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The Envelope Protein of a HERV-W family Human Retrovirus Activates Innate Immunity Through CD14/TLR4 and Promotes Th1 Like Responses.

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Running title: The HERV family MSR/V retrovirus activates innate immunity

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Abstract

MSRV is a retroviral element the sequence of which served to define the W family of human endogenous retroviruses (HERV). MSRV viral particles display pro-inflammatory activities both in vitro in human mononuclear cell cultures and in vivo in a humanized SCID mice model. To understand the molecular basis of such properties, we have investigated the inflammatory potential of the surface unit of the MSRV envelope protein (ENV-SU), the fraction that is poised to naturally interact with host cells. We report here that MSRV ENV-SU induces, in a specific manner, human monocytes to produce major pro-inflammatory cytokines through engagement of CD14 and Toll Like Receptor 4 (TLR4) that are pattern recognition receptors of primary importance in innate immunity. ENV-SU could also trigger a maturation process in human dendritic cells. Finally, ENV-SU endowed dendritic cells with the capacity to support a Th1-like type of T helper cell differentiation. The data are discussed in the context of immune responses and chronic pro-inflammatory disorders.

Introduction

HERV (Human Endogenous RetroViruses) represent about 8% of the human genome and result from integration of exogenous retroviruses that have infected the germline of their host during primate evolution (1). Although most HERV elements are partly or completely deleted following integration, the human genome does contain HERV sequences with open reading frames encoding functional proteins (2). For instance, the so called W family of HERV possesses a copy on chromosome 7 well known to express an envelope protein called syncytin that plays an important role in the placenta physiology through syncytiotrophoblast formation (3-5). Our knowledge of the possible influence of HERV-encoded components in human disorders remains rather limited. In particular, little is known about the effects of HERV proteins on the innate immune system which represents the first line of defense against viruses and operates largely through detection of invariant microbial molecular patterns by pattern recognition receptors (PRR) expressed on antigen presenting cells (APC) such as monocytes/macrophages and dendritic cells as well as other cell types. Examples of PRR are the trans-membrane Toll-Like Receptors (TLR) that can sense distinct microbial products and are central in innate immune response to various classes of pathogens (6-9).

MSRV is an enveloped virus with reverse transcriptase activity (10) that represents the prototype genome which defined the HERV-W family in human DNA (3, 11) and was initially isolated in cell cultures from patients affected by the severe inflammatory demyelinating disorder of the central nervous system (CNS) multiple sclerosis (12, 13). The origin of MSRV particles is still unclear. They could originate from a modified endogenous HERV-W provirus or from a transmissible exogenous member of the same family (11, 14, 15). It was previously observed that MSRV virions trigger the secretion of IL-6 and TNF- α pro-inflammatory cytokines by human peripheral blood mononuclear cells in culture (16). In addition, in severe combined immunodeficiency (SCID) mice grafted with human peripheral

blood cells, intra-peritoneally-injected MSRV virion caused an over-expression of TNF- α leading to death by brain hemorrhages within few days (17). Thus, MSRV particles exert potent pro-inflammatory effects both in mononuclear cells in culture and in vivo in a humanized-SCID mice model.

In the present study, we have investigated the mechanisms of the pro-inflammatory properties of the surface unit (ENV-SU) of MSRV envelope protein. This fraction contains the binding site to the cellular receptor and allows the virus to naturally interact with host cells. We report that ENV-SU is able to specifically activate cells of the innate immune system, such as monocytes, through pattern recognition receptors CD14 and TLR4. This activation is associated with the production of major pro-inflammatory cytokines such as IL-1 β , IL-6 or TNF- α . Moreover, we also show that ENV-SU can activate dendritic cells and promote the development of Th1 like responses.

Materials and methods

Proteins and toxins.

ENV-SU is a 33 KDa and 293 amino acids fraction of the full length MSR (Multiple Sclerosis-associated Retro-Viral element) envelope protein (ENV pV14; genbank AF331500). ENV-SU and ENV pV14 organizations are presented in Figure 1A and amino acid sequence of ENV-SU is presented in Fig. 1B. Recombinant ENV-SU protein was produced and purified by Protein'expert (Grenoble, France) by using the pET-15b expression vector (Novagen, Merck Biosciences, UK) and AD494 (DE3) *E. Coli* cells. Briefly, isopropyl-beta-D-thiogalactopyranoside (IPTG)-treated cultures grown at 37°C were centrifuged and the pellet was washed. After centrifugation, the pellet was resuspended in wash buffer in the presence of a cocktail of protease inhibitors (Roche-Diagnostics) and subjected to sonication. The cell lysate was then centrifuged and the pellet, containing the inclusion bodies, was resuspended in buffer plus protease inhibitors. After centrifugation, the pellet was washed in the presence of 2% TritonX100, then washed again to remove detergent. Inclusion bodies were solubilized by resuspension in the presence of 8 M Urea prior to centrifugation. The supernatant was diluted in Tris HCl pH 8, 1 mM β -mercaptoethanol and loaded onto a Chelating Sepharose Fast Flow column (Amersham Biosciences) for purification under denaturing conditions. ENV-SU was refolded by dialysis against Tris 50 mM pH 7, NaCl 300 mM, β -mercaptoethanol 1 mM, Sucrose 2%, Glycerol 2%, 2 M urea. Under these conditions, the protein remained soluble. Amino acid sequence determination of purified ENV-SU based on Edman degradation was in accordance with the known sequence. Fifty μ g/ml aliquots were flash frozen into liquid nitrogen and stored at -80°C. SDS-PAGE and mass spectrometry (MALDI-TOF) analysis profiles of recombinant ENV-SU (Protein'expert) are shown in Fig. 1C-D. A mock protein, casein kinase, was used as negative control in experiments and was synthesized and purified under the same conditions as ENV-SU. Both proteins were tested for the presence of

endotoxins by a Lymulus Amebocyte Lysate (LAL) test performed by CleanCells (Bouffere, France) and all fractions were below the detection level of 5 UI/ml. Staphylococcal enterotoxin B (SEB) was obtained from Toxin Technology (Sarasota, FL) and was 95 % pure. Lipopolysaccharide (LPS) from E. coli strain 026:B6 was obtained from Sigma (St Louis, Mi).

Cell isolation and preparation.

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donor buffy coats by density gradient centrifugation over Ficoll Paque. Unlabeled monocytes were isolated from PBMCs by depletion of T cells, B cells, dendritic cells, basophils and NK cells by using a Monocyte Isolation Kit II from Miltenyi Biotec (Bergisch Gladbach, Germany). Briefly, cells were incubated with a cocktail of biotinylated antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and CD235a prior to addition of Anti-Biotin MicroBeads. The cell suspension was applied onto a MACS Column placed in the magnetic field of a MACS Separator and the effluent was collected along with fractions corresponding to three washes. The purity of monocytes was assessed by immuno-staining (anti-CD14 MOP9 mAb) and flow cytometry and ranged from 96 to 98%. For the generation of monocyte-derived dendritic cells (MDDC), purified monocytes were cultured for 5 days in the presence of recombinant IL-4 (25 ng/ml) and GM-CSF (100 ng/ml) (both from R&D systems, Minneapolis, MN). Cells were fed at day three of culture with full amount of cytokines. As assessed by morphology and flow cytometry analysis the resulting cell preparation contained more than 90 % of CD1a positive dendritic cells. Similar results were obtained in experiments when MDDC culture were used directly or after depletion of possible residual CD14+ cells by immuno-magnetic separation. Naïve CD4+CD45RA+ T cells were isolated from total PBMC using a naïve T

cell enrichment cocktail (StemCell Technologies, Vancouver, CA). The recovered CD4+ T cell populations were always more than 90 % pure.

