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A high-resolution mass cytometry analysis reveals a delay of cytokines production after TLR4 or TLR7/8 engagements in HIV-1 infected humans

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ABSTRACT

HIV infection is associated with chronic inflammation in both non-treated and treated patients. TLR-dependent mechanisms are strongly involved in the maintenance of this inflammation. Indeed, the residual replication of HIV, the potential viral co-infections, or the products issued from microbial translocation provide TLR ligands, which contribute to trigger innate immune responses. Maintaining this chronic inflammation leads to an exhaustion of the immune system. Therefore, the TLR-dependent responses could be altered in HIV-infected patients. To investigate this hypothesis, we performed high-resolution phenotyping using a mass cytometry panel of 34 cell markers. Whole blood cells from healthy, non-treated HIV-infected and ART-treated HIV-infected subjects were stimulated with LPS, R848 or Poly(I:C). We observed the immune responses induced in T-cells, B-cells, polymorphonuclear cells, NK cells, basophils, monocytes and dendritic cells. We observed that, for either LPS or R848 stimulations, the production of cytokines in monocytes and conventional dendritic cells was delayed in treated or non-treated HIV-infected patients, compared to healthy individuals. These results suggest that leukocytes from chronic HIV-infected patients are slower to respond following the sensing of pathogens and danger signals, which may be an important feature of HIV infection.

1. Introduction

Once a microorganism invades the host, its recognition is performed by a large range of cell populations thanks to germline-encoded receptors called pattern recognition receptors (PRR) [1]. These receptors recognize pathogen-associated molecular patterns (PAMP) expressed by the microorganism. Toll-like receptors (TLR) were the first PRR to be discovered and have been extensively studied in healthy and disease settings [2–4]. TLR engagement by specific ligands usually triggers intracellular signals that initiate innate immune responses. These latter in turn, help the establishment of an adaptive response directed specifically towards the invading microorganism [1,3,5]. They include both the production of pro-inflammatory cytokines, and changes in the expression levels of Fc receptors, adhesion and activation markers [4,6].

Although detected by different TLR, such as TLR7, TLR8 and TLR9, the Human Immunodeficiency Virus (HIV) is not cleared by the immune system [7–10]. Therefore, HIV establishes a chronic infection that leads to acquired immune deficiency syndrome (AIDS) when not treated with antiretroviral therapy (ART) [9,11]. Nowadays, thanks to ART, progression to AIDS is a rare event. However, the persistence of HIV in the organism results in chronic inflammation that eventually leads to the development of cancers and cardiovascular diseases [12,13].

Several causes account for the persistent inflammation. Even in individuals successfully treated with ART, HIV persists as integrated DNA in rare latently infected CD4 T and in “tissue sanctuary sites”. In tissues as gut or lymph nodes, a residual replication is then observed, which leads to a continuous induction of immune responses by TLR-dependent mechanisms [14,15]. HIV infection can also be associated with reactivation of other latent viruses such as the hepatitis virus or the cytomegalovirus which in turn provide additional TLR ligands [16,17]. Finally, damages induced by HIV infection in gut mucosa, especially in

Abbreviations: TLR, toll-like receptors; HIV, human immunodeficiency virus; PAMP, pathogen-associated molecular patterns; LPS, lipopolysaccharides; Poly(I:C), polyinosinic: polycytidylic acid; R848, resiquimod; SPADE, spanning-tree progression analysis of density-normalized events; HIV-NT, non-treated HIV infected patients; ART, antiretroviral therapy; HIV-ART, HIV-infected patients on antiretroviral therapy

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mucosa-associated lymphoid tissues (MALT), also contribute to maintain an inflammatory environment. These alterations facilitate the entry of microbial products into the organism; leading to pro-inflammatory immune responses [18–22].

This continuous harnessing of the innate response provokes an inflammation and an exhaustion of the immune system [23]. In this context, the reactivity of the innate immune cells could be affected. Although several studies had already focused on this hypothesis, data on the potential impacts of chronic HIV infection on the abilities of innate immune cells to produce cytokines after TLR engagements are still limited. Currently, studies have shown that the percentage of pDCs producing IFN-α after TLR engagement by TLR7, TLR7/8 or TLR9 ligands are lower in HIV-infected patients compared to uninfected subjects [24–26]. Inversely, the percentages of PBMC producing TNF-α after TLR engagements by TLR4 or TLR7/8 ligands are greater in non-treated HIV-1 patients [27]. Such overproduction of TNF-α has been associated in part to M-DC8$^+$ monocytes, which are both more numerous and higher producer of this cytokine in HIV-infected patients [28].

