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SARS-CoV-2 induces transcription of human endogenous retrovirus RNA followed by type W envelope protein expression in human lymphoid cells.

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Research Article

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Abstract

Patients with COVID-19 may develop abnormal inflammatory response and lymphopenia, followed in some cases by delayed-onset syndromes, often long-lasting after resolution of the initial SARS-CoV-2 infection. As viral infections may activate human endogenous retroviral elements (HERV), we studied the effect of SARS-CoV-2 on HERV-W and HERV-K envelope (ENV) expression, known to be involved in immunological and neurological pathogenesis of human diseases. We demonstrate here that an initial exposure to SARS-CoV-2 virus activates early HERV-W and K transcription in peripheral blood mononuclear cell (PBMC) cultures from healthy donors. Within a week of primary PBMC culture, only HERV-W ENV protein expression was detected in lymphoid cells of some donors, although SARS-CoV-2 infection of PBMC was not observed. HERV activation was reproduced with UV-inactivated virus and with a recombinant spike protein. Interestingly, exposure to SARS-CoV-2 protein induced a significant production of interleukin 6 in PBMC, independently from detectable HERV expression. Altogether, these results show that SARS-CoV-2 viral protein could induce HERV-W ENV expression in lymphocytes from some individuals, underlying the importance to further address the implicated molecular pathways, to understand patients' genetic susceptibility associated to the activation of HERV-W and its possible relevance for targeting therapeutic intervention in COVID-19 associated syndromes.

Introduction

The present COVID-19 pandemic has raised many questions about the underlying biological mechanisms of the many symptoms or syndromes associated with SARS-CoV-2 infection¹⁻⁶. Most of them are debated, while others have been explained by, e.g., immune-mediated inflammation and/or activation of blood clotting pathways^{7,8}. However, such pathways may themselves be triggered by still unidentified factors linking coronavirus infection to a dysregulation of physiological signaling pathways.

Such an indirect pathological activation of receptors and their signaling pathways are known to take place when infectious agents have the potential to lift the epigenetic control and/or to directly activate endogenous retroviral elements present in the human genome^{9,10}. The resulting production of an endogenous protein of retroviral origin with pathogenic effects may generate clinical symptoms corresponding to the organ, tissue or cells, in which it is expressed and determined by the tropism of the triggering infectious agent¹¹⁻¹⁹. Abnormal expression of human endogenous retroviruses (HERV) may become self-sustained, thus creating lifelong chronic expression from host's genome copies in affected tissues²⁰, e.g., with cytokine-mediated feedback loops²¹ or possibly mediated by their envelope proteins²². This has been shown to be involved in brain lesions and in their expansion in patients with multiple sclerosis^{20,23-25}.

Abnormally expressed HERV envelope proteins revealed to display major immunopathogenic²⁶⁻³² and/or neuropathogenic^{23,28-35} effects *in vitro* and *in vivo*, associated with pathognomonic features of human diseases. We therefore studied whether SARS-CoV-2 could activate HERV, considered as 'dormant

enemies within³⁶, to evaluate their potential pathogenic contribution in COVID-19 associated syndromes. This question became critical after a recent study revealed the significant expression of HERV-W envelope protein (ENV) in lymphoid cells from COVID-19 patients, correlating with disease outcome and markers of lymphocyte exhaustion or senescence³⁷.

The present study shows that (i) HERV RNA and known pathogenic HERV-W envelope protein expression can be triggered in naive human peripheral blood mononuclear cells (PBMC) by SARS-CoV-2 virus itself or by its trimeric bioactive spike protein, with analogies to previously known examples of interactions between exogenous viruses and HERV¹², (ii) only about one-third of healthy blood donors have PBMC responding with HERV activation under SARS-CoV-2 exposure, however, (iii) all donors responded with similar interleukin 6 (IL-6) secretion kinetics after incubation with SARS-CoV-2 protein but without HERV activation in non-responders.

Materials And Methods

Cells

PBMC from healthy donors were obtained from the “Etablissement Français du Sang” (EFS) of Lyon (France). PBMC were isolated by Ficoll separation (Ficoll-Plaque PLUS) (GE Healthcare, 17-1440-02) from blood samples and cultured in RPMI-1640 medium (Gibco, 61870-010) completed with 5% of decompemented Human AB serum (Sigma, H4522). Healthy donors signed a written Informed Consent Form, documented at the EFS, allowing the commercial use of their blood and blood components for medical research after definite anonymization.

Astrogliaoma cell line U87-MG (U87) (U-87 MG ATCC®HTB-14™) and Vero E6 (ATCC® CRL-1586™) cell line were cultured in Dulbecco’s Modified Eagles Medium (DMEM, Gibco™), complemented with 10% heat inactivated Fetal Calf Serum (FCS), 1% glutamine and 1% penicillin-streptomycin.

U87 cells expressing both the human Angiotensin-converting enzyme 2 (ACE2)-receptor and the serine protease TMPRSS2 were generated using a second-generation lentiviral system. A lentivirus producer cell line (HEK293T cells: ATCC® CRL-3216™) was transiently transfected with a transfer plasmid encoding the transgene (ACE-2 or TMPRSS2), simultaneously with two other plasmids encoding either the vesicular stomatitis virus (VSV)-G envelope glycoprotein and a lentiviral packaging plasmid to generate the lentiviral particles³⁸. At 48 hours post transduction the cell supernatant was replaced again by fresh medium with Hygromycin at a concentration of 50µg/ml for antibiotic selection. The ACE-2 transduced U87 cells were observed daily and the supernatant replaced every 2-3 days by fresh Hygromycin-containing medium until the generation of the stable U87 ACE-2-expressing cell line. This new cell line was submitted to the same procedure using the TMPRSS-2 encoding lentivirus and then selected with Neomycin G418 at 250µg/ml to generate U87 cell line stably expressing both ACE-2 and TMPRSS2 proteins. The monitoring of *ACE2/TMPRSS2* expression by RT-qPCR revealed that both genes were efficiently expressed.

SARS-CoV-2 serology of blood donors

SARS-CoV-2 serology of blood donors was determined on plasma diluted 10 times using Simple Western technology, an automated capillary-based size sorting and immunolabeling system (ProteinSimple™). The SARS-CoV-2 Multi-Antigen Serology Module (SA-001) was used with Wes device and all procedures were performed according to manufacturer's protocol. Wes device was associated with Compass software for device settings and raw data recording (ProteinSimple/Biotechne).

Exposure to wt SARS-CoV-2, inactivated wt SARS-CoV-2 or recombinant active trimer Spike SARS-CoV-2

SARS-CoV-2 strain (BetaCoV/France/IDF0571/2020, GISAID Accession ID = EPI_ISL_411218) was cultured on Vero E6 cell line (ATCC®CRL1586™) for virus production. PBMC, U87 and Vero cells were infected with SARS-CoV-2 at a multiplicity of infection (MOI) 0.1. PBMC infection was performed in RPMI-1640 medium (Gibco, 61870-010) completed with 2% of heat inactivated Human AB serum (Sigma, H4522), U87 and Vero infections were performed in DMEM medium (DMEM, Gibco™) completed with 2% of heat inactivated FCS. 2 h later, the concentrations of AB-human serum or FCS were respectively increased to 5 and 10%. Recombinant non-stabilized trimer Spike SARS-CoV-2 (ACROBiosystems, USA; Ref. SPN-C52H8) treatment was performed at 0.5 and 2.5 µg/mL.