Cell stimulation.

Cells (PBMC, monocytes or MDDC) were plated in 24-well plates at a concentration of 1×10^6 per well in 1 ml of medium consisting in RPMI 1640 (Gibco, Rockville, MD) supplemented with 1 % L-glutamine, 1 % penicillin/streptomycin, 1 % sodium-pyruvate, 1 % non-essential amino acids (all from Sigma), and 10 % heat inactivated FCS (BioWest, Nouaille, France). After stimulation with ENV-SU, LPS, SEB or Mock, cells were incubated at 37°C in 5 % CO₂ in humidified atmosphere for various periods of time. Where indicated, proteins and toxins were pre-treated for 30 minutes at 37°C with 25 µg/ml of polymyxin B (PB) (Sigma) prior to stimulation. In some cases, cells were pre-incubated before stimulation with either 20 µg/ml of anti-CD14 (Polyclonal sheep IgG; R&D systems), anti-TLR4 (Monoclonal mouse IgG2a; clone HTA125, eBioscience, San Diego, CA)(18, 19) or anti-TLR2 antibodies (Monoclonal mouse IgG2a; clone TL2.1, eBioscience). In some experiments, ENV-SU, Mock, LPS and SEB were boiled for 30 min before cell stimulation. To determine the specificity of our results, prior to cell stimulation, 1 µg of ENV-SU, Mock and LPS were pre-incubated for 45 min at 4°C with 30 µg/ml of monoclonal antibodies (mAbs) directed against either ENV-SU (Monoclonal mouse IgG1; clone 13H5A5 and 3B2H4; bioMérieux, Marcy l'Etoile, France) or GAG (Monoclonal mouse IgG1; clone 3H1H6; BioMérieux) recombinant proteins. 13H5A5, 3B2H4 and 3H1H6 mAbs were obtained by immunization of mice with respectively ENV-SU and GAG recombinant proteins. The specificity of the antibodies was controlled by ELISA and only 13H5A5 and 3B2H4 mAbs specifically bound ENV-SU recombinant protein.

Immunofluorescence staining and flow cytometry.

Cells were harvested, washed in PBS and immuno-stained for surface expression of distinct markers. The following mAbs were obtained from Becton-Dickinson (San Jose, CA): anti-CD1a allophycocyanin (HI149-APC), anti-CD14 fluorescein isothiocyanate (MOP9-FITC), CD40 phycoerythrin (5C3-PE), CD80 phycoerythrin (L307.4-PE), CD86 phycoerythrin (IT2.2-PE) and HLA-DR peridin chlorophyll (L243-PerCP). Briefly, direct immunofluorescence staining of cells was performed in ice cold PBS supplemented with 2 % of FCS in the presence of mAbs at concentrations recommended by the manufacturers. After 30 min at 4°C, cells were washed and analyzed using a FACS Calibur and the software CellQuest (Becton-Dickinson).

Cytokine production and T cell polarization assays.

Culture supernatants were harvested and preserved at -20°C before evaluation of cytokine production by ELISA. OptEIA ELISA kits from PharMingen (San Diego, CA) for detection of human IL-1 β , IL-6, IL-12p40, IL-12p70 and TNF- α , were used according to the manufacturer's instructions.

For T cell polarization assay, stimulated MDDC were used as stimulators. Responder cells were purified allogeneic CD4⁺CD45RA⁺ T cells used at 1×10^5 per well (96-well round bottom microtiter plates). Stimulatory cells were added to T cells in graded doses and cultures were set up in triplicates in a final volume of 200 μ l of medium supplemented with 10% of human AB serum (Sigma). After 5 days of incubation at 37°C, cell supernatants were collected and tested for the production of T cell cytokines by ELISA.

Results

ENV-SU stimulates the production of pro-inflammatory cytokines in human PBMC cultures. We first studied ENV-SU abilities to stimulate cytokine production in PBMC cultures from healthy controls. Human PBMC were incubated with graded doses of ENV-SU for 24 hours and secretions of TNF- α , IL-1 β and IL-6 were analyzed by ELISA. We observed that ENV-SU induced the production of all three cytokines in a dose/dependent manner even at doses as low as 10 ng/ml (Fig. 2A). In parallel, a mock control protein (Mock), produced and purified under the same conditions as ENV-SU, was also tested and no cytokine production was observed (not shown). We then studied the kinetics of cytokine secretion induced by ENV-SU in PBMC cultures and compared it with Mock, SEB (a well characterized bacterial SA α) and LPS. All proteins and toxins were used at a concentration of 1 μ g/ml, found to be the optimal concentration for pro-inflammatory cytokine production. As shown in figure 2B, ENV-SU-mediated kinetics of cytokine secretion was more similar to that of LPS rather than SEB. Both ENV-SU and LPS induced the secretion of high amounts of TNF- α , IL-6 and IL-1 β already after 24 hours. TNF- α and IL-1 β reached their peak of production at this time point and then decreased while IL-6 levels constantly increased following stimulation. SEB did not induce any IL-1 β or IL-6, but a constant TNF- α secretion was observed. Mock-induced cytokines remained below the detection threshold. Finally, separate experiments have revealed that secretion of IFN- γ and IL-10 are marginal in PBMCs cultures treated for 24 hrs with soluble ENV-SU (20). Thus, ENV-SU induces the secretion of major pro-inflammatory cytokines in human PBMC cultures from healthy donors.

ENV-SU-mediated pro-inflammatory properties are specifically inhibited by monoclonal antibodies and are not due to endotoxin contaminations. The possibility that the pro-inflammatory effect of ENV-SU essentially reflected endotoxin contamination was

ruled out by successive analysis. As a first control, the protein preparations were tested in a FDA-approved LAL assay (see methods) and were negative. Indeed, it was unlikely that trace amounts (<5 UI/ml) of endotoxin could cause a higher release of IL-6 by PBMC than the secretion observed in the presence of 1 µg/ml of pure LPS (Fig. 2B) and doses of LPS corresponding to the detection threshold of the LAL assay effectively did not cause such effects (not shown). In order to establish the specificity of ENV-SU pro-inflammatory properties, we studied the effects of anti-ENV-SU monoclonal antibodies (mAb) on the cytokine production previously described. PBMC were incubated for 24 hours with Mock, ENV-SU or LPS pre-incubated with antibodies directed against either MSR V ENV-SU or MSR V GAG. As shown in figure 3A, two of the anti-ENV-SU monoclonal antibodies (13H5A5 and 3B2H4) tested specifically blocked ENV-SU-mediated TNF- α secretion but not that induced by LPS. In contrast, cytokine secretion was not affected in either case by treatment with anti-GAG mAb. Since the 13H5A5 and 3B2H4 mAbs had no effect on LPS-induced TNF- α secretion, the data make the additional point that the inhibition of ENV-SU pro-inflammatory activity cannot be explained by a hypothetical toxic effect of these antibodies on PBMC. These results definitely demonstrate that ENV-SU can specifically cause PBMC activation and cytokine release.