Since TLR engagement studies have always been performed on a limited number of cell populations and markers, we took advantage of mass cytometry to achieve a comprehensive profile of the changes associated with TLR triggering [29,30]. To characterize TLR engagement in HIV-infected patients, we stimulated whole blood cells from healthy and HIV-1 infected subjects with different TLR ligands. We used LPS, Poly(I:C) and R848, acting as natural or synthetic ligands for TLR3, TLR4, and TLR7/8 respectively. LPS was chosen because it has been found in the blood of chronic HIV-infected patients as a result of microbial translocation [13]. Poly(I:C) and R848 were chosen because they can mimic viral derived ligands. Indeed, they are analogues of double and single strand RNA, respectively [31,32].

Following TLR triggering by LPS or R848, we observed the production of TNF-α, MIP-1β, IL-8, IL-6, and IL-1α in monocytes and conventional dendritic cells (cDCs) from healthy donors. Plasmacytoid dendritic cells produced TNF-α, MIP-1β, IL-8, and IFN-α only after R848 stimulation. Moreover, we didn't observe any production of cytokines in T-cell, B-cell, NK-cell, polymorphonuclear cells (PMN) or basophils. Interestingly, in HIV-1 infected individuals, the production of cytokines by monocytes and cDCs was delayed compared to healthy subjects for both LPS and R848 stimulations. In addition, we noted that the responses induced by a mixture of LPS, R848, and Poly(I:C) were different to those induced by the stimulation using a single TLR ligand. Together, these results underlined the usefulness of CyTOF strategy to describe dysfunctions of myeloid cells to TLR triggering.

2. Materials and methods

2.1. Blood collection

Whole blood samples from healthy, non-treated (NT), and treated HIV-1 infected donors were collected in lithium heparin tubes by the Etablissement Français du Sang (EFS, Hôpital Saint Louis, Paris, France) and by the Hôpital du Kremlin Bicêtre. The gender, age, infection routes, viral load, year of detection, year of the beginning of treatments, the adherence to treatments, and the type of treatments were provided for each HIV-infected patient (Table 1). Briefly, the group of HIV-NT patients was composed by two male and one female (n = 3). The age was ranging between 25 and 47 years, the CD4 cell count was ranging between 2 and 132 cells/μL, and the plasma HIV RNA level was ranging between 48,153 and 5,323,991 copies/mL. All HIV-ART patients were male (n = 3). The age was ranging between 51 and 60 years, the CD4 cell count was ranging between 324 and 1451 cells/mm^3, and the median plasma HIV RNA level was < 40 copies/mL. The prescribed ART regimens were shown in Table 1. For the whole set of HIV infected patients, no HBV nor HCV coinfection was detected.

This experiment was approved by the Comité de Protection des Personnes (Ile de France VII), under protocol number PP 14-003.

2.2. Stimulation, fixation, and storage

Fresh whole blood samples were stimulated during 2 or 6 h at 37 °C with 5% CO_2 in 50 ml plastic tubes (BD Biosciences) with either LPS (Invivogen) at 1 μg/ml, R848 (Invivogen) at 3.14 μg/ml, Poly(I:C) (Invivogen) at 100 μg/ml, or a mixture of the three TLR ligands. Brefeldin A (Sigma-Aldrich) in dimethyl sulfoxide (Sigma-Aldrich) was added after 1 h of stimulation at a final concentration of 1 μg/ml. Stimulations were stopped by the addition of a fixation mixture (FM). For 1 ml of blood, 10 ml of FM was used. FM was composed of 36% paraformaldehyde (VWR BDH Prolabo) and contained 18.5% glycerol (Sigma-Aldrich) in 1X-Dulbecco's phosphate buffered saline (DPBS), without CaCl2 or MgCl2, pH 7.4 (Gibco by Life Technologies). After an incubation of 10 min at 4 °C, samples were centrifuged at 800 × *g* for 5 min at room temperature (RT). Red cells present in the pellets were lysed by adding 10 ml Milli-Q water (and by pipetting) at RT for 20 min. After two washes with 1X DPBS (centrifugation at 800 × *g* for 5 min at RT), cells were counted and distributed in 200 μl aliquots containing 3×10^6 cells. Cells were stored at −80 °C in FM.

FM used to fix and store the cells was prepared the day before the experiment and conserved at 4 °C. This solution allowed freezing and

Table 1
Characteristics of HIV-infected patients and healthy donors. The gender, current age, infection routes, viral load, year of detection, the beginning of treatments, the adherence to treatments and the type treatments were provided for each treated and non-treated HIV-infected patient. In addition, the gender and the current age of healthy donors were also provided.