Inactivated SARS-CoV-2 virus used to treat U87 and ACE2+/TMPRSS2+U87 was obtained by Ultraviolet light irradiation at 254 nm during 30 minutes and inactivation was confirmed by viral titration.

Immunofluorescence

Cells in suspension were pelleted by centrifugation and deposited on 3 well epoxy microscope slides (Thermo Scientific, 30-12A-BLACK-CE24) while adherent cells were manipulated directly in 48 wells plates. Suspension and adherent cells received the same following steps. Cells were fixed in paraformaldehyde 4% during 15 minutes at RT. Cells were washed three times in PBS 1X and permeabilized 15 min in 0.2% Tween20, PBS 1X. Saturation was performed using 2.5% horse serum, 0.2% Tween20, PBS 1 X, during 30 minutes at RT before incubation with a mix of primary antibodies during 1 hour or overnight: 3µg/mL of anti-HERV-W ENV (GeNeuro, GN_mAb_Env01, murine antibody) or 10 µg/mL anti-HERV-K ENV (GeNeuro, GN_mAb_Env-K01, murine antibody), together with either anti-N SARS-CoV-2 diluted 1/500 (SinoBiological, 40143-T62, rabbit antibody) or anti-S SARS-CoV-2 diluted 1/500 (SinoBiological, 40590-T62, rabbit antibody). Antibody solutions were prepared in the previously described saturation buffer. After three washes in PBS 1X, cells were incubated during 1 hour with secondary antibodies mix containing 1µg/mL goat anti-mouse Alexa Fluor 488 (ThermoFisher Scientific, A11029), 1µg/mL donkey anti-rabbit Alexa Fluor 647 (ThermoFisher Scientific, A31573) and DAPI 1/2000 (Sigma, D9542) diluted in the previously described saturation buffer. Finally, cells were washed three times in PBS 1X and mounted using the Fluoromount-G mounting medium (Southern Biotech, 0100-01). Pictures were acquired on NIKON Eclipse TS2R microscope and analyzed on ImageJ software.

Cytofluorometry

At 24 or 72 h post infection, cells were pelleted by centrifugation. PBMC were incubated with FcR Blocking Reagent according to manufacturer's protocol (Miltenyi Biotec, 130-113-199) and stained with 1/10^e CD3-PE (BD Biosciences, 552127) whereas U87 and ACE2+/TMPRSS2+ U87 were stained with 10 µg/mL anti-S SARS-CoV-2 Alexa Fluor 594 (R&D Systems, FAB105403T). Following cell surface staining, cells were fixed and permeabilized using Cytotfix/Cytoperm kit (BD Biosciences, 554714) according to manufacturer's instructions. Then, PBMC were stained with 10 µg/mL of an anti-HERV-W ENV-FITC (GeNeuro, GN_mAb_Env01-FITC) or mouse IgG1 FITC-labeled isotype control (Miltenyi Biotec, 130-113-199). U87 and lentifected U87 cells were stained with 10 µg/mL of anti-HERV-W ENV (GeNeuro, GN_mAb_Env01, murine antibody) followed by incubation with 2 µg/mL of secondary antibody goat anti-mouse Alexa Fluor 488 (ThermoFisher Scientific, A11029). Stained cells were acquired on a BD LSR Fortessa, fluorochrome emissions from the pool of antibodies were compensated using OneComp Beads (Invitrogen, 01-1111-42) and data analyzed with FlowJo software (v.10).

Quantitative RT PCR (RT-qPCR)

At several time points after infection or recombinant Spike exposure, cells were harvested and total RNA extracted. 200 ng of DNase-treated RNA were reverse-transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, 1708891) according to the manufacturer's protocol. A control with no-RT was prepared in parallel, to confirm the absence of contaminating DNA in PCR experiments. An amount of 5 ng of initial RNA in RT reaction has been used to quantitatively evaluate the transcriptional levels of HERV-W ENV, HERV-K ENV, SARS-CoV-2 (Ferren et al. preprint 2021. DOI: [10.21203/rs.3.rs-122126/v1](https://doi.org/10.21203/rs.3.rs-122126/v1)) and ACE2 genes by RT-qPCR (primer sequences are shown in Table 1). The assays were performed in a StepOnePlus instrument (Applied Biosystems) using Platinum SYBR Green (Invitrogen, 11744-500). The housekeeping gene beta-2 microglobulin (*B2M*) was used to normalize the results in PBMC experiments whereas glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used in Vero cells experiments. Each experiment was completed with a melting curve analysis to confirm the specificity of amplification and the lack of any non-specific product and primer dimer. Quantification was performed using the threshold cycle (Ct) comparative method: the relative expression was calculated as follow: $2^{-[D_{Ct}(\text{sample}) - D_{Ct}(\text{calibrator})]} = 2^{-DD_{Ct}}$, where $D_{Ct}(\text{sample}) = [Ct(\text{target gene}) - Ct(\text{housekeeping gene})]$ and the $D_{Ct}(\text{calibrator})$ was the mean of D_{Ct} of non infected/non treated cells.

Table 1. Primer sequences

Gene	Primer sequence (5' to 3')	
	forward	reverse
HERV-W ENV	GTATGTCTGATGGGGGTGGAG	CTAGTCCTTTGTAGGGGCTAGAG
HERV-K ENV	CTGAGGCAATTGCAGGAGTT	GCTGTCTCTTCGGAGCTGTT
<i>B2M</i>	TTACTCACGTCATTCAGCAG	GATGGATGAAACCCAGACAC
<i>GAPDH</i>	CACCCACTCCTCCACCTTTGAC	GTCCACCACCCTGTTGCTGTAG
SARS-CoV-2 N	AAACATTCCCACCAACAG	CACTGCTCATGGATTGTT
ACE2	TCCATTGGTCTTCTGTCACCCG	AGACCATCCACCTCCACTTCTC

Quantification of IL-6 secretion

IL-6 secretion was assessed in PBMC culture supernatant 2, 15 and 24 h after recombinant Spike exposure, by ELISA using BD Opt EIA Set Human IL-6 (BD, 555 220) according to supplier's recommendations.

Cell viability assay

PBMC viability was analyzed using CellTiter-Glo 2.0 Assay kit (Promega, G9241) according to the manufacturer's protocol. Viability percentage was calculated using the following equation:

$$\% \text{ of viability} = (\text{RLU}_{\text{experimental}} - \text{RLU}_{\text{background}}) / [\text{Mean} (\text{RLU}_{\text{control}} - \text{RLU}_{\text{background}})] \times 100$$

Background: wells containing medium without cells; Control: untreated cells at 24 h post treatment.

