD54, HLA-DR, HLA-ABC, and ILT3 did not change during the course of LV infection in DC and MP (Fig. 5B) even 7 days after infection (data not shown). Similar results were obtained if both types of cell were stimulated with inactivated LV (data not shown). Thus, LV did not activate DC or MP, and did not induce the maturation of DC, as suggested by the absence of CD83 at the surface of infected DC (Fig. 5B). The expression of these molecules was similar in LV-infected, inactivated LV-stimulated, and mock-treated cells if DC

FIGURE 3. LV infection of DC and MP is productive. Infectious viruses produced in the culture supernatants were quantified by determining the number of FFU on a lawn of Vero E6 cells. A, Kinetics of viral particle production by $10^6$ DC or by $10^6$ MP at MOIs of 0.2, 2, or 10. Results are expressed as the mean ± SD of six independent experiments. Viral production by Vero E6 cells infected at a MOI of 0.01 is also shown. B, Production of LV by mature DC. Infectious virus was quantified in the day 3 culture supernatants of DC activated with LPS 24 h before LV infection at various MOIs (0.2, 2, and 10) and the results obtained were compared with those for LV production by immature DC. Results are expressed as the mean ± SD of three independent experiments for LPS-activated DC. *, Significant differences ($p < 0.05$) in viral titers between the two groups.

FIGURE 4. The viability of DC and MP is not altered by LV infection. Mock- or LV-infected DC (A) and MP (B) were harvested various numbers of days after infection (MOI = 2) and doubled stained with Annexin VFITC and 7AAD. The stained cells were then analyzed by flow cytometry. For DC, cell viability was also determined after stimulation of the cells with sCD40L 2 h after infection (A). The upper right quadrants of each panel show nonviable necrotic cells (annexin V+, 7AAD+). The lower right quadrants show apoptotic cells (annexin V+, 7AAD−), and the lower left quadrants show viable cells (annexin V−, 7AAD−). The percentage of all cells in lower and upper right quadrants is indicated. Results are representative of four independent experiments.

FIGURE 5. Expression of surface molecules by LV-infected DC and MP. A, CD1a and CD14 surface expression on DC and MP, after 6–7 days of culture in the presence of GM-CSF plus IL-4 and M-CSF, respectively, was analyzed by flow cytometry. Results are representative of eight independent experiments. B, Cell surface expression of several molecules in DC and MP mock-infected (gray lines), LV-infected (black lines), mock-infected and activated 2 h after infection with sCD40L (DC) or LPS (MP) (dotted gray lines), or LV-infected and sCD40L- or LPS-activated (dotted black lines), was analyzed by flow cytometry 24 and 72 h after infection. Isotype controls are indicated (shaded histograms). Results are representative of four independent experiments.
and MP were activated 2 h after infection with sCD40L and LPS, respectively, suggesting that LV infection did not interfere with these exogenous activation signals (Fig. 5B and data not shown).

LV does not induce the maturation of DC

We studied the functional properties of infected DC to investigate whether DC matured in response to LV infection. As shown in Fig. 6, the infection of immature DC with LV neither modified their capacity to take up dextran-FITC (85 ± 12% dextran-positive in mock-DC and 82 ± 14% in Lassa-DC) by phagocytosis, nor changed the rate of disappearance of the phagocytic properties of these cells in response to sCD40L treatment 2 h postinfection (p.i.) (36 ± 9% dextran-positive in mock-DC and 38 ± 12% in Lassa-DC). Similar results were obtained with inactivated LV (data not shown).