Nevertheless, we performed complementary control experiments either by boiling or treating the proteins and toxins with the cationic antibiotic polymyxin B (PB) that neutralizes LPS activity, prior to their addition to the culture. Figure 3B shows that while PB treatment completely abolished LPS-induced TNF- α secretion, it only marginally affected ENV-SU-induced TNF- α production. Conversely, boiled ENV-SU lost most of its stimulatory capacity whereas LPS stimulatory potential remained unaffected by boiling. Taken together, the results indicate that residual endotoxin contamination cannot account for the pro-inflammatory activity of ENV-SU and that the component responsible for this activity is a protein.

ENV-SU directly activates purified human monocytes.

While analysing total PBMC cultures exposed to ENV-SU for 24 and 48 hours, we observed that the frequency of both CD25⁺ and CD69⁺ T lymphocytes remained at the background level. By intracellular staining, CD3⁺ mononuclear cells were also devoid of TNF- α , IFN- γ and IL-4 production (data not shown). Thus, the data failed to reveal any signs of T cell activation in response to soluble ENV-SU in short term cultures. In addition, the profile of cytokine production induced by ENV-SU was comparable to that induced by LPS and is reminiscent of the pattern observed upon monocyte activation. We therefore examined whether ENV-SU was able to directly activate monocytes. Unmanipulated CD14⁺ mononuclear cells isolated by negative immuno-magnetic separation (>96 % purity) were stimulated with Mock, ENV-SU or LPS for 24 hours and the expression of the activation marker CD80 was evaluated by flow cytometry. When compared to Mock, ENV-SU induced the up-regulation of CD80 expression at a level similar to that induced by LPS (Fig. 4A). Several other markers such as CD40, CD86 and HLA-DR were also tested but no differences were observed at this time point (not shown). We then studied the profile of cytokine secretion induced by ENV-SU in purified monocytes and observed that high amounts of TNF- α , IL-1 β , IL-6 and IL-12p40 were produced in response to ENV-SU (Fig. 4B) while only marginal levels of IL-12p70 were obtained (not shown). Similar observations were made when using heat-inactivated MSR_V particles indicating that the pro-inflammatory effects of recombinant ENV-SU on purified monocytes is recapitulated by viral particles (Fig. 5). Taken together these observations indicate that ENV-SU induces a rapid and direct monocyte activation associated with pro-inflammatory cytokine secretion.

CD14 and TLR4 pattern recognition receptors are involved in the pro-inflammatory response to ENV-SU.

The detection of pathogen-associated molecular patterns by antigen presenting cells relies largely on a group of trans-membrane PRRs named Toll-Like Receptors (TLR) (6, 7, 9). For instance, TLR4 engagement mediates monocytes/macrophages activation in response to Gram-negative bacteria through LPS detection (21). Findings supporting a role for TLR in virus detection include the sensing of double stranded viral RNA by TLR3 (22), a role for TLR2 in IL-6 secretion by macrophages in response to the measles virus (23) and the identification of TLR4 as an essential component of the response to respiratory syncytial virus (RSV) fusion protein (24) and possibly Mouse Mammary Tumor Virus (MMTV) (25). To determine whether ENV-SU is susceptible to engage activation pathways similar to those triggered by LPS or RSV fusion protein, (namely TLR4 and the accessory glycosyl phosphatidylinositol anchored protein CD14), we pre-treated purified monocytes with anti-CD14, anti-TLR4 and TLR2 neutralizing antibodies prior to activation with ENV-SU, LPS or PMA which activates monocytes in a TLR4/CD14 independent manner. The levels of TNF- α secreted were then measured (Fig. 6). No significant inhibition of TNF- α production was observed with any blocking antibody tested when monocytes were stimulated with PMA. In contrast, when tested on ENV-SU- or LPS-stimulated monocytes, both CD14 and TLR4 blocking antibodies caused substantial inhibition of TNF- α secretion. The inhibition of ENV-SU-induced cytokine production by anti-CD14 antibodies was consistently stronger than that observed in the presence of the weak affinity anti-TLR4 HTA125 antibody. Such inhibitory effects were found to be dose sensitive (not shown). TLR2 blocking antibody, which was used as an isotype control did not induce any inhibition under any conditions tested. In addition, TLR4, but not TLR2, blocking antibody was also shown to interfere with cytokine production induced by heat-inactivated MSRVP particles (Fig. 7). These results indicate that both TLR4

and CD14 pattern recognition receptors are involved in the pro-inflammatory effects of ENV-SU on human monocytes.

ENV-SU directly activates Monocyte Derived Dendritic Cells and confer them the potential to support the development of Th1 like responses.

Dendritic cells (DC) are professional antigen presenting cells that express a variety of TLRs. TLR engagement induces DC to up regulate antigen presenting and costimulatory molecules expression as well as to secrete pro-inflammatory cytokines. These maturation events render them highly potent at activating naïve T cells and promoting their differentiation in effector cells (26, 27). We therefore examined whether ENV-SU was able to directly activate monocyte-derived DC (MDDC) and whether these MDDC would show any capacity to polarize naïve T cell responses. MDDC were generated in vitro from highly purified human monocytes by using IL-4 and GM-CSF and stimulated during 24 hours with Mock, ENV-SU or LPS. In contrast to the differentiation marker CD1a whose expression level remained unaltered, we observed that ENV-SU induced a substantial increase in the surface expression level of CD80, CD86, CD40 and HLA-DR molecules, indicating that ENV-SU triggers a phenotypic maturation process in MDDC (Fig. 8A). Regarding the secretion of pro-inflammatory cytokines, IL-6, TNF- α , IL-12p40 and IL-12p70 were produced at rather high levels only in the presence of ENV-SU or LPS (Fig. 8B).