Patients	Non-treated PATIENT-1	Non-treated PATIENT-2	Non-treated PATIENT-3	Treated PATIENT-1	Treated PATIENT-2	Treated PATIENT-3
Gender	Female	Male	Male	Male	Male	Male
Current age	25	47	36	58	60	51
Infection routes	Sexual	Sexual	Sexual	Sexual	Transfusion	Sexual
CD4$^+$ T-cells (cells/mm^3)	132	2	47	324	521	1451
Viral load (copie/ml)	1,740,324	48,153	5,323,991	< 40	< 40	< 40
Year of HIV diagnosis	2011	2015	2015	2002	1984	1990
Treatments starting	2011	–	–	2002	2002	1997
Adherence to treatments	No	–	–	Yes	Yes	Yes
Treatments	Ritonavir Darunavir Emtricitabine Ténofovir	–	–	Lopinavir Ritonavir Atazanavir	Raltegravir Emtricitabine Ténofovir	Lopinavir Ritonavir Atazanavir

Healthy donors	HEA-1	HEA-2	HEA-3
Gender	Female	Male	Male
Current Age	57	58	25

recovery of all blood leukocytes, especially polymorphonuclear cells, which are highly labile and cryopreservation-sensitive [30].

2.3. Staining and acquisition

For each sample, three million cryopreserved fixed cells were washed twice with staining buffer (PBS/0.5% BSA (Sigma-Aldrich)), then labeled with conjugated antibodies according to the following procedures. Cells were incubated at 4 °C for 30 min with a mixture of the metal-labeled surface antibodies (Abs) in staining buffer. After two washes with 1X DPBS, cells were incubated in fixation solution (PBS/1.6% PFA (Electron Microscopy Sciences Hartfield)) at RT for 20 min and permeabilized with 1X Perm/wash buffer (BD Biosciences) at RT for 10 min. Staining with metal-labeled intracellular Abs and an iridium nucleic acid intercalator in 1X Perm/Wash was carried out as for extracellular staining. Cells were stored overnight with 0.1 μM iridium nucleic acid intercalator in fixation solution. The following day cells were washed with Milli-Q water, resuspended in 1 ml Milli-Q water and filtered using a 35-μm nylon mesh cell strainer (BD Biosciences), before the addition of EQ Four-Element Calibration Beads (Fluidigm), according to the manufacturer's instructions. Acquisition of each sample was manually performed two times in succession on a CyTOF-1 instrument (Fluidigm). Panels, antibody concentrations, clone names and antibody tags of all antibodies were shown in Tables 2 and 3.

Table 2
Antibodies and cell markers used to stain cells from healthy and non-treated HIV-infected patients. The metal isotopes, markers, antibody clones and the used antibody concentrations are indicated. *The clone of IL-12 used to stain cells from healthy donors corresponds to C11.5 whereas the clone used for cells from non-treated HIV-infected patients correspond to C8.6. Therefore, the IL-12 marker was excluded from the analysis.

Label	Antibody	Clone	Concentration (μg/μl)
141Pr	CD66	TET2	0.3
142Nd	HLA-DR	L243 (G46-6)	1
143Nd	CD3	UCHT1	0.3
144Nd	CD64	10.1.1	1
146Nd	IL-6	MQ2-13A5	1
147Sm	CD123	7G3	1
148Nd	IL4	7A3-3	1
149Sm	CD11a	HI111	1
150Nd	CD11b	ICRF44	1
151Eu	IL-8	NAPII	1
152Sm	CD16	B73.1	1
153Eu	CD23	M-L233	1
154Sm	CD86	2331 (FUN-1)	1
155Gd	CD32	2E1	1
156Gd	MIP-1β (CCL4)	D21-1351	1
158Gd	IP10	6D4	1
159Tb	TNF-α	MAb11	1
160Gd	IL-1α	364/3B3-14	1
161Dy	Perforine	DTA G9	1
162Dy	IL-12	C11.5 or C8.6*	1
164Dy	CD184 (CXCR4)	12G5	1
165Ho	TLR2	REA109	1
166Er	CD195 (CCR5)	3A9	1
167Er	CD28	CD28.2	1
168Er	CD11c	B-ly6	1
169Tm	IFN-α	LT27:295	1
170Er	CD14	M5E2	1
171Yb	IL10	JES3-9D7	1
172Yb	TLR7	IMG4G6	1
173Yb	Granzyme B	GB11	1
174Yb	CD19	HIB19	1
175Lu	IL1-RA	1H5	1
176Yb	NFkB-pS529	K10-892.12.50	1
191/193Ir	DNA intercalator	–	1

Table 3
Antibodies and cell markers used to stain cells from treated HIV-infected patients. The metal isotopes, markers, antibody clones and the used antibody concentrations are indicated.