Results

1. Exposure to SARS-CoV-2 virus triggers HERV-W and -K *ENV* mRNA early transcription from PBMC of healthy donors

We initially analyzed whether infectious SARS-CoV-2 could modulate the expression of HERV W and HERV-K genes in lymphocytes from healthy individuals. RT-qPCR was performed using specific primers for HERV-W and HERV-K envelope genes, as already validated in patients with HERV-associated diseases^{33,39,40}, using B2M mRNA as a suitable reporter gene for PBMC⁴¹. PBMC of 11 blood donors were cultured or not with infectious SARS-CoV-2 (MOI = 0.1) and RNA was collected at 2H post-inoculation (**Figure 1**). In PBMC from 3 (Donors # 28, 29 and 40) out of 11 (27%) donors, HERV-W RNA transcription was increased at 2H after exposure to wild type SARS-CoV-2 virus (MOI=0.1); a low response for HERV-W was seen with one donor (#27), as shown in Figure 1 and supplementary Table S1. The same donors also showed relative transcriptional activation of HERV-K *ENV* (Figure 1). For further kinetics analysis, RNA from PBMC of 4 representative donors with various RNA results at 2H, was collected at 2H, 19H and 24H and tested in parallel (**Supplementary Figure S1**) Interestingly, the transcriptional level of both HERV W and HERV-K RNA was decreased at 19H and 24H to a lower level than the baseline of cultures "not inoculated" (NI) with the infectious virus from the same donors at the same time points. A similar decrease of HERV-W and -K *ENV* transcription, when compared to NI control wells, was observed from the initial time point (2H) in PBMC from of the "non-responding" donors after exposure to SARS-CoV-2 virus (supplementary Figure S1 A, B).

RT-qPCR analysis of the kinetics of SARS-CoV-2 *N*RNA only showed abundant RNA load from the inoculum with a significant decrease at 19h post-infection (inoculation), which confirmed the absence of viral replication in PBMC (supplementary Figure S1 C).

2. Exposure to SARS-CoV-2 triggers HERV-W envelope (*ENV*) production from PBMC of healthy donors

We next analyzed whether the HERV RNA expression is followed by HERV protein production. PBMC cultures of 8 new blood donors, inoculated or not with SARS-CoV-2 at 0.1 MOI, were collected at either 3

or 7 days post-exposure. Cells were stained with specific monoclonal antibodies raised against envelope proteins of HERV-W, HERV-K and against SARS-CoV-2 N protein, then analyzed by immunofluorescence (**Figure 2**). Cells were maintained for up to 7 days in culture.

All tested conditions are presented in parallel with an example from donor # 12: HERV-K ENV staining was not detected in the cells after exposure to SARS-CoV-2 (**Figure 2D and Supplementary Figure S2 D'**), despite specific HERV-K *ENV* RNA detection and even when HERV-W ENV protein was detected in the same cells (**Figure 2B and Supplementary Figure S2 B'**).

HERV ENV protein expression was donor-dependent. HERV-W ENV protein was detected in a variable proportion of PBMC from cultures inoculated with SARS-CoV-2, numerous in donor #7 (**Figure 2 E'**), less in #12 (**Figure 2 B**), or few in #9 (**Figure 2 F'**), whereas not detected in non-responders (**Figure 2G'**, donor #11). However, active production was seen in positive cells as shown with high magnification (**Fig. 2 H-L**). SARS-CoV-2 antigen was not detected in any of the conditions. In all immunofluorescence analyses, neither HERV-W ENV, nor HERV-K ENV protein expression was detected in control cultures without SARS-CoV-2 (**Figure 2 A- C- E, F and G**).

The same immunofluorescence conditions showed clear expression of SARS-CoV-2 N protein, in parallel with its spike protein, in positive control cells (Vero) used for productive replication (Supplementary **Figure S3**). The specificity of the anti HERV-W antibodies was also confirmed with the absence of staining in the same SARS-CoV-2-infected Vero cells, in parallel with negative RT-qPCR for HERV-W and HERV-K but with increased SARS-CoV-2-N mRNA transcription (Supplementary **Figure S3 A-D and E-G**). Positive controls for anti-HERV antibodies sensitivity and specificity on cells transfected with expression plasmids for HERV-W and -K *ENV* (Supplementary **Figure S4**) and negative control staining with secondary antibodies (Supplementary **Figure S5**) were performed in parallel. The specificity of anti-HERV-W ENV antibody used here was also established in different conditions with a previous study (Charvet et al. 2021, *Viol. Sin.* doi: 10.1007/s12250-021-00372-0 *in press*). Finally, the SARS-CoV-2 serological status of donors was controlled (Supplementary, **Figure S6**).

3. Exposure to SARS-CoV-2 triggers HERV-W envelope production in T-cells of healthy individuals

HERV-W ENV protein expression having been observed in CD3⁺ T-lymphocytes of COVID-19 patients³⁷, we next analyzed whether SARS-CoV-2 could induce HERV-W ENV in T lymphocytes. We analyzed HERV-W ENV expression in PBMC cultures from three healthy donors, with or without exposure to SARS-CoV-2, using cytofluorometry analysis. As illustrated in **Figure 3**, CD3⁺T lymphocytes were identified with the gating strategy in non infected (NI) cultures, showing an increased detection when inoculated with SARS-CoV-2 at MOI=0.1. However, at 24H post-viral exposure, a CD3-low population originally shown to be characteristic of superantigen activity⁴² and tentatively associated to the spike protein of SARS-CoV-2⁴³ was observed (**Figure 3B**), whereas only few such cells were seen in NI cultures (**Figure 3A**). When cells were double-labeled with anti-HERV-W ENV specific antibody an important proportion of these CD3-low T-cells was positive for HERV-W ENV (**Figure 3B CD3+ top panel**), compared to NI cultures (**Figure 3A**

CD3⁺ top panel). Fewer CD3-high were found to express HERV-W ENV after exposure to the virus at this time-point (**Figure 3B**, CD3⁺ bottom panel). The percentage of HERV-W ENV positive cells in each condition is represented in **Figure 3E**. A significant increase in CD3⁺ low/HERV-W⁺ lymphocytes exposed to SARS-CoV-2, compared to those from NI cultures was confirmed in at 72H post-inoculation (**Figure 3F**) and the same was observed versus all control cells from cultures without virus (**Figure 3 E-F**).

4. Human cell-line expressing *ACE2/TMPRSS2* produces HERV-W ENV protein following SARS-CoV-2 infection

U87 astrogliaoma cells were previously shown to respond to HHV-6A with an activation of HERV-W expression leading to the production and secretion of bioactive ENV protein in cell-free culture medium causing TLR4 activation¹². However, these cells cannot be infected by SARS-CoV-2 and, expression of HERV-W and K was not observed after exposure to this coronavirus (**Figure 4 A-F and M-P**). To obtain a cell line permissive to SARS-CoV-2 infection, U87 cells were transduced with *ACE2* and *TMPRSS2* expressing lentivector (supplementary **Figure S8**). U87-*ACE2*⁺/*TMPRSS2*⁺ cells did not spontaneously express HERV-K or HERV-W (**Figure 4 G, I, K and Q, S**). However, after inoculation with infectious SARS-CoV-2, N and S viral proteins, as well as HERV-W ENV protein were detected in the same cells. This showed SARS-CoV-2 infection and replication, in parallel with HERV-W activation and ENV protein production (**Figure 4 H, J, L and J'-L'**). Interestingly, no HERV-K ENV protein was detected in any cell from these cultures (**Figure 4 R,T and T,T'**).