IL-12 is a major cytokine for committing T cells to Th1 lineage differentiation (28). Th1 and Th2 subsets can develop upon interaction of mature DC with the same T cell precursor, which is a naïve CD4 T cell. Thus, in order to evaluate the ability of stimulated MDDC to polarize naïve T lymphocytes, MDDC were co-cultured with purified allogeneic CD4⁺ CD45RA⁺ T cells and cytokine production was measured. MDDC pulsed with ENV-SU were much more efficient at inducing naïve T cells to secrete IFN- γ than IL-4 (Th1 and Th2 associated

cytokines respectively). High amounts of IFN- γ (Fig. 8C, left panel) were indeed produced even at responder/stimulator ratios as low as 1/100 while the amount of IL-4 (right panel) obtained when MDDC were stimulated with ENV-SU was consistently below the background level of the Mock control. By performing inhibition experiments, we found that anti-CD14 antibodies could detectably interfere with the phenotypic activation of immature MDDC induced by ENV-SU (Fig. 9A). Inhibition by the anti-TLR4 HTA125 mAb was marginal (not shown). Nevertheless, we consistently noticed that HTA125 was able to reinforce the inhibitory effect of the anti-CD14 antibodies on ENV-SU-induced cytokine secretion by MDDCs. This effect was indeed reminiscent of that observed in experiments performed with purified monocytes (see Figure 6). Altogether, the data indicate that ENV-SU is able to induce phenotypic and functional maturation of dendritic cells and confer them the potential to support the development of Th1-like effector T lymphocytes.

Discussion

The present study provides evidence that the surface unit of the envelope protein (ENV-SU) of MSR/V, a retroviral element of the HERV-W family is capable of activating innate immunity through pattern recognition receptors TLR4/CD14. This conclusion arises from the observation that ENV-SU induced human monocytes to produce major pro-inflammatory cytokines in a CD14 and TLR4 dependent fashion. The specificity of such phenomenon was unequivocally shown by the inhibitory effect of two anti-MSR/V-ENV monoclonal antibodies. ENV-SU also induced dendritic cell maturation and conferred them the capacity to support a T helper 1 type of T cell differentiation.

CD14 and TLR4 are evolutionary conserved receptors with critical roles for the activation of antigen presenting cells and pro-inflammatory cytokine production in response to lipopolysaccharide (21, 29, 30). In addition, Respiratory Syncytial Virus (RSV) induce cytokine secretion by human monocytes via CD14 and TLR4 engagement and TLR4 deficient mice infected with RSV showed deficient IL-12 production and NK cell response as well as an increased titer of viral particles in the lung relative to control mice (31). Thus, CD14 and TLR4 are PRR that cooperate for the activation of innate immunity in response to both bacteria and viruses. Our observations that CD14 and TLR4 are involved in mediating the pro-inflammatory effect of the MSR/V envelope protein identify proteins of HERV-W family as a putative new class of viral ligand for these PRR. As dendritic cells do not express CD14 at their surface, their capacity to react to MSR/V ENV-SU most certainly relies on the recruitment of soluble CD14 that is present in the serum as it is known to be the case for the response of dendritic cell to LPS stimulation (32, 33).

The activation of the innate immune system might contribute to the development of neurodegenerative diseases and a possible role for CD14 and TLR4 in autoimmune/pro-inflammatory disorders has been evoked. It was thus shown that the level of soluble CD14

naturally present in plasma was elevated in diseases such as multiple sclerosis (34), rheumatoid arthritis (35) or systemic lupus erythematosus (36). Moreover, specific activation of CNS innate immunity through TLR4 can lead to neuro-degenerative phenomena (37) via activation of brain-resident macrophages (microgliaocytes) which are the only glial cells with TLR4 expression. Finally, TLR4 is necessary for LPS-induced oligodendrocyte injury in the CNS (38). These results indicate that the engagement of TLR4 expressed on CNS resident, and/or peri-vascular, macrophages by its ligands might contribute to oligodendrocytes damage and neuro-degeneration.

Since MSR/V was first isolated in choroid plexus/leptomeningeal cell cultures from multiple sclerosis patients (12, 13), and virion-associated MSR/V RNA can be detected in sera and/or cerebrospinal fluids of such patients (39, 40), it is plausible that its envelope protein can exert its CD14/TLR4 dependent pro-inflammatory effect within the CNS and therefore initiates, and/or substantially exacerbates the disorder. This idea is in line with the findings that (i) HERV-W ENV and GAG glycoproteins are expressed in the white matter of patients with multiple sclerosis (41-43), and that (ii) the virus load detected in the CSF increases with MS progression and thus may have prognosis value (38). HERV-W 7q ENV expression is enhanced relative to tissues from healthy controls or patients with other neurological disorders and has the potential to cause inflammation and oligodendrocyte death through the induction of redox reactants in astrocytes (41).

A role for TLR has also been argued in initiation of autoimmunity. One possible way by which MSR/V-mediated activation of the TLR4 signalling pathway could contribute to the development of autoimmunity is by interference with the immune suppression naturally mediated by a subclass of CD4⁺ T lymphocytes named regulatory T cells (Treg). For instance, Treg can readily suppress the development of auto-immune responses in Experimental Autoimmune Encephalomyelitis (EAE), an animal model for multiple sclerosis

(44). A recent report documented that IL-6 produced following TLR-mediated recognition of microbial products renders naive CD4⁺ T cells insensitive to the suppressive activity of CD4⁺ CD25⁺ Treg (45). We have here demonstrated that via ENV-SU, MSRV could induce the production of massive amounts of IL-6 in human monocyte and MDDC cultures. Thus, through the TLR4 dependent secretion of IL-6, ENV-SU could interfere with the suppressive activity of Treg cells and therefore, facilitate the priming of auto-reactive T cells.

DC are antigen-presenting cells, which upon activation, have the unique ability to induce primary specific immune responses. Various pathogen products can induce the maturation of dendritic cells through TLR signalling. In the present study, MDDC pulsed with ENV-SU displayed all features of a mature phenotype. Interestingly, in the context of multiple sclerosis, some DC were shown to express the maturation marker CD83 in active lesions (46) and to secrete pro-inflammatory cytokines such as IL-6 and TNF- α in peripheral blood (47). Depending on the maturation stimuli, MDDC can induce the activation/polarization of naive T cells towards either Th1 or Th2 lineage. MDDC stimulated with ENV-SU produced large amounts of pro-inflammatory cytokines including IL-12p70, and were able to promote the development of naive CD4⁺ CD45RA⁺ T cells into IFN- γ secreting Th1 like cells. Induction of Th1 cells insufficiently counterbalanced by Th2 cells has indeed been proposed in the pathogenesis of demyelinating disorders (48).

Altogether, our observations support the notion that ENV-SU of the HERV-W family MSRV element, can activate the innate immune system through a TLR4/CD14 dependent pathway and is susceptible to promote the development of a Th1 type of immune responses upon DC activation. The data are compatible with the idea that, through the pro-inflammatory properties of its surface envelope protein, MSRV could be involved in the immunopathological cascades associated with chronic inflammatory and/or neurodegenerative diseases.

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Footnotes:

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Figure legends

Figure 1. ENV-pV14 and ENV-SU organizations and amino acid sequences.