Label	Antibody	Clone	Concentration (μg/μl)
141Pr	CD66	TET2	0.3
142Nd	HLA-DR	L243 (G46-6)	1
143Nd	CD3	UCHT1	0.3
144Nd	CD64	10.1.1	1
145Nd	CD8	37006	1
146Nd	IL-6	MQ2-13A5	1
147Sm	Granzyme A	CTLA-3	1
148Nd	IL-1β	H1b-98	1
149Sm	CD14	M5E2	1
150Nd	CD123	7G3	1
151Eu	IL-8	NAPII	1
152Sm	CD16	B73.1	1
153Eu	CD23	M-L233	1
154Sm	CD86	2331 (FUN-1)	1
155Gd	CD32	2E1	1
156Gd	MIP-1β (CCL4)	D21-1351	1
158Gd	IP10	6D4	1
159Tb	TNF-α	MAb11	1
160Gd	IL-1α	364/3B3-14	1
161Dy	CD141	MAB3947	1
162Dy	IL-12	C8.6	1
164Dy	CD184 (CXCR4)	12G5	1
165Ho	TLR2	REA109	1
166Er	CD195 (CCR5)	3A9	1
167Er	CD28	CD28.2	1
168Er	CD11c	B-ly6	1
169Tm	IFN-α	LT27:295	1
170Er	CD45RA	T6D11	1
171Yb	IFN-γ	25723	1
172Yb	CD4	L200	1
173Yb	Granzyme B	GB11	1
174Yb	CD19	HIB19	1
175Lu	IL1-RA	AS17	1
176Yb	MCP-1	5D3-F7	1
191/193Ir	DNA intercalator	–	1

2.4. Cytometry data processing

Cytometry data were acquired by using EQ Four-Element Calibration Beads, normalized using Rachel Finck's MATLAB normalizer [33], concatenated using the FCS file concatenation tool (Cytobank). SPADE analyses were performed on Cytobank platform, whereas FlowJo software (TreeStar version 9.9) was used to determinate the percentages of cells producing cytokines.

2.5. Statistical analysis

Statistical analyses of cell population abundances were performed using the R software, and were based on a nonparametric permutation tests [34]. This choice was based on the fact of permutation tests are more adapted for studies having a small number of samples.

3. Results

3.1. TLR4 or TLR7/8 engagement: non-treated HIV infection disturbed the production of cytokines in monocytes and dendritic cells

To evaluate the potential impact of chronic HIV infection on the ability of immune cells to respond to stimulation involving TLR ligands, leukocytes from non-treated HIV-infected (HIV-NT) and healthy individuals were stimulated with PBS (control), Poly(I:C), R848, or LPS for 2 or 6 h.

3.1.1. Phenotype profiling

To compare the immune responses obtained from each individual

group, stimulated leukocytes were labeled with the mass cytometry panel shown in Table 2. After the acquisition, a Spanning-tree Progression Analysis of Density-normalized Events (SPADE) was performed using the whole dataset of cytometry profiles [35]. The SPADE analysis was parameterized to identify 100 cell populations using a down-sampling parameter of 5%. The clustering was based on the expression of all extracellular markers, granzyme B, and perforin. Cell clusters were annotated according to the expression of CD3, CD11c, CD14, CD16, CD19, CD64, CD66, CD123 and HLA-DR to identify T-cells, B-cells, PMN, NK cells, monocytes, and dendritic cells (Supplementary Fig. 1). These cell populations were computationally isolated to be analyzed independently. Thereafter, for each cell type, the percentages of cells producing cytokines were determined by manual gating.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.08.018.

In leukocytes from both HIV-NT and healthy individuals, and in comparison to the control, we did not observe production of cytokines following stimulation with Poly(I:C). More in general, we did not observe cytokine production in T-cells, B-cells, NK cells, PMN, and basophils. Conversely, productions of cytokines by monocytes, conventional dendritic cells and plasmacytoid dendritic cells were detected after stimulation with LPS or R848 (Supplementary Fig. 2). However, a strong production of cytokines by plasmacytoid dendritic cells was exclusively detected after stimulation with R848. Therefore, we focused our analysis on these three populations.