Cytofluorometry analysis confirmed the co-expression of coronavirus S-protein and HERV-W ENV showing HERV-W activation and both protein production in the same cells (**Figure 5**). Nevertheless, only a minor percentage of cells co-expressed HERV-W ENV and SARS-CoV-2 S proteins in the present conditions. The total number of HERV-W ENV positive and/or of SARS-CoV-2 spike positive cells was significantly elevated compared to mock-infected cells (**Figure 5 A**; HERV-W⁺, SARS-CoV-2 S⁺, and positive for both: $p < 0.0001$). Not all SARS-CoV-2 infected cells expressed HERV-W ENV and vice-versa (Cf. examples in **Figure 5 C-D**), thereby indicating that some cells could express HERV-W ENV without being infected by SARS-CoV-2 and/or that some infected cells had not induced HERV-W ENV expression at the moment of analysis. Exposure of *ACE2*⁺/*TMPRSS2*⁺ U87 cells to inactivated SARS-CoV-2 virus also induced HERV activation with significant HERV-W ENV production (**Figure 5 A, E-F**; HERV-W⁺: $p < 0.0001$) similar to those observed with the infectious virus but, as expected, without infection nor detection of N-protein in the same cells (data not shown).

5. Exposure to SARS-CoV-2 virus recombinant trimeric spike protein triggers HERV-W ENV protein production in PBMC of healthy individuals.

We previously observed a rapid response to SARS-CoV-2 virus with an early peak of RNA followed by HERV-W ENV protein expression in cells maintained in culture (≤ 5 days) (**Figures 1-3**). This immediate RNA response in the absence of detectable infection of PBMC by SARS-CoV-2 prompted us to investigate a possible direct stimulation by SARS-CoV-2 proteins and, most of all, by its surface spike protein. The

same question was also raised following the activation of HERV-W ENV expression in U87-ACE2⁺/TMPRSS2⁺ cells by the UV-inactivated coronavirus, in the absence of any replication and without S-protein detection by double labeling of cells, using cytofluorometry.

To further address the activation of HERV-W in the absence of infection, a recombinant trimeric spike protein was added into the culture medium of PBMC from 4 new healthy donors, in parallel to mock-control, corresponding to the buffer only. Cell viability was measured and did not vary significantly within the culture period of experiments (≤ 5 days; Cf. Supplementary Material **Figure S7**).

PBMC RNA from 4 new donors were collected at 2H, 15H and 24H post-inoculation and analyzed by RT-qPCR for HERV-W and HERV-K envelope gene expression. Donor #31 showed transcriptional increase for both HERV-W and HERV-K *ENV* at 2H post-inoculation, donor #30 showed a peak of HERV-W and HERV-K *ENV* RNA at 15h, while both RNA levels remained rather stable or decreased in donors #32 and #33 (**Figure 6 A-B**). RNA levels for both HERV-W and HERV-K also decreased below the baseline of identical non-exposed cells after having shown a peak of transcription. This was again seen in the absence of significant cell death that would anyhow have been compensated by the differential quantification with B2M RNA. Of interest, donor #30 was the only one tested positive for anti-SARS CoV-2 antibodies (supplementary Figure S6), which did not prevent HERV transcriptional activation by this recombinant spike trimer but coincides with slightly delayed peak of HERV RNA.

IL-6 secretion was significantly increased at 15H and 24H PE ($p < 0.01$ and $p < 0.05$) in PBMC from all donors, including non-responding donors (without HERV activation) after exposure to SARS-CoV-2 S antigen (**Figure 6 C**). HERV activation independently from IL-6 production was confirmed by immunofluorescence analysis at 72H, which showed HERV-W ENV positive lymphoid cells in cultured PBMC of 2 out the four donors inoculated with spike trimers (0.5 $\mu\text{g}/\text{mL}$) but not in mock-control cultures (**Figure 6 D**).

Of note, as seen with the infectious virus, few HERV-W ENV positive cells were detected but an increase in HERV RNA was seen despite this low proportion of activated cells within the cultures. Also, even in the presence of IL-6 within the culture media of all cultures (**Figure 6 C**), HERV-W ENV was expressed in few cells only within PBMC cultures from responders, but not detected in PBMC from non-responders at both RNA and protein levels.

Discussion

Unlike physiological effectors, the transcription of which is normally upregulated to produce active molecules when needed, HERV activation by environmental factors does not reflect a physiological response and seldom results in protein expression⁴⁴. Exceptions are known with a few “domesticated” HERV genes, known to encode physiological proteins⁴⁵ or at the transcriptional level only, with the high number of non-coding RNA types involved in major post-genomic regulations⁴⁶. Thus, though representing about 8% of the human genome (whereas physiological genes represent much less), HERV

are mostly defective and non-coding remnants of ancestral integrations of retroviral genomes in the germ line of individuals infected by exogenous retroviruses. Non-physiological HERV envelope protein expression has been shown to be detrimental in certain human diseases and cannot be considered as just another response to environmental triggers like, e.g., an immune response with cytokines release, though a parallel modulation mediated by certain HERV RNA is conceivable. The expression of HERV-K or HERV-W envelope proteins may have trophic roles in the placenta⁴⁵ or in stem cells⁴⁷, but envelope proteins from the same HERV families were rather shown to be involved in disease pathogenesis when expressed in adult cells^{34,39}.

This study shows that SARS-CoV-2 is able to trigger both HERV-W and HERV-K RNA transcription, while only HERV-W ENV protein was detected during these short-term primary cultures of non-stimulated PBMC from about 30% of healthy donors. However, a quite potent expression is made consistent from the observed ENV-protein immunofluorescent labeling in producing cells, which lasted for several days after exposure to SARS-CoV-2, and from the detectable HERV-W ENV RNA increase above the baseline, though expressed in few cells only. but.

Most importantly, after addition of SARS-CoV-2 spike protein trimer in PBMC cultures, the dosage of IL-6 showed that its secretion occurred in the supernatants from all donors, either responding or non-responding with HERV activation. The fact that this IL-6 production was induced by SARS-CoV-2 in the absence of detectable HERV RNA or protein expression provides a supplementary evidence that the presently observed HERV activation is not induced by cytokines, or by inflammation due to viral infection, but rather by SARS-CoV-2 spike protein itself.