(A) Organizations of ENV-pV14 (full length MSRNV envelope protein) and ENV-SU. ENV-SU is a 293 amino acids fraction, representing the extracellular surface unit cleaved at K316 of the full length ENV pV14 protein. (B) Amino acid sequence of ENV-SU (bold characters). (C) SDS-12,5% PAGE analysis of recombinant ENV-SU after purification by affinity chromatography under denaturing conditions (molecular mass is in kDa). (D) Mass spectrum of the recombinant ENV-SU protein. The profile represents the transformation of the mass to charge ratio on the real mass scale. Vertical axis: % relative intensity; horizontal axis: molecular weight.

Figure 2. ENV-SU induces the production of pro-inflammatory cytokines in PBMC cultures.

(A) PBMC from healthy donors were stimulated for 24 hours with graded doses of ENV-SU and culture supernatants were then analyzed by ELISA for TNF- α , IL-1 β and IL-6 production. (B) PBMC were stimulated with 1 μ g/ml of Mock, ENV-SU, LPS or SEB and incubated for 24, 48 and 72 hours before analysis of cytokine secretion by ELISA. Results are presented as the mean +/- standard error of three independent experiments.

Figure 3. ENV-SU mediated TNF- α production is inhibited by specific monoclonal antibodies and is not due to endotoxin contamination.

(A) PBMC were stimulated for 24 hours with 1 μ g/ml of mock control, ENV-SU and LPS pre-incubated or not for 45 min at 4°C with mAbs (30 μ g/ml) specific for ENV-SU (Clones 13H5H5 and 3B2H4) or GAG (Clone 3H1H6) proteins. Culture supernatants were harvested and analyzed for TNF- α secretion. (B) Where indicated, the proteins and toxins were pre-

treated for 30 minutes at 37°C with 25 µg/ml of PB prior to PBMC stimulation. In parallel, cells were also incubated with proteins and toxins boiled for 30 min (100°C). Culture supernatants were then harvested and tested by ELISA for TNF-α release. Results represent the mean +/- standard error of three independent experiments.

Figure 4. ENV-SU directly activates purified human monocytes.

(A) Human monocytes were purified from human PBMC (> 95% purity) and then stimulated with Mock, ENV-SU or LPS at 1 µg/ml for 24 hours. Cells were harvested and surface expression of the activation marker CD80 was analyzed by flow cytometry. One representative experiment of Mock, ENV-SU and LPS stimulation is presented. (B) Cytokine production (TNF-α, IL-1β, IL-6 and IL-12p40) was analyzed by ELISA. Results represent the mean +/- standard error of three independent experiments.

Figure 5. MSRV particles directly activate purified monocytes.

(A) Purified monocytes were stimulated for 24 hours with 2×10^3 MSRV particles or MOCK control and the expression of several activation markers such as CD80 and CD86 were assessed by flow cytometry. The surface expression of both CD80 and CD86 were up regulated following MSRV treatment. MSRV particles were obtained from MSRV infected immortalized B cell cultures after ultra-centrifugation. MSRV particles were quantified by reverse transcriptase activity measurement. All MSRV particles preparations were mycoplasma free. The MOCK control was prepared from MSRV negative B cell cultures.

(B) The supernatants were then tested for pro-inflammatory cytokine secretion by ELISA.

The values are the mean +/- standard error of replicate from one experiment representative of three.

Figure 6. CD14 and TLR4 are involved in ENV-SU mediated pro-inflammatory properties.

Purified monocytes were pre-incubated at 37°C for one hour with or without neutralizing antibodies to CD14 (polyclonal IgG), TLR4 (HRTA125 mAb, functional grade) and TLR2 (TL2.1 mAb) (all at 20 µg/ml) and then further stimulated for 24 hours with PMA at 100 ng/ml, ENV-SU at 200 ng/ml or LPS at 50ng/ml. The effects of PB were also tested in parallel as a control. Culture supernatants were harvested and TNF-α release was evaluated by ELISA. Isotype controls (Functional grade mouse IgG2a and polyclonal sheep IgG) were also tested and no inhibitory effects were observed (not shown). The values represent the mean +/- standard error of replicate from one experiment representative of three.

Figure 7. MSRV particles activate purified monocytes through TLR4.

Purified monocytes were pre-incubated at 37°C for one hour with or without (x) neutralizing antibodies to TLR4 (α TLR4) or TLR2 (α TLR2) (both at 20 µg/ml) and then further stimulated for 24 hour with 2×10^3 MSRV particles. Culture supernatants were harvested and TNF-α release was evaluated by ELISA. The values correspond to the mean +/- standard error of three independent experiments.

Figure 8. MDDC activated with ENV-SU secrete IL-12 and promote the development of Th1 like responses.

(A) MDDC were generated from purified monocytes and then stimulated with Mock, ENV-SU (1 µg/ml) or LPS (100 ng/ml) for 24 hours. Surface staining of activation markers HLA-DR, CD80, CD86 and CD40 was made after Mock (dotted line), ENV-SU (shaded histogram) and LPS (bold line) treatments and then analyzed by flow cytometry. One representative experiment is presented. (B) Cytokine productions (TNF-α, IL-6, IL-12p40 and IL-12p70)

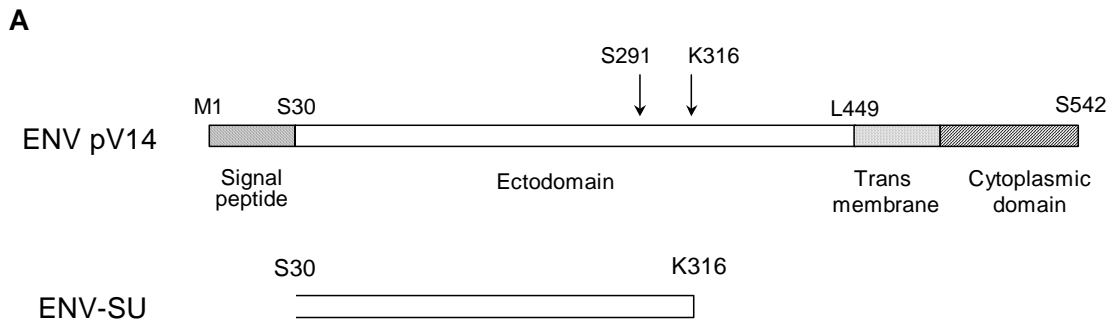
were analyzed by ELISA. (C) Various concentrations of MDDC stimulated either with Mock (dotted line), ENV-SU (bold line) or LPS were co-cultured for 5 days with purified allogeneic CD4⁺ CD45RA⁺ T cells. Supernatants were then tested for IFN- γ (left panel) and IL-4 release (right panel). The values represent the mean \pm standard error of three (B) and two (C) independent experiments.

Figure 9. CD14 and TLR4 receptors are involved in ENV-SU-mediated activation of immature MDDC.

Monocytes-derived MDDC were stimulated with ENV-SU (5 ng/ml) and analyzed for phenotypic maturation and cytokine secretion.

(A) Inhibition of ENV-SU-induced MDDC phenotypic maturation by anti-CD14 mAbs. Surface expression of CD40, CD86, CD80, and HLA-DR were analyzed by immunostaining and flow cytometry (vertical axis: cell count, horizontal axis: Log fluorescence intensity). Vehicle (closed histograms), ENV-SU (open histograms, bold line), ENV-SU + 20 μ g/ml anti-CD14 (open histograms, dotted line), and ENV-SU + 20 μ g/ml control IgG (open histograms, regular line). The histograms shown are representative of three independent experiments.