3.1.2. Monocytes and conventional dendritic cells from non-treated HIV-infected patients were less reactive to LPS stimulation

To evaluate the potential impact of chronic HIV infection on the ability of monocytes and dendritic cells to respond to LPS stimulation, the percentages of monocytes and dendritic cells producing cytokines (after stimulation) in HIV-NT patients were compared to those obtained in healthy subjects.

As shown in Fig. 1A, after 2 h of stimulation, the percentages of monocytes producing IL-8$^+$ (p-value = 0.0491) and MIP-1β$^+$ (p-value = 0.0479) were significantly higher in healthy donors than in HIV-NT patients. Similarly, after 6 h of stimulation, the percentages of monocytes producing IL-8$^+$ (p-value = 0.0490) and MIP-1β$^+$ (p-value = 0.0493) were also significantly higher in healthy donors. We did not observe significant differences for the production of TNF-α, IL-6 and IL-1α.

In conventional dendritic cells, at 2 and 6 h of stimulation, the percentages of cDCs producing IL-8$^+$ (p-value at 2 h = 0.0476 and p-value at 6 h = 0.0488) and MIP-1β$^+$ (p-value at 2 h = 0.0465 and p-value at 6 h = 0.0498) were significantly higher in healthy donors than in HIV-NT patients (Fig. 1B). In addition, after 6 h of stimulation, a significantly higher percentage of cDCs producing IL-6 (p-value = 0.0495) was also observed in healthy donors.

In summary, following stimulation with LPS, HIV-NT individuals showed a delayed production of IL-8 and MIP-1β by both monocytes and cDCs. A similar delay was observed for IL-6 in cDCs. Therefore, these results suggest that monocytes and cDCs from HIV-NT patients are less reactive to LPS stimulation.

3.1.3. Monocytes, conventional and plasmacytoid dendritic cells from non-treated HIV-infected patients were less reactive to R848 stimulation

Our previous data suggested that innate immune responses induced following TLR4 engagement were delayed in HIV-NT patients. To know if the chronic HIV infection had also impacted other TLR-dependent immune responses, we performed the same analysis with data from leukocytes stimulated with R848.

After 2 h of stimulation, the percentages of monocytes producing IL-8$^+$ (p-value = 0.0491), MIP-1β$^+$ (p-value = 0.0496) and TNF-α$^+$ (p-value = 0.0499) were higher in healthy donors than in HIV-NT patients. Additionally, after 6 h of stimulation, a higher percentage of IL-1α$^+$ producing monocytes (p-value = 0.0495) was observed in healthy donors (Fig. 2A).

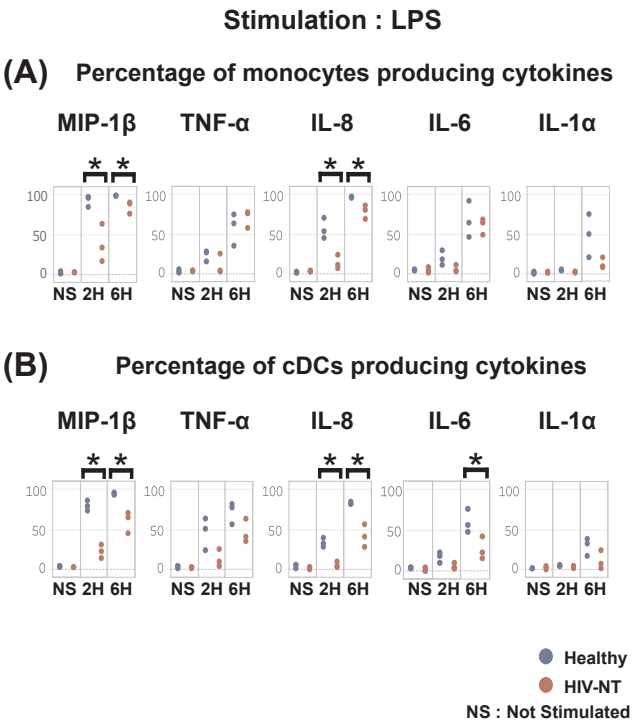


Fig. 1. Monocytes and cDCs from HIV-NT patients are less reactive to LPS stimulation compared to cells from healthy subjects. Whole blood cells from healthy (n = 3) and HIV-NT (n = 3) donors were stimulated for 2 or 6 h with LPS. Controls (samples not stimulated) were performed for each simulation time point. However, to simplify the figure, only the control done after 2 h was represented. The percentages of (A) monocytes and (B) cDCs producing MIP-1β, TNF-α, IL-8, IL-6, and IL-1α after LPS stimulation were represented. Blue points correspond to percentages of cells obtained from healthy subjects whereas red points correspond to those from HIV-NT patients. [*means p-value ≤ 0.05]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A delay in the kinetics of cytokine production was also observed in cDCs. Indeed, after 2 h of stimulation, the percentages of cDCs producing IL-6$^+$ (p-value = 0.0484), IL-8$^+$ (p-value = 0.0495), MIP-1β$^+$ (p-value < 0.0001) and TNF-α$^+$ (p-value = 0.0493) were significantly higher in healthy donors compared to HIV-NT patients. Additionally, after 6 h of stimulation, the percentages of cDCs producing TNF-α$^+$ (p-value = 0.0469) but also IL-1α$^+$ (p-value = 0.0487) were significantly higher in healthy donors (Fig. 2B).