Cytofluorometry analysis from these PBMC cultures also confirmed that HERV-W ENV protein expression was effectively induced in T-lymphocytes. A noticeable sub-group of CD3-low T-lymphocytes appeared in which HERV-W ENV protein was predominantly expressed, whereas it remained minimal and HERV-W negative in control cultures not exposed to the virus. These observations may corroborate previous works describing superantigen motifs of SARS-CoV-2 spike protein⁴³, and may involve cellular mechanisms associated to lymphopenia and hyperinflammation already characterized for other emerging viruses (e.g., Ebola or Lassa)^{48,49}. However, because HERV-W ENV was also shown to display superantigen-like effect²⁸, the origin of this effect on T-lymphocytes or potentially combined effects are questioned. Indeed, this work now calls for further analyses of possible markers of exhaustion and for studying the fate of these cells in kinetics analyses with, e.g., markers of apoptosis versus markers of early activation. The present *in vitro* observation is consistent with the co-stained HERV-W ENV positive CD3 lymphocytes shown to express either exhaustion or terminal differentiation markers in leukocytes from COVID-19 patients with severe clinical evolution³⁷. Thus, despite an expected parallel stimulation of T-cell antiviral reactivity, the present experimental results consistently raise the question of a possible role of this HERV protein expression in the induction of anergy or in the depletion of lymphocytes. The frequent lymphopenia in COVID-19 patients corroborates a defect of adaptive immunity and an imbalance with innate immune reactivity, compared to usual viral infections⁵⁰. This question may now be addressed

under the new angle of HERV expression in lymphocytes, in parallel with a mechanism of induction in T-lymphocytes that remains to be characterized. Moreover, since HERV-W ENV (previously named MSRV-ENV), is already known to be a potent TLR4 agonist and itself induces inflammation via TLR4 signaling pathway^{30,51}, its superimposed or synergistic role in the hyperactivation of innate immunity should also be considered in parallel with a potential contribution to the impairment of adaptive immunity as seen in COVID-19.

However, the presently observed HERV activation occurs without signs of infection of lymphocytes or monocytes by SARS-CoV-2. Furthermore, UV-inactivated virus and a recombinant trimer of its spike protein appeared sufficient to reproduce similar HERV-W and -K ENV RNA stimulation as well as HERV-W ENV protein production in lymphoid cells. This type of HERV activation mediated by an interaction between a triggering virus and a specific receptor on certain cells has already been described for HHV-6A and U87 cell-line¹². Thus, the question of another cellular receptor bound by SARS-CoV-2 spike trimers that would trigger a signaling pathway activating HERV-W ENV protein production in T-cells is raised and should be addressed in a comprehensive study. Considering the present results, an unstable variable conformation of this trimeric spike antigen and/or an induction of a receptor in few cells only, would then explain the low proportion of HERV expressing cells in PBMC cultures of responding donors in the present *in vitro* conditions.

SARS-CoV-2 did not infect normal U87 cells and, unlike HHV-6A, did not induce HERV-W ENV expression in normal U87 cells either. When lentifected with ACE2/TMPRSS2 receptor, U87 cells became susceptible to SARS-CoV-2 virus infection and co-expressed spike and N proteins. This human cellular model showed that the productive coronavirus infection also induced HERV-W ENV expression but, here again, HERV-K ENV protein and RNA were not detected. This provides an additional control in-between HERV-W activation without SARS-CoV-2 infection in human lymphoid cells and SARS-CoV-2 infection and replication without HERV-W expression in simian Vero cells.

SARS-CoV-2 virus, unlike HHV-6A, did not activate HERV-W expression in U87 cells naturally devoid of ACE2/TMPRSS2 receptors, but did so after ACE2/TMPRSS2 receptors expression in transduced U87 cell membrane. Because SARS-CoV-2 induced HERV-W expression in human lymphoid cells that neither express ACE2 (confirmed with specific RT-qPCR) nor TMPRSS2 receptor and were not infected, these cannot be expected to be the receptors through which SARS-CoV-2 would trigger a signaling pathway resulting in HERV activation. This activation was also obtained with a recombinant trimer tested for an effective binding to ACE2 receptors, but not stabilized in a specific conformation. Pre- and post-fusion variable conformations of this spike protein may confer divergent properties⁵², whereas vaccine preparations are likely to stabilize its structure⁵³⁻⁵⁵ with a good safety profile^{54,56}. Here again, further studies are needed to define sequence-related and protein conformational properties involved in a protein-receptor interaction leading to HERV activation in susceptible individuals. This may further provide relevant information for viral constructs or preparation envisaged for SARS-CoV-2 vaccines.

At the transcriptional level, within the present short-term PBMC cultures after a single exposure to SARS-CoV-2 or to its active protein, a single peak of HERV RNA is seen when occurring in responding donors. The absence of recurrent stimulation and/or of renewed PBMC comprising susceptible cell-type(s) may explain this single peak nonetheless followed by active HERV-W protein synthesis. Thus, an increase in the number and percentage of lymphoid cells with HERV-W activation strongly expressing the envelope protein under persisting SARS-CoV-2 virion release would be consistent with observations from COVID-19 patients³⁷.

A feed-back inhibitory loop triggered by abnormal HERV activation is probably limiting the yield of HERV expression in targeted PBMC in the present cultures. When HERV activation was not seen in PBMC cultures from 7 out of 11 healthy blood donors exposed to the virus and from 1 out of 4 exposed to the spike trimers, this coincided, with constantly decreased RNA levels in PBMC exposed to the virus or to the recombinant spike trimer, compared to non-exposed PBMC. Thus, an inter-individual variability in the rapidity and potency of expression of HERV inhibitors, with a genetic or epigenetic origin, may offer an avenue of research to understand why some individuals do not activate this HERV expression in PBMC. Symptoms potentially superimposed or synergized by the pathogenic effects of HERV envelope protein expression may consequently occur in certain individuals only. This should also be investigated in COVID-19 patients for it may provide possible prognostic information on individuals who may present an enhanced reaction to the infection with severe evolution or long-lasting post-infectious syndromes.

Finally, since the HERV-W ENV protein was shown to have multifaceted pathogenic effects with a common TLR4 activation⁵¹ and appeared consistent with pathognomonic features of diseases in the lifelong course of which it was detected within tissue lesions^{23-25,28-30,32,35,39,40}, its induction by SARS-CoV-2 in lymphocytes is not expected to be neutral. Observation of the induction of HERV-W⁺ CD3-low lymphocytes and the significant detection of exhaustion markers on HERV-W⁺ T-cells from patients with COVID-19, correlating with parameters of severity in disease outcome³⁷, raise the question of a possible contribution of this HERV expression in the frequently observed lymphopenia. Thus, beyond a possible lymphotoxicity, previous studies also suggest a potential impact on endothelial cells³² and on Schwann cells⁴⁰, as well as on microglia and remyelinating oligodendrocyte precursor cells^{23,25}, which would be relevant with known vascular and neurological syndromes associated with COVID-19 during the acute infection or in the so-called post-COVID period^{3,5-7,31}. This does not exclude promoting autoimmunity as already shown in a humanized mouse model²⁴, when autoimmune features are often observed in COVID-19 or post-COVID patients⁵⁷⁻⁶⁰. Altogether, these data call for evaluating HERV-W ENV as a potential target for therapeutic intervention in COVID-19 associated syndromes.

In conclusion, this study aimed at elucidating the possible origin of HERV-W ENV expression in lymphocytes of COVID-19 patients and revealed that SARS-CoV-2 via its spike protein may trigger HERV expression in human PBMC and, in particular, in T-lymphocytes, which could play a role in the immunopathogenesis of COVID-19.