(B) Additive inhibitory effect of anti-TLR4 and anti-CD14 antibodies on cytokine secretion by ENV-SU-treated immature MDDC. Cytokine productions (TNF- α , IL-6 and IL-12p40) were analyzed by ELISA after stimulation in the presence of anti-CD14, anti-TLR4, anti-TLR2 or anti-CD14 + anti-TLR4. The data are representative of two independent experiments.



B

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 NSATLCMHANTHYWTGKMINPSCPGLGATVCWTYFHTSMSDGGGIQQQAREKQVKEAISQLTRGHSTPS
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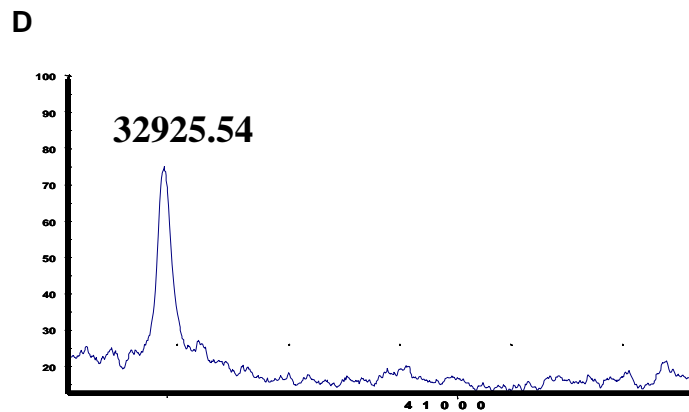
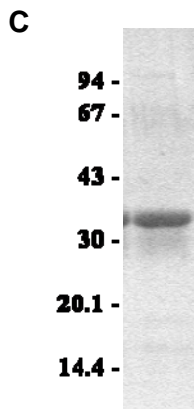