Unlike LPS, R848 induced a strong production of cytokines in pDCs. This result was expected since pDC population expresses predominantly TLR7 and TLR9 but not TLR4 [29]. Using this TLR ligand, we tested if chronic HIV infection had also modified the kinetics of cytokine production in this population. To do so, the productions of cytokines by pDCs from HIV-NT patients were compared to those from healthy donors. After 2 h of stimulation, the percentage of pDCs producing IFN-α$^+$ (p-value = 0.0500) was significantly higher in healthy donors. After 6 h of stimulation, no differences between HIV-NT and healthy individuals were identified (Fig. 2C). Moreover, we did not observe significant differences for the production of TNF-α, MIP-1β and IL-8.

Together, these results suggest that in chronic HIV infection the production of cytokines in monocytes and dendritic cells stimulated with R848 is delayed.

3.2. TLR7/8 engagements: antiretroviral therapy did not fully restore the kinetics of cytokine productions

To evaluate the effects of antiretroviral therapies on the capacity of

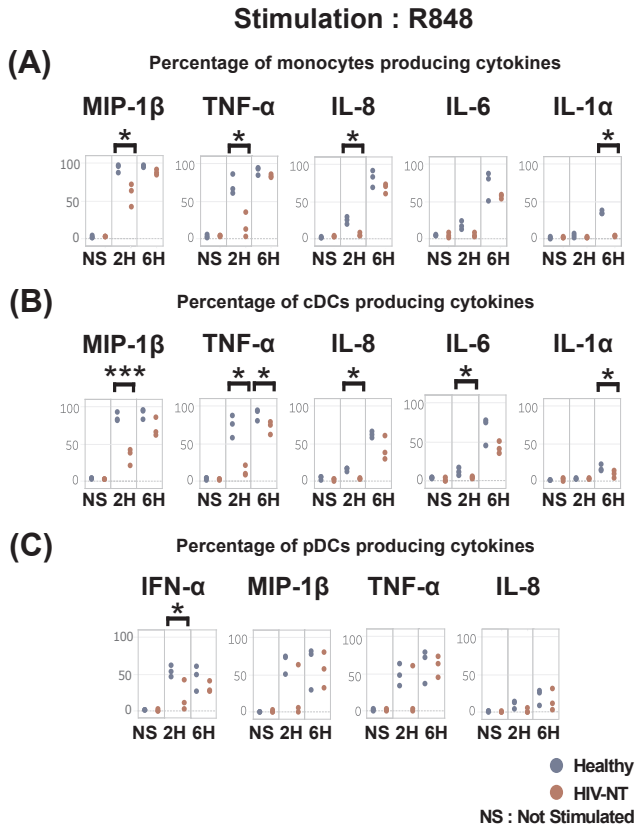


Fig. 2. Monocytes, cDCs, and pDC from HIV-NT patients are less reactive to R848 stimulation compared to cells from healthy subjects. Whole blood cells from healthy (n = 3) and HIV-NT (n = 3) donors were stimulated for 2 or 6 h with R848. Controls (samples not stimulated) were performed for each simulation time point. However, to simplify the figure, only the control done after 2 h was represented. The percentages of (A) monocytes, (B) cDCs and (C) pDCs producing MIP-1β, TNF-α, IL-8, IL-6, IL-1α, and IFN-α were represented. Blue points correspond to percentages of cells obtained from healthy subjects whereas red points correspond to those from HIV-NT patients. [*means p-value ≤ 0.05; ***means p-value ≤ 0.001]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