Declarations

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Figures

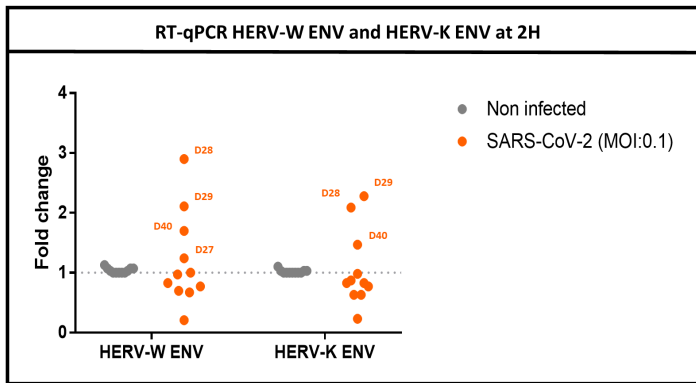


Figure 1

Figure 1

Quantification of HERV-W ENV and HERV-K ENV RNA in PBMC from healthy blood donors exposed to SARS-CoV-2. The level of mRNA of HERV-W ENV and HERV-K ENV of PBMCs cultures from 11 healthy blood donors, exposed or not to infectious SARS-CoV-2 (MOI:0.1), was analyzed by RT-qPCR. The graph presents mean results from triplicate cultures of 11 donors at 2H post-exposure. Numbers of donors with

increased expression of HERV-W and HERV-K are indicated and values of each sample is presented on the Supplementary table S1.

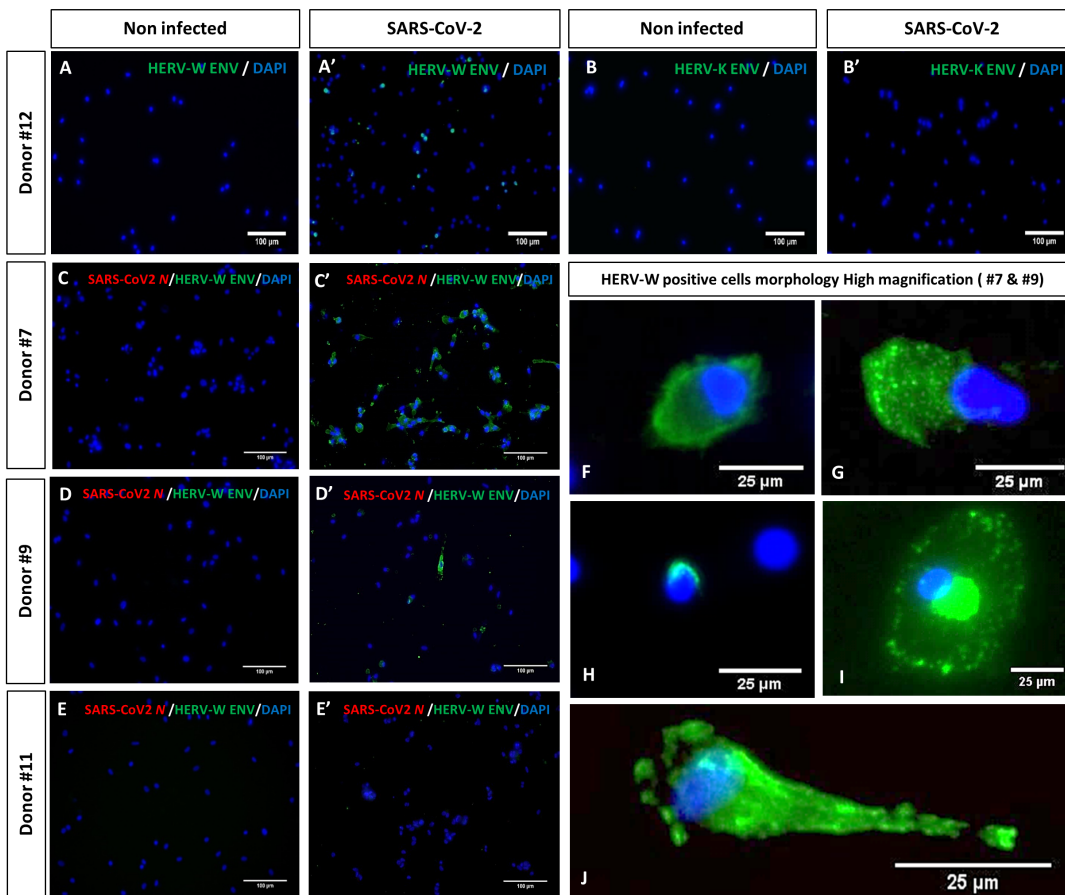


Figure 2

Figure 2

Immunodetection of HERV-W ENV in primary PBMC from healthy individuals following the exposure to SARS-CoV-2. PBMCs from healthy blood donors were inoculated or not with SARS-CoV-2 virus (MOI: 0.1) and collected after 3 or 7 days. For each culture, GN_mAb_Env01 and anti-N-SARS-CoV-2 antibodies were

used respectively to detect HERV-W ENV (green labelling) and nucleocapsid protein of SARS-CoV-2 (red labeling). (A-C) PBMCs from healthy blood donor #12 were inoculated (B, D) or not (A, C) with SARS-CoV-2 virus (MOI: 0.1). GN_mAb_Env01 (A, B') or GN_mAb_Env-K01 (C, D) antibodies were respectively used to detect HERV-W ENV or HERV-K ENV (green labelling). (E-L) PBMC cultures from 3 representative donors with variable number of positive cells: Donor # 7 at day 7 (E, E') and Donor # 9 at day 3 (F, F') and a non-responding culture from Donor # 11 at day 7 (G, G'). The different morphological aspects of HERV-W ENV positive cells are presented with high magnifications (H-L). DAPI was used to stain nuclei (blue staining).

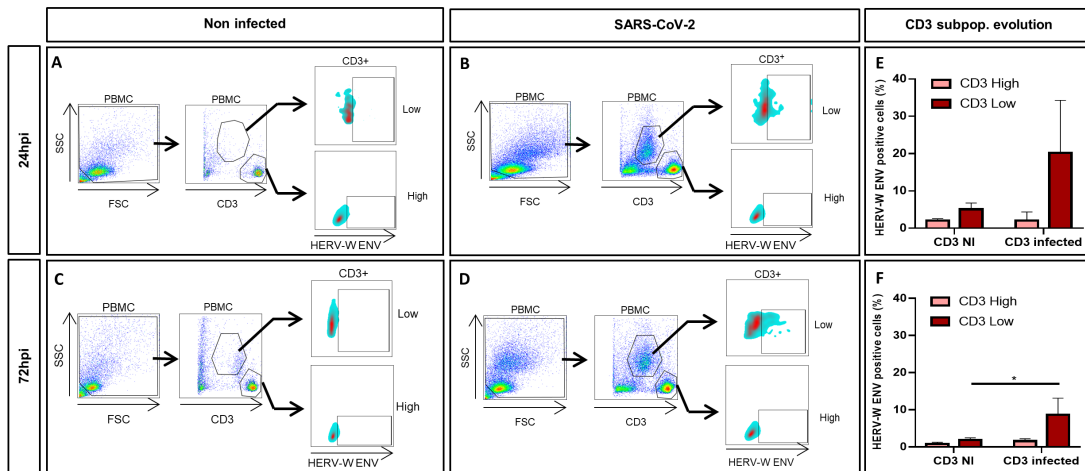


Figure 3

Figure 3

SARS-CoV-2 induces the expression of HERV-W ENV in CD3^{low} T cells. PBMCs from 3 healthy donors were either incubated with SARS-CoV-2 (MOI=0.1) for 24H (B) or 72H (D), or remained non exposed to the virus (A, C). Cells were stained using anti-CD3 and GN_mAb_Env01 antibodies and analyzed by flow cytometry. A-D, Gating strategy for the separation of CD3 high and CD3 low expressing T cells is presented. E,F The percentage of HERV-W ENV positive cells from subpopulations within CD3 T lymphocytes at 24Hpi and 72Hpi (average from 3 donors +SD) is presented with histograms. Statistical analysis was performed using Tukey's multiple comparisons test.