Figure 1

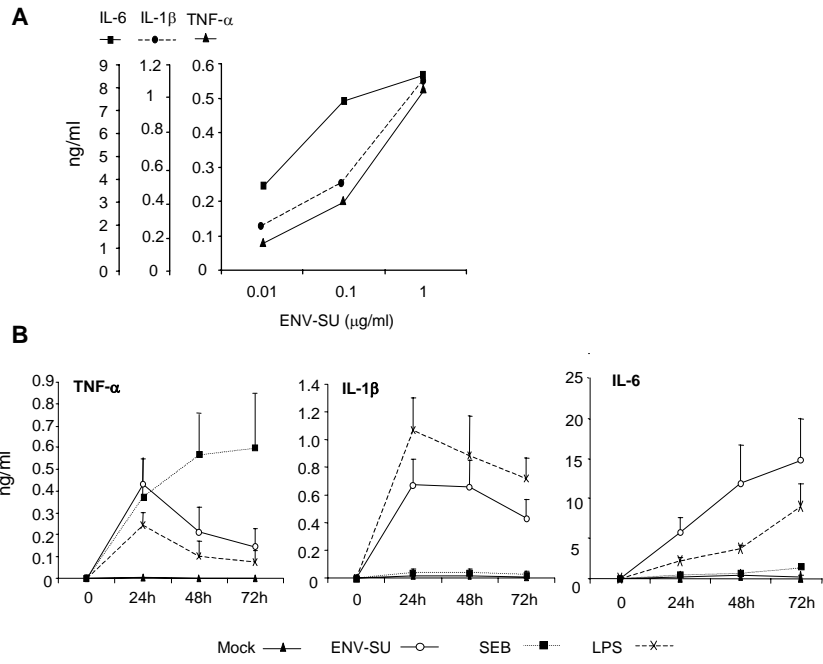


Figure 2

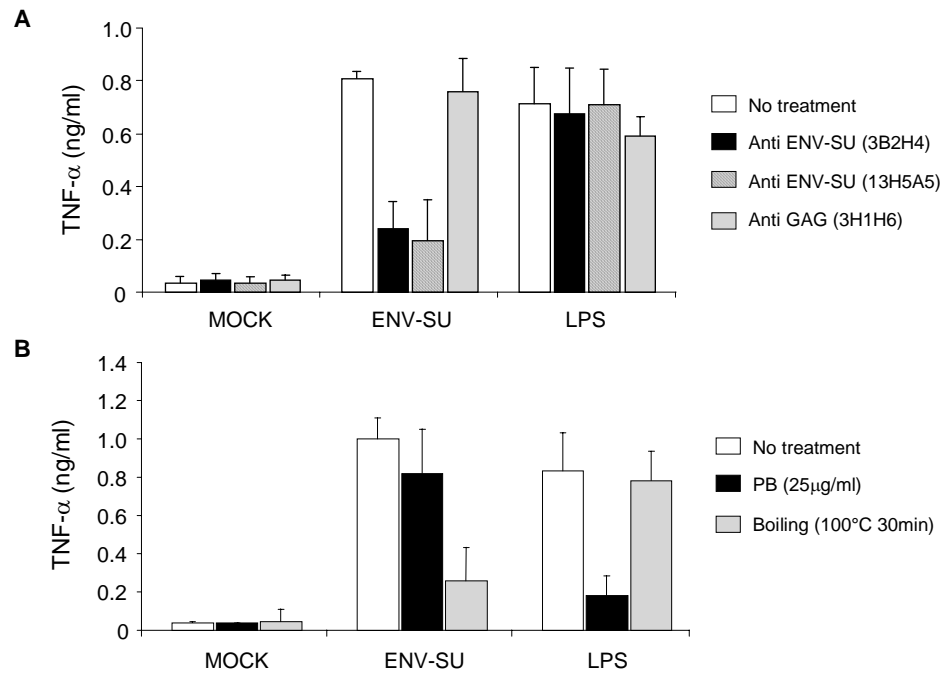


Figure 3

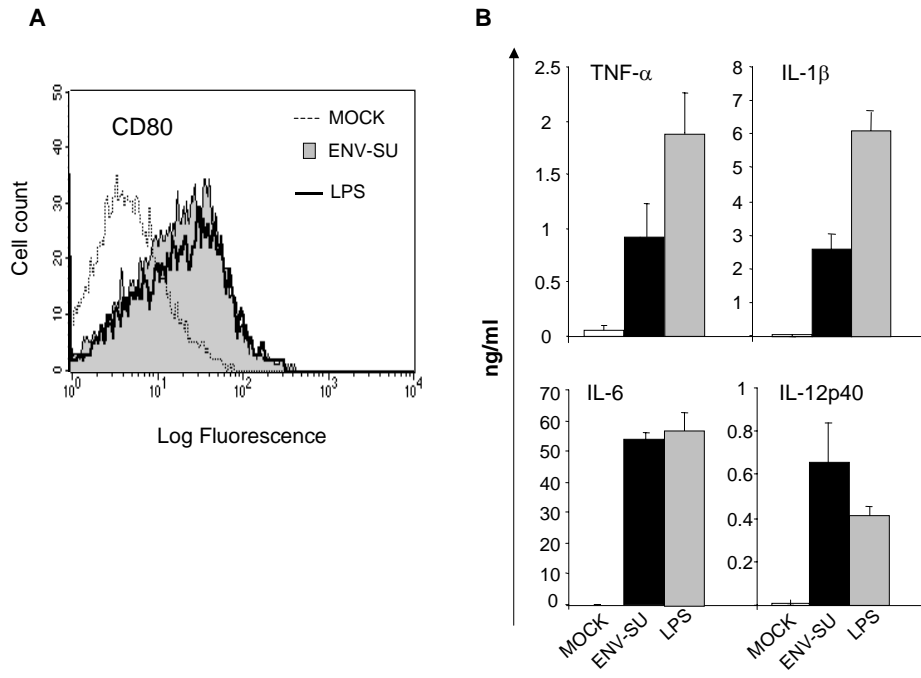
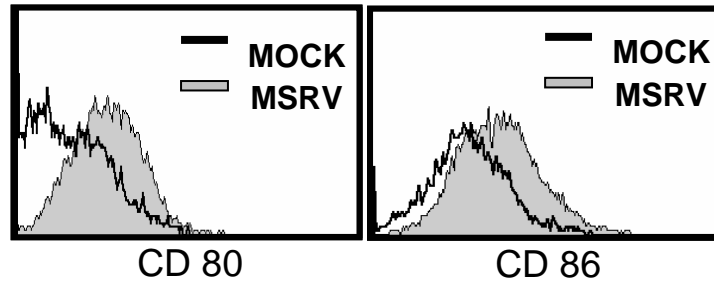


Figure 4

A



B

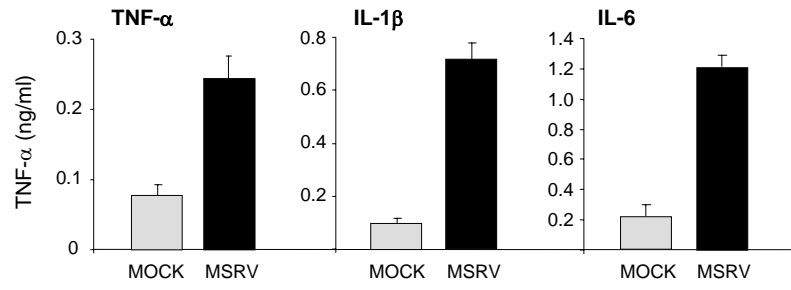


Figure 5

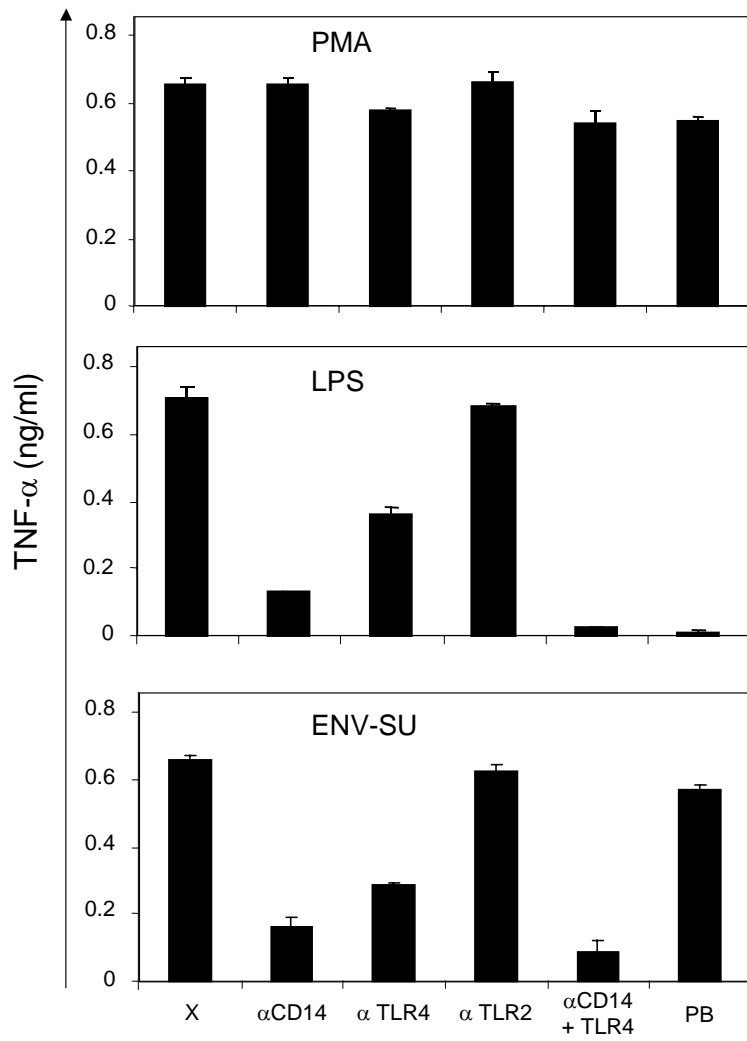


Figure 6

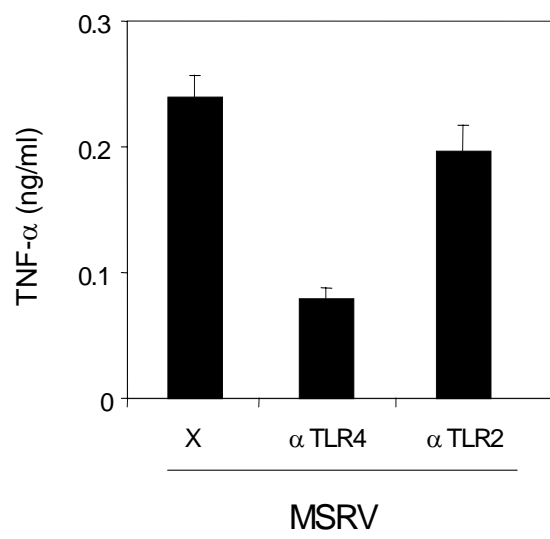


Figure 7

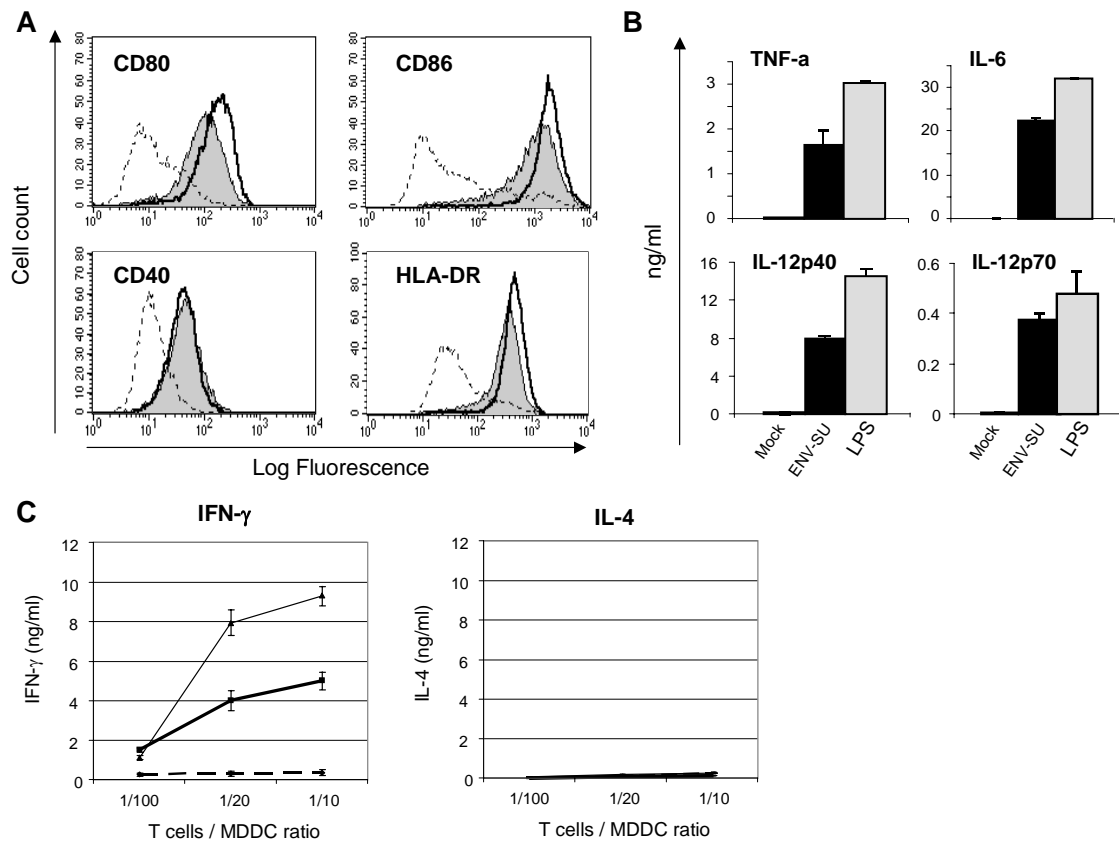
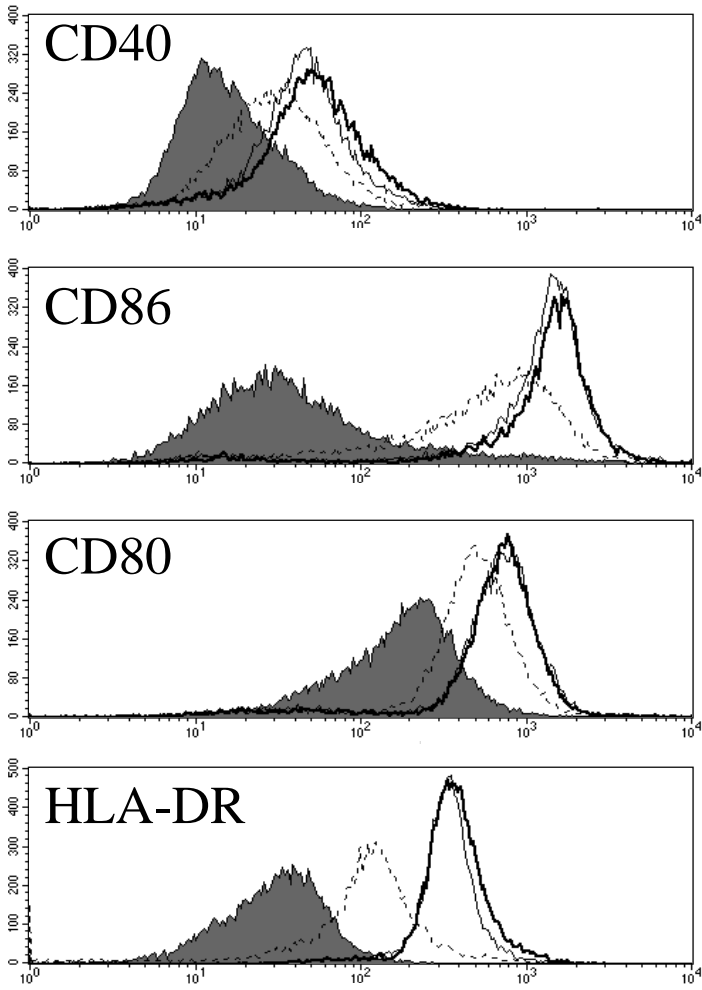


Figure 8

A



B

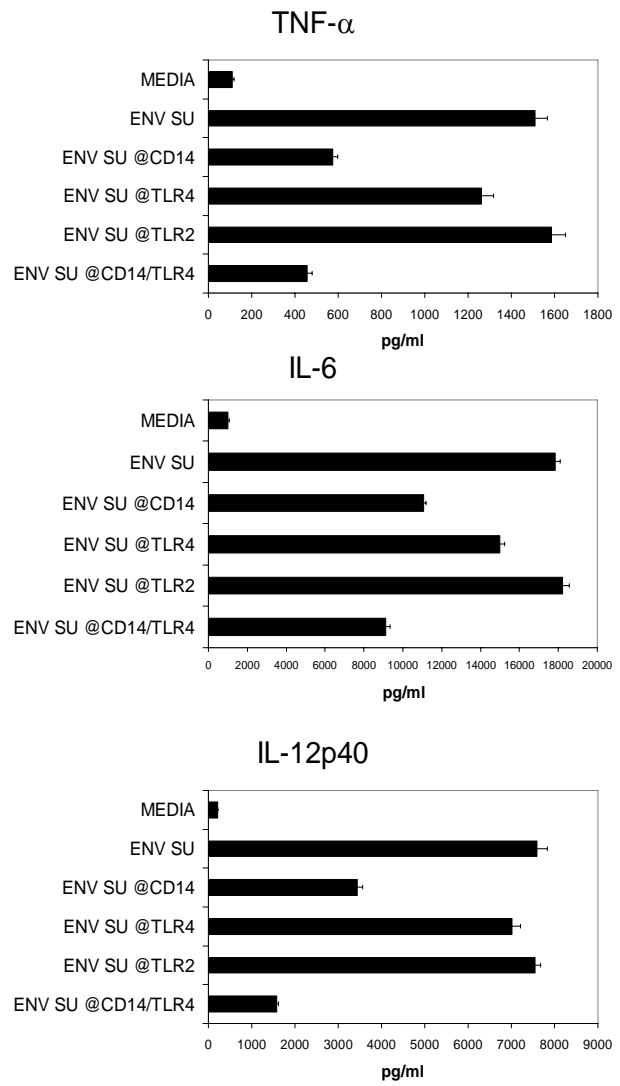


Figure 9