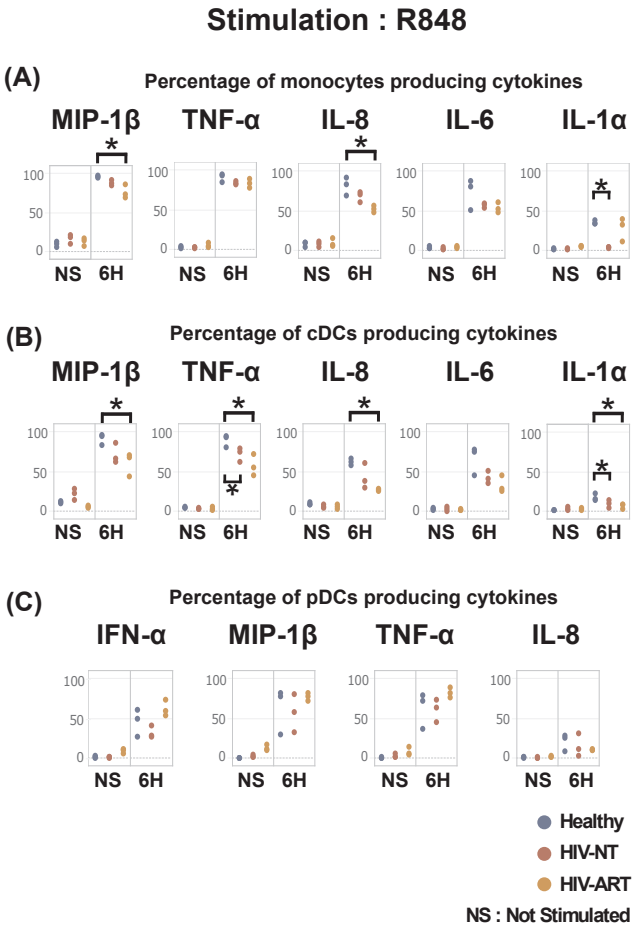


Fig. 3. Antiretroviral treatments do not fully restore the ability of monocytes, cDCs, and pDC to produce cytokines. Whole blood cells from healthy (n = 3), HIV-NT (n = 3) and HIV-ART (n = 3) donors were stimulated (or not) for 6 h with R848. The percentages of (A) monocytes, (B) cDCs and (C) pDCs producing MIP-1β, TNF-α, IL-8, IL-6, IL-1α, and IFN-α were represented. Blue, red and orange points correspond to percentages of cells obtained from healthy subjects, HIV-NT and HIV-ART individuals, respectively. [*means p-value ≤ 0.05]. Significant differences showed in Fig. 2 are not depicted again. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cells to produce cytokines after TLR engagement, whole blood cells from ART-treated HIV-infected (HIV-ART) patients were stimulated with R848 during 6 h. Thereafter, immune responses obtained in this individual group were compared with those obtained in HIV-NT and healthy subjects.

3.2.1. Phenotype profiling

After stimulation, leukocytes from HIV-ART patients were labeled with the mass cytometry panel shown in Table 3. Similar to the previous analysis, a SPADE analysis was parameterized to obtain 100 clusters using a down-sampling parameter of 5%. The clustering was based on the expression of all extracellular markers, granzyme B, and perforin. Afterwards, each cluster was annotated on the same principle as previously described in Supplementary Fig. 1. Monocytes and dendritic cells were computationally isolated in order to be analyzed independently. The percentages of cells producing cytokines were determined by manual gating, and each cytokine was analyzed independently. Because the panel used to stain cells from HIV-ART donors was different from the one used for cells from HIV-NT and healthy donors; the comparison of these results with the previously obtained data was based exclusively on the percentages of cells producing cytokines. This comparison was possible because the performed analyses were based on the study of whole cell populations. It is important to highlight that the antibody clones used for each cytokine were identical in both panels.

3.2.2. Monocytes and conventional dendritic cells from HIV-ART patients were less reactive to TLR7/8 stimulation

To determine if antiretroviral therapies restored the ability of monocytes to normally produce cytokines, the immune responses obtained in monocytes from HIV-ART patients were compared to those from healthy individuals. As shown in Fig. 3A, after 6 h of R848 stimulation, the percentages of monocytes producing IL-8$^+$ (p-value = 0.0477) and MIP-1β$^+$ (p-value = 0.0488) were significantly lower in HIV-ART patients compared to healthy individuals. However, unlike monocytes from HIV-NT patients, a production of IL-1α (p-value = 0.0450) was observed in HIV-ART subjects. More precisely, the percentage of IL-1α$^+$ monocytes in HIV-ART and healthy individuals were similar whereas they were significantly different between HIV-ART and HIV-NT patients (p-value = 0.0496) (Fig. 3A). These results suggest that ART restore only partially the ability of monocytes to produce cytokines in HIV-ART patients.