LV-infected DC and MP do not produce cytokines or IL-2R

The synthesis of several pro- and anti-inflammatory cytokines and of the IL-2R (CD25) was studied at the RNA and protein levels (except for CD25). The levels of several mRNA species were assessed by real-time RT-PCR, with normalization according to β-actin mRNA levels. Certain other mRNA species were detected purely by qualitative RT-PCR. Neither the infection of DC nor the stimulation of these cells with inactivated virus induced the synthesis of mRNA for TNF-α, IL-1β, IL-12p35, IL-10, TGF-β, IFN-γ, or CD25 (Table I). In contrast, we observed a decrease (nonsignificant in most cases) in the production of mRNAs encoding TNF-α and IL-1β in response to LV. Similarly, the synthesis of mRNAs for IL-6 and IL-12p40 was not induced in response to LV infection or to stimulation with inactivated virus (data not shown). These results were confirmed by the absence of TNF-α, IL-1β, IL-10 (Fig. 7A), and IL-12p70 (below the detection thresh-

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### Table 1. Expression of mRNA by infected DC by real-time RT-PCR

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* DC were either mock-infected (mock), inactivated LV-infected (Lassa-γ), or LV-infected (Lassa, MOI = 2), and mRNA were extracted 24 and 72 h after infection for real-time RT-PCR.
+ DC were stimulated with sCD40L 2 h p.i.
* Expression of each cytokine mRNA was normalized with β-actin mRNA, and results of each sample were expressed as the ratio of mRNA expression of sample on mRNA expression of mock-infected DC at 24 h (see Materials and Methods).
+ A significant difference (p < 0.05) in mRNA expression between sample and mock-infected DC at the same time after infection.
old, not shown) in supernatants of infected DC. It is interesting to note that neither LV infection nor inactivated LV-stimulation of DC affected the response to sCD40L activation (2 h after infection), in terms of either mRNA (Table I) or protein (Fig. 7A) levels. Similarly, neither the infection of MP nor the stimulation of these cells with inactivated LV induced the production of mRNAs encoding TNF-$\alpha$, IL-1$\beta$, IL-12p35 and p40, IL-10, IL-6, IFN-$\gamma$, TGF$\beta$, or CD25 mRNA (Table II and data not shown). These results were confirmed by the lack of TNF-$\alpha$, IL-1$\beta$, IL-10 (Fig. 7B), and IL-12p70 (undetected, not shown) proteins in the supernatants of LV-infected MP.

Expression of chemokines and their receptors by LV-infected DC

To determine whether LV infection of DC lead to an induction of the expression of chemokines and their receptors, the expression of mRNA coding for MIP-1$\alpha$, MIP-1$\beta$, IL-8, CCR5, CCR6, and CCR7 was analyzed by real-time RT-PCR. The expression of the mRNA coding for CCR6 was unchanged after infection of DC, activated or not with sCD40L, with LV or stimulation with inactivated LV. On the contrary, infection of DC with LV, and in a lesser extent with inactivated LV, led to a 2-fold drop (nonsignificant) and a 4-fold drop ($p < 0.05$) of the synthesis of CCR5 and CCR7 mRNA, respectively (Table III). LV infection of DC led to a 2-fold enhancement (nonsignificant) of the expression of the mRNA coding for MIP-1$\alpha$ and MIP-1$\beta$ 1 and 3 days after infection, while sCD40L-stimulated LV-infected DC expressed significantly more MIP-1$\beta$ mRNA 3 days p.i than their mock-infected counterparts (Table III). Interestingly, the expression of IL-8 mRNA was significantly up-regulated ($p < 0.05$) following LV infection but not after stimulation with inactivated LV, and the same results have been obtained when DC are stimulated with sCD40L 2 h after infection (Table III). However, the constitutive levels of the respective chemokines in supernatants of DC were unchanged following LV infection, as observed in the Table IV.

Chemotaxis responses of LV-infected DC

The capacity of LV-infected DC to migrate toward several chemokines was studied 3 days after infection. Whereas 24% of mock-infected DC migrated toward MIP-1$\alpha$, only 14% of LV-infected DC have kept this capacity (Fig. 8). The stimulation of DC with LPS led to the disappearance of the response to MIP-1$\alpha$. Mock-infected DC migrated poorly toward MIP-3$\beta$ (6.5% of input cells). Interestingly, infection of DC with LV did not lead to an enhancement (5.1%) in the number of cells that have migrated toward MIP-3$\beta$. Activation of DC with LPS induced a strong migration in response to MIP-3$\beta$ stimulation (52 and 48% for mock- and LV-infected DC, respectively), confirming that LV infection do not interfere with exogenous activation signals. Finally, migration toward MIP-3$\alpha$ was negligible in all conditions tested (Fig. 8). To determine whether LV infection of DC induce the production of chemokines able to attract uninfected DC, the capacity of immature DC to migrate toward LV-infected DC culture supernatants has been observed in response to mock- and LV-infected DC culture supernatants (migration of immature DC toward day 3 mock-infected DC supernatant, 7.1%; toward day 3 LV-infected DC supernatant, 5.1%).

Discussion

In this study, we demonstrated that both DC and MP are susceptible to LV infection and compared the responses of these two cell types to LV infection in vitro. DC and MP are permissive to LV, and the infection is productive in both cell types. More particles were produced at low MOI, and similar results were obtained with Vero E6 cells (data not shown). Although most DC and MP were infected by day 2, as shown by the detection of NP, immature DC produced 30 times more viral particles than MP, almost reaching

Table II. Expression of mRNA by infected MP by real-time PCR$^a$

| Medium | Mock | Lassa | Lassa | +LPS (2 h p.i.)$^b$
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$^a$ Results are expressed as for Table I.

$^b$ MP were stimulated with LPS 2 h after infection.

Table III. Expression of mRNA coding for chemokines and their receptors by infected DC by real-time RT-PCR$^c$

<table>
<thead>
<tr>
<th>Medium</th>
<th>Mock</th>
<th>Lassa</th>
<th>Lassa</th>
<th>+sCD40L (2 h p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
<td>24 h</td>
<td>72 h</td>
</tr>
<tr>
<td>CCR-5</td>
<td>1</td>
<td>1.6</td>
<td>1.5</td>
<td>0.4</td>
</tr>
<tr>
<td>CCR-6</td>
<td>1</td>
<td>2</td>
<td>1.1</td>
<td>2.1</td>
</tr>
<tr>
<td>CCR-7</td>
<td>1</td>
<td>1.8</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>MIP-1$\alpha$</td>
<td>1</td>
<td>3</td>
<td>1.2</td>
<td>2.2</td>
</tr>
<tr>
<td>MIP-1$\beta$</td>
<td>1</td>
<td>3.6</td>
<td>1.3</td>
<td>2.7</td>
</tr>
<tr>
<td>IL-8</td>
<td>1</td>
<td>1.2</td>
<td>0.8</td>
<td>1</td>
</tr>
</tbody>
</table>

$^c$ Results are expressed as for Table I.
the numbers of particles produced in Vero E6 cells. The susceptibility of human MP and of the U937 monocyte line to LV has been reported elsewhere (9, 24). Productive LV-infection of DC was also described in a recent study (25). The numbers of particles found in our culture supernatants were similar to those described in other studies (9, 25). This high-level, persistent production of LV suggests that MP, and particularly DC, may be crucial targets for LV at early stages of the disease. Indeed, given that humans are infected via mucosal/cutaneous contacts with infected body fluids or excreta (1, 6), DC and MP are probably the first target cells encountered by LV, in which the early cycles of replication of LV are likely to take place. The viral particles produced in situ may be disseminated via blood and lymph vessels. Mature DC are also susceptible to LV infection, although to a lesser extent than their immature counterparts. Thus, APC from the spleen and lymph nodes may also act as a reservoir for LV during the course of the disease, which is consistent with the presence of LV Ags in APC and the high viral load observed in the spleen and lymph nodes of LV-infected monkeys (26, 27). Furthermore, other Arenaviruses, such as LCMV and Junin virus, are known to infect splenic DC and MP (17, 20).

LV infection of DC and MP did not affect cell viability. This may be due to binding of the Z protein of LV with promyelocyte leukemia protein, an oncoprotein induced by and involved in type I IFN responses (28, 29). This binding leads to the redistribution of promyelocyte leukemia protein to the cytoplasm (30) and inhibition of its pro-apoptotic activity (31). Thus, the deregulation of host cell apoptotic machinery may allow Arenaviruses to establish persistent infections.

DC and MP were not activated by LV infection. Indeed, the expression of several surface molecules involved in the costimulation of T cells, adhesion, and Ag presentation was not modified during the course of LV infection, even at day 7 (data not shown). Similarly, the lack of synthesis of proinflammatory cytokines by infected DC and MP, observed at both the mRNA and protein levels, confirmed that these cells were not activated. The absence of TNF-α production in LV-infected MP and DC has been reported in previous studies (9, 25). In this study, we observed that none of the proinflammatory cytokines normally induced in virus-infected DC and MP (i.e., TNF-α, IL-1β, IL-6, and IL-12 (32)) was up-regulated. On the contrary, LV infection may even have inhibited the constitutive synthesis of these cytokines, given the slight, but not significantly lower levels of mRNA and protein for TNF-α and IL-1β in LV-infected DC. This finding was unexpected, as most RNA viruses activate DC and MP due to the production of dsRNA, an intermediate product of viral replication that appears to trigger many of the cellular responses to virus infection (33). The recognition of dsRNA is mediated by Toll-like receptor 3, the IFN-inducible protein kinase, and 2’-5’ oligoadenylate synthetase, and results in NF-κB activation (33, 34). The Z protein of LV may be responsible for the lack of cell activation as this protein has been reported to interact with certain host cell translation factors in the LCMV system (35). The lack of proinflammatory cytokine production by LV-infected DC and MP suggests that these cells may not directly be involved in the increase in permeability of the endothelium observed in LV infection, in contrast to previous suggestions (36). Nevertheless, we cannot exclude the possibility that factors other than those analyzed here are produced by infected APC.

LV infection of DC is associated with a moderate induction of the expression of the mRNA coding for the chemokines IL-8, MIP-1α, and MIP-1β, as previously described (25). However, the expression of these mRNA does not lead to a significant release of chemokines in our hands, as we did not observe difference in the levels of the three chemokines in supernatants of mock- and LV-infected DC. Furthermore, no migration of immature DC was detected toward supernatants of LV-infected DC, confirming that MIP-1α and MIP-1β were not significantly released after LV-infection. The discrepancy between our results and the significant levels of IL-8, MIP-1α, and MIP-1β observed by Mahany et al. (25) may be related to the different strains of LV used in both studies, but more probably to the different methods used to prepare the viral stocks. Rather than obtaining LV after 14 days of culture on Vero E6 and disruption of the cell-monolayer, we harvested the LV-containing Vero E6 cell supernatant after 3 days of culture. Viral titers are maximal at this time point in Vero E6 cells. Thus, the viral preparation of Mahany et al. (25) probably contained a significant quantity of noninfectious viral products in addition to infectious particles. Indeed, we found with the AV strain of LV that the ratio of viral RNA copies to infectious viral particles was 100 times higher 14 days after infection of Vero cells than 3 days after infection (unpublished results). These noninfectious viral products may lead to the LV replication-independent release of chemokines by DC, consistent with the finding that chemokine release was induced by both infectious and inactivated LV (25). LV-infected DC do not express CCR6 neither acquire the capacity to migrate toward MIP-3α, a chemokine produced in inflamed tissues. This lack of expression is probably linked to the lack of TGFβ production by LV-infected DC, as monocyte-derived DC express CCR6 only when TGFβ is present in the culture medium (37).

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**Table IV. Production of chemokines by LV-infected DC**

<table>
<thead>
<tr>
<th></th>
<th>Mock 24 h</th>
<th>Mock 72 h</th>
<th>Lassa 24 h</th>
<th>Lassa 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>10.1 ± 3.1</td>
<td>6.6 ± 1.6</td>
<td>10.4 ± 2.0</td>
<td>6.6 ± 0.9</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>67 ± 15</td>
<td>60 ± 23</td>
<td>70 ± 14</td>
<td>70 ± 23</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>395 ± 133</td>
<td>306 ± 155</td>
<td>510 ± 260</td>
<td>420 ± 217</td>
</tr>
</tbody>
</table>

* DC were either mock- (mock) or LV-infected (Lassa, MOI = 2), and supernatants were harvested 24 and 72 h after infection. IL-8, MIP-1α, and MIP-1β were quantified by using commercial ELISA kits. Results were expressed as the mean ± SD of five independent experiments, in nanograms per milliliter for IL-8 and picograms per milliliter for MIP-1β.

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**FIGURE 8.** Chemotaxis responses of LV-infected DC. The capacity of mock- (DC-Mock) and LV- (DC-Lassa) infected DC activated (DC-LPS) or not with LPS 2 h after infection to migrate toward 100 ng/ml rhMIP-1α, MIP-3α, and MIP-3β was evaluated 3 days after infection (see Materials and Methods). Bars represent the percentage of input cells (10⁵) that have migrated in the bottom chamber minus the percentage of input cells that migrated toward medium alone. The absence of chemokinesis has been evaluated by adding a similar concentration of chemokine in the upper chamber. Two independent experiments with different donors with duplicate samples in each have been performed. The data presented are from an experiment representative of the other.
DC do not mature in response to LV infection, as shown by the lack of CD83 expression and of changes in the ability of these cells to take up dextran-FITC. This lack of maturation was confirmed by the lack of induction of allogeneic lymphocyte proliferation by LV-infected DC (25). Furthermore, LV-infection of immature DC did not induce the capacity to migrate toward MIP-3β, a chemokine involved in the chemotaxis of mature DC in secondary lymphoid organs (38), and CCR-7 mRNA levels were significantly decreased by the LV infection of DC. Together with the absence of maturation in LV-infected DC, these results suggest that an immature DC infected in the periphery is unlikely to migrate to the secondary lymphoid organs and induce T cell responses (32, 39), and that splenic immature DC that seem to represent the main population of splenic DC (40), will be massively infected without maturation. This hypothesis is supported by the findings that Ag presentation by immature DC results in tolerance and the induction of regulatory T cells (41, 42), and that proinflammatory cytokines are crucial to the induction of adaptive immunity (43). Similarly, the lack of activation of MP following LV infection may be involved in the pathogenicity of the disease, favoring viral spread, as the activation of MP is known to increase the microbicidal action (44). This is consistent with the finding that infection of the guinea pig with a virulent strain of Pichinde virus, a new-world Arenavirus, leads to a lack of MP activation, whereas the attenuated counterpart of this virus does induce MP activation (45). Furthermore, the infection of MP with Mopeia virus, a non-pathogenic Arenavirus closely related to LV (46), induces the activation of cells (D. Pannetier, manuscript in preparation). Thus, the absence of activation and maturation in LV-infected DC and MP may be associated with viral pathogenicity and with the absence of effective inflammatory and immune responses observed in fatal Lassa infection (14, 16).

Interestingly, LV does not modify the responses of DC and MP to exogenous activation signals such as sCD40L and LPS. This was demonstrated by the lack of change in the modifications induced in cells by these stimuli: up-regulation of surface molecule expression and of cytokine production, and acquisition of the mature phenotype by DC including the capacity to migrate toward MIP-3β. LV infection only slightly inhibits activation as this virus is able to prevent cellular responses by its own products without changing other activation pathways. Most viruses known to inhibit APC activation also affect responses to other stimuli (47, 48). In another report, LV was reported to inhibit the production of TNF-α and IL-8 induced by LPS in MP (9). The reasons for these differences are unclear, but may be related to differences in the experimental conditions used in the two studies. The dose of LPS and the time after LV infection at which LPS was added to cultures differed, as well as the LV strains used. These results suggest that LV probably cannot inhibit the activation of APC by other pathogens and that mature LV-infected DC residing in secondary lymphoid organs may be able to interact with resident T cells via CD40-CD40L signals and to induce effective T cell responses.

We found that immature DC produced significantly more LV particles than MP and mature DC. We cannot yet account for immature DC being the most permissive cells for LV, but investigations are underway. This observation suggests that immature DC may be a better reservoir than MP and mature DC for LV production in vivo.

In conclusion, we have demonstrated that DC and MP are susceptible to a human isolate of LV and that these cells probably play an important role in the early burst of LV replication from the initial site of infection. LV infection did not lead to activation of DC and MP, but the virus did not interfere with exogenous activation signals or with cell viability. Thus, the tropism of LV for APC is probably crucial to the pathogenicity and immunosuppression associated with this severe disease.

Acknowledgments

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References