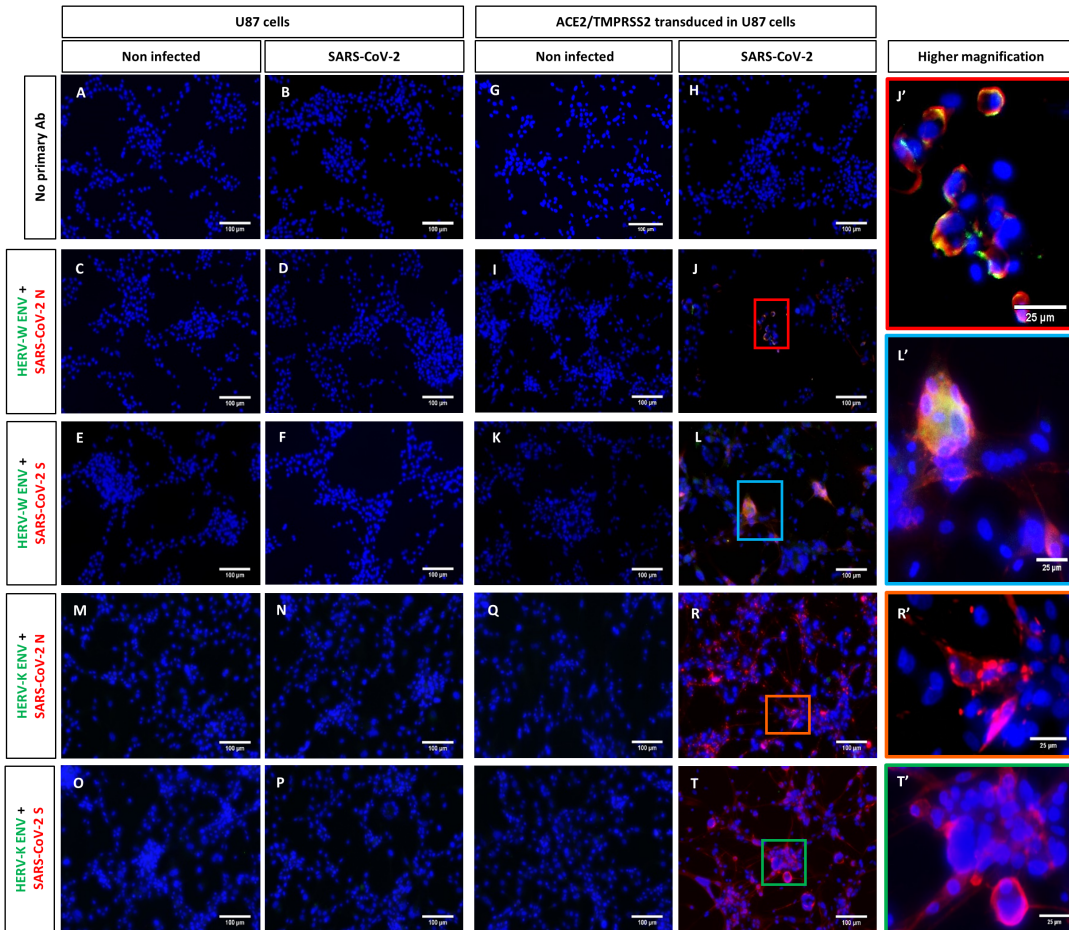


Figure 4

Figure 4

Expression of HERV-W ENV in a U87 cell line expressing ACE2/TMPRSS2. U87 human astrogliaoma cells do not express ACE2/TMPRSS2 and are not susceptible to SARS-CoV-2 infection. This was confirmed by the absence of staining using anti-N and anti-S-SARS-CoV-2 antibodies 24 h after SARS-CoV-2 infection (MOI: 0.1) (D, F, red staining), similarly to the non-infected cells (C, E, red staining). HERV-W ENV was not constitutively expressed by U87 cells (C, E) and exposure to SARS-CoV-2 did not induce

HERV-W ENV (D, F) expression in these cells. Lentifection allowing the ACE2/TMPRSS2 expression at the surface of U87 did not induced HERV-W ENV activation by itself (I, K). SARS-CoV-2 replication in ACE2/TMPRSS2-expressing U87 was confirmed by a strong SARS-CoV-2 N and S proteins detection at 24H pi (respectively J and L, red labeling). The expression of HERV-W ENV was also detected after SARS-CoV-2 infection of lentifected U87 cells (J, L; green labelling or yellow when merged with red fluorescence of SARS CoV-2 proteins). Higher magnification of pictures presenting double N- or S-SARS-CoV-2 and HERV-W ENV positive staining are also presented (J' and L'). The background staining possibly generated by the secondary antibodies was assessed and shown not to occur in the absence of primary antibodies (A, B, G, H). DAPI was used to stained nuclei (A-T', blue staining). HERV-K ENV (green labeling) was not detected in original U87 cells with or without exposure to SARS-CoV-2 (M-P). HERV-K ENV was not detected in U87-ACE2/TMPRSS2 cells with or without exposure to SARS-CoV-2 (Q-T). SARS-CoV-2 replication in ACE2/TMPRSS2 expressing U87 was confirmed by staining of SARS-CoV-2 N and S proteins at 24H pi (respectively R and T, with corresponding high magnification in R', T'; red labeling).