To continue our investigations, the ability of both cDCs and pDCs to produce cytokines was studied using the same method as for

monocytes. After 6 h of stimulation, the percentages of cDCs IL-1α$^+$ (p-value = 0.0500), IL-8$^+$ (p-value = 0.0499), MIP-1β$^+$ (p-value = 0.0500) and TNF-α$^+$ (p-value = 0.0495) were significantly higher in healthy donors compared to HIV-ART patients (Fig. 3B). Moreover, the treatment was not able to restore the capacity of cDCs to produce IL-1α.

Finally, after 6 h of stimulation, the production of cytokines by pDCs from HIV-ART patients was not statistically different compared to the production observed in pDCs from HIV-NT or healthy subjects (Fig. 3C). Because the delay in the production of IFN-α was only observed after 2 h of stimulation, we cannot exclude a potential impact of ART on the ability of pDCs to produce cytokines in the first hours.

Together, these results showed that despite ART, the immune responses induced by TLR engagement could still be affected in this individual group.

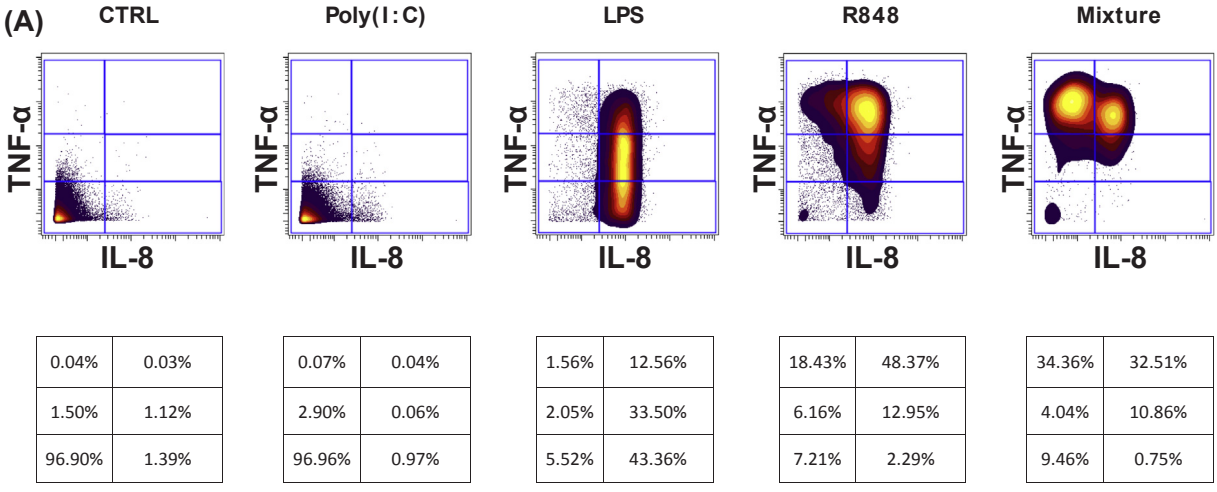
3.3. Stimulation by a mixture of TLR ligands

In vivo, the organism can be simultaneously exposed to TLR3, TLR4 and TLR7/8 ligands. Indeed, both bacteria and viruses can be present on the mucosal barriers. When these barriers are weakened, the transmucosal passage of microbial products can include both LPS and viral RNA. To better understand the development of innate immune responses induced by leucocytes after simultaneous exposure to different TLR ligands, whole blood cells from healthy individuals were stimulated with Poly(I:C), R848, LPS or a mixture of the three TLR ligands for 6 h. In this analysis, leukocytes were labeled with a mass cytometry panel shown in Table 2, allowing the study of different pro-inflammatory cytokines. Monocytes from healthy individuals were computationally isolated using SPADE on the same principle as shown in Supplementary Fig. 1. We focused the analysis on monocytes because they express all the TLRs of interest.

As previously observed, poly(I:C) did not induce production of cytokines in monocytes. These monocytes were mainly IL-8$^+$ TNF-α^{mid} after stimulation with LPS whereas monocytes stimulated with R848 were mainly IL-8$^+$ TNF-α^{high} (Fig. 4A). Moreover, the percentage of monocytes being IL-8$^+$ TNF-α^{-mid} was significantly higher after LPS stimulation compared to R848 stimulation (p-value = 0.0494). Inversely, the percentage of monocytes being IL-8$^+$ TNF-α^{-high} was significantly higher after R848 stimulation compared to LPS stimulation (p-value = 0.0482) (Fig. 4B). Finally, a higher percentage of monocytes being IL-8$^+$ TNF-α$^-$ was observed after LPS stimulation compared to R848 stimulation (p-value = 0.0481) (Fig. 4A and B).

Interestingly, the distribution of monