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Lymphocytic choriomeningitis virus infection of dendritic cells interferes with TLR-induced IL-12/IL-23 cytokine production in an IL-10 independent manner

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ABSTRACT

Dendritic cells produce IL-12 and IL-23 in response to viral and bacterial infection and these cytokines are responsible for successful pathogen clearance. How sequential viral and bacterial infections affect the production of IL-12 and IL-23 is currently not known. Our study demonstrates that in dendritic cells infected with Lymphocytic choriomeningitis virus (LCMV), TLR activation with bacterial PAMPs resulted in reduced IL-12 and IL-23 expression compared to non-infected cells. Furthermore, expression of other proinflammatory cytokines, TNF-α and IL-6, were not inhibited under these conditions. We discovered that TLR-induced phosphorylation of p38 was significantly inhibited in LCMV-infected cells. We detected enhanced expression of suppressor of cytokine signalling (SOCS)-3 and IL-10. Yet, neutralizing IL-10 did not restore IL-12/IL-23 expression. Taken together, these results show that virus infection interferes with the magnitude of TLR-mediated inflammatory responses by repressing specific cytokine expression.

1. Introduction

Virus infection can modulate the immune response such that immunity to a second bacterial pathogen is compromised [1,2]. During such infections, dendritic cells (DCs) secrete cytokines that serve to activate the adaptive immune response. In particular, DCs produce the IL-12 cytokine family members, IL-12 and IL-23, in response to TLR ligation by bacterial products such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA) [3–5]. While IL-12 induces naïve T cell proliferation and supports Th1 development, IL-23 acts on memory T cells and supports Th17 development [6]. Thus, a lack of efficient IL-12 and IL-23 cytokine production can impact CD4+ T cell differentiation, and impair the immune responses, ultimately affecting pathogen clearance [7].

Due to their effects on CD4 T cell differentiation, the IL-12 family of cytokines is important to the clearance of several bacterial and viral infections [2,7]. It is known that HIV infection inhibits IL-12 and IL-23 expression [8,9], whereas Sendai virus enhances their production after infection [10]. However, exactly how virus infection influences the ability of DCs to produce IL-12/IL-23 in response to TLR activation, akin to what could occur in a secondary bacterial infection, have not been fully described.

In order to elucidate the impact of virus infection on IL-12/IL-23 expression, we used an LCMV-CL13 *in vitro* infection model system, a well-characterized model commonly employed to study mechanisms of immune suppression [11–13]. In this study, we investigated the effects of LCMV-CL13 infection in bone marrow derived dendritic cells (BMDC) on TLR ligand-induced IL-23 and IL-12 production. Our data demonstrate that LCMV-infected BMDC exhibit decreased LPS or LTA-induced IL-23 and IL-12 production. This inhibition was attributed to reduced activation of p38 MAPK in the infected BMDC. Other inflammatory cytokines induced by TLR4 ligation such as IL-6 and TNF-α were upregulated in response to virus infection implying a selective inhibition of cytokine expression.

Taken together, our results demonstrate that virus infection can inhibit production of two key proinflammatory cytokines, IL-12 and IL-23, in response to subsequent TLR stimulation, which is mediated by induction of negative cytokine regulators.

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2. Materials and methods

2.1. Mice, cells and virus preparations

C57BL/6 mice, 6–8 weeks old were purchased from Jackson laboratory's (Bar Harbor, Maine, USA). Bone marrow-derived dendritic cells (BMDC) were generated after culturing for 7 days as we previously described in our work comparing their cultures with macrophages [14]. The BMDC were cultured in RPMI containing 10% FBS, and 10 ng/mL GM-CSF (Cedarlane) followed by surface marker analyses with flow cytometry as described below. For virus infection, LCMV strains (CL13 and WE) [15], originally obtained from F. Lehmann-Grube [16] (Hamburg, Germany), were titrated and propagated as previously described by our group [17]. Mock preparations served as controls for virus infection and were prepared in the same manner but do not contain virus.

2.2. Virus infection and TLR stimulation of BMDC

After 7 days of culturing the bone marrow-derived cells, the non-adherent BMDC cells that express $CD11c^+$ and $MHC\text{-}II^+$ were cultured in RPMI medium with 10% FBS at 37 °C. The cells were then infected with LCMV (MOI = 3) for 6 h prior to LPS (1 μg/ml) or LTA (1 μg/ml) exposure for another 4 h. LPS (*E. coli* 055:B5), which binds TLR4 was purchased from Sigma-Aldrich and purified LTA (*S. aureus*), which binds TLR2 were purchased from Cedarlane.

2.3. Detection of de novo LCMV-NP protein expression

LCMV-NP detection was determined using intracellular staining and flow cytometry analyses. Input virus is not enough to allow for detection and only after replications of the virus, we can detect protein signal. LCMV-infected BMDC were fixed with 4% Formalin for 30 min at room temperature, then permeabilized with 1% Triton-X for 20 min at room temperature and incubated for 1 h with rat anti-LCMV-NP Ab (clone VL4) [17]. The cells were then further incubated with FITC-conjugated goat anti-rat IgG Ab (1 μg/ml, Cedarlane) for 1 h at room temperature after washed twice with PBS. The data were acquired with the Epics XL-MCL flow cytometer and analysed with the Expo 32 Advanced Digital Compensation Software package (Beckmann Coulter, USA).

2.4. Flow cytometry analysis of surface antigens

The phenotypic characteristics of BMDC following 4 h of treatment with TLR ligands or medium alone, with and without virus infection were evaluated by flow cytometry. The cells were stained for 20 min at 4 °C with fluorochrome-labeled anti-mouse Abs (Cedarlane) - specific for MHC-II, I-Ab (clone 25-9-17s), CD11c (clone N418), TLR2 (cloneT2.5) TLR4/MD2 complex (clone MTS510). Background staining with obtained using non-specific antibody isotype controls. The surface expression on the cells was detected by flow cytometry (Beckmann Coulter).

2.5. Cytokine quantification by ELISA

BMDC were incubated with the various stimuli over different time points and the supernatants from those experimental procedures were collected and stored at −80 °C until the enzyme-linked immunosorbent assays (ELISA) were performed as per manufacturer's instructions. The mouse IFNα, IL-23, the IL-12p70 and IL-10 ELISA kits were purchased from Invitrogen and e-Biosciences, USA. The IL-23 and IL-12p70 kits detect both heterodimer subunits for each cytokine and the IFNα kit detects IFNα2 and IFNα4 isoforms.

2.6. IL-10 neutralizing antibody treatment of BMDC

BMDC were treated with anti-mouse IL-10 polyclonal goat IgG antibody (10 μg/ml; R&D Systems, Inc.) or control polyclonal goat IgG isotype antibody (10 μg/ml; R&D Systems, Inc.) 1 h after LCMV infection. After LCMV infection of BMDC for 6 h, LPS from *E. coli* (1 μg/ml) was added to the culture system followed by a second dose of anti-mouse IL-10 Polyclonal Goat IgG antibody (10 μg/ml) or the control polyclonal goat IgG isotype antibody (10 μg/ml) for an additional 4 h in the assay. After the total 10 h of the experimental procedure, IL-10 and IL-23 ELISA assays were carried out on the supernatants as per manufacturer's instructions to quantify their levels.

2.7. Western blot analysis

Protein cell lysates (50 μg of protein per lane) from BMDC cells were separated on 12% SDS-PAGE, transferred onto polyvinylidene fluoride (PVDF) membranes (Pall Corporation) and then blocked 1 h in 2.5% bovine serum albumin (BSA) (Bioshop Canada, Inc.). The blots were stained with different primary antibodies: phosphorylated p38 (Santa Cruz), pan p38 (Cell Signalling), phosphorylated ERK (Santa Cruz), pan ERK (Santa Cruz) and suppressor of cytokine signalling (SOCS3, Santa Cruz) for overnight at 4 °C, followed by secondary staining (1h at room temperature) with peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz). Hsp90 (Santa Cruz) was used as a loading control as indicated. The protein bands in membranes were visualized using ECL Advance Detection Kit (Amersham Biosciences) and detected with HD2 Alphalnnotech imaging system (Fisher Scientific).

2.8. RNA isolation and reverse transcriptase-polymerase chain reactions (RT-PCR)

Total RNA was extracted using the TRI-Reagent method (Bioshop Canada, Inc.), and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen). Aliquots of 2 μl cDNA equivalent to 1 μg of RNA were amplified by PCR for LCMV-NP [18], IL-23p19, IL-12/IL-23p40, IL-10, IFN-α, 18s rRNA, and SOCS3. The primer sequences used for this study are summarized in Table 1. The PCR products were separated on 2% agarose gels (Bioshop Canada Inc) containing ethidium bromide (Sigma) and were observed using the HD2 Alphalnnotech imaging system.

2.9. Reporter gene assay for NF-κB and AP-1 assay

RAW-Blue cells (InvivoGen), derived from RAW264.7 macrophages, stably express a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-κB and AP-1. After selection in Zeocin-supplemented DMEM + 10%FCS, cells were infected with LCMV-CL13 at an MOI of 3 and cultured for 6 h in a 6-well plate. After 6 h, cells were collected and seeded in a flat bottom 96-well plate at a density of 1×10^5 cells/well, then stimulated with LPS or LTA for 18 h before collecting

Table 1
RT-PCR primers.

IL-23p19 forward	5′-TGCTGGATTGCAGCGCAGTAA-3′
IL-23p19 reverse	5′-AGTCCTTGTGGGTCACAACC-3′
IL-12p40 forward	5′-GAGGTGGACTGGACTCCCGA-3′
IL-12p40 reverse	5′-CAAGTTCTTGGGCGGGTCTG-3′
IFN-α forward	5′-TGTCTGATGCAGCAGGTGG-3′
IFN-α reverse	5′-AAGACAGGGCTCTCCAGAC-3′
SOCS3 forward	5′-TGCGCCATGGTCACCCACAGCAAGTTT-3′
SOCS3 reverse	5′-GCTCCTTAAAGTGGAGCATCATACTGA-3′
IL-10 forward	5′-ATTTGAATTCCCTGGGTGAGAA-3′
IL-10 reverse	5′-ACACCTTGGTCTTGGAGCTTATTAA-3′
18s rRNA forward	5′-AAACGGCTACCACATCCAAG-3′
18s rRNA reverse	5′-CCTCCAATGGATCCTCGTTA-3′