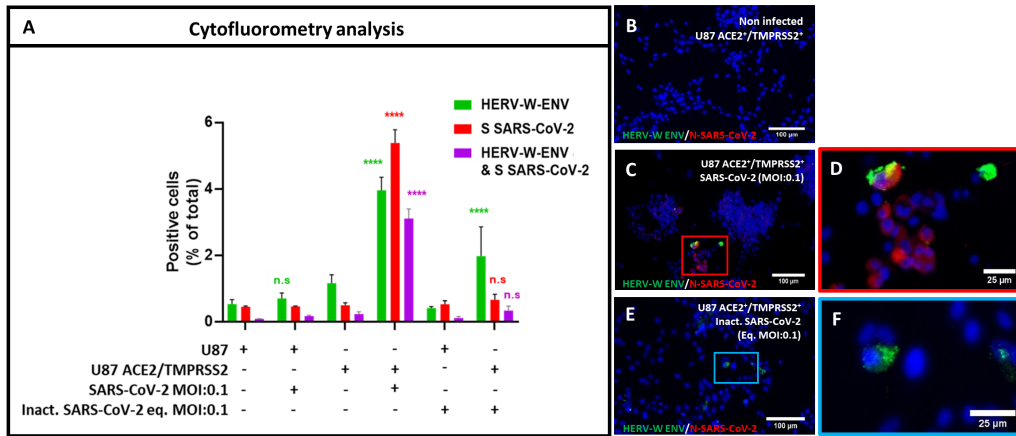


Figure 5

Figure 5

Effect of infectious and UV-inactivated SARS-CoV-2 on HERV-W expression in U87 ACE2⁺/TMPRSS2⁺ cells U87 and U87-ACE2⁺/TMPRSS2⁺ cells were infected or not with SARS-CoV-2 (MOI: 0.1). In parallel, U87-ACE⁺/TMPRSS2⁺ cells were exposed to UV-inactivated SARS-CoV-2 (equivalent MOI: 0.1). After 24 h, cytofluorometry analyses were performed using anti-HERV-W ENV and anti-S-SARS-CoV-2 antibodies. (A) Percentages of HERV-W ENV and/or S-SARS-CoV-2 positive cells are illustrated on histograms. (B-F)

Immunofluorescence analysis of corresponding cells. Examples of cells from actively infected U87 ACE+/TMPRSS2+ cultures are presented: positive for both HERV-W ENV and S-SARS-CoV-2 (C and seen in green + red with high magnification in D), for HERV-W ENV only (from C and seen in green only with high magnification in D) and for S-SARS-CoV-2 only (from C and seen in red only with high magnification in D). The same type of presentation was done for U87 ACE+/TMPRSS2+ cells exposed to the inactivated virus (E), with higher magnification of HERV-W ENV+ green cell (blue square, F). DAPI was used to stained nuclei (B-F, blue staining). Statistical analysis was performed using Tukey's multiple comparison. "Infected" or "exposed to inactivated virus" conditions were compared to their corresponding "non-infected"/"non-exposed" condition. (n.s.: $p > 0.5$; *: $0.5 < p < 0.01$; **: $0.01 < p < 0.005$; ***: $0.005 < p < 0.0001$; ****: $p < 0.0001$). Of note, low SARS-CoV-2 signals quantified in the absence of virus are not significant and correspond to the background noise of the technique.

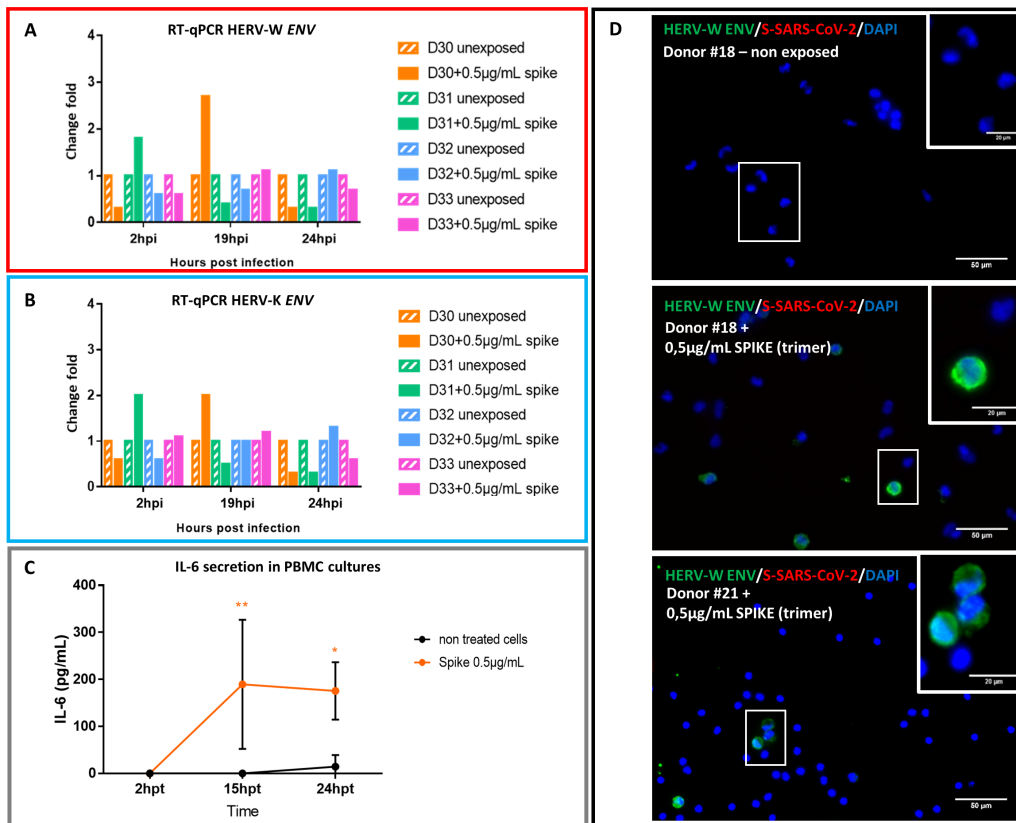


Figure 6

Figure 6

Induction of HERV-W ENV and HERV-K ENV mRNA expression and IL-6 secretion in PBMCs cultures from healthy donors after exposure to the recombinant Spike trimer. PBMCs from 4 healthy blood donors were exposed during 2, 15 and 24 h to 0.5µg/mL of active trimer Spike recombinant protein. (A) HERV-W ENV and (B) HERV-K ENV mRNA levels were assessed by RT-qPCR using specific primers. Results were presented as fold change versus the corresponding non infected condition. Hatched histograms

correspond to the non-exposed conditions and plain histograms to conditions with inoculation of spike protein in the cultures. pi: post-inoculation. (C) PBMCs isolated from 3 healthy blood donors (donors #30 to 32) were incubated, or not, with 0.5µg/mL of recombinant spike trimer. IL-6 secretion was monitored in culture at 2, 15 and 24 h post treatment (hpt) using the BD® Opt EIA Set Human IL-6® ELISA dosage. Statistical analysis: Sidak's multiple comparison. Here, not treated and treated condition were compared at each time point 2hpt, 15 and 24hpt. (n.s.: $p>0.5$; *: $0.5<p<0.01$; **: $0.01<p<0.005$; ***: $0.005<p<0.0001$; ****: $p<0.0001$). (D) PBMCs from healthy blood donors were exposed during 24 h with 0.5 µg/mL of active trimer Spike recombinant protein. Here, results obtained on 2 responding donors (donor # 18 and donor # 21) are presented. The recombinant Spike protein was not detected following the staining with anti-S-SARS-CoV-2 antibody (red staining). When expressed, HERV-W ENV was detected in few cells of cultures exposed to the spike trimer (green labeling). Higher magnification of HERV-W ENV positive cells is presented in white squares. Non exposed cells do not present any HERV-W ENV labelling using GN_mAb_Env01 antibody. DAPI was used to stained nuclei (blue staining).

Supplementary Files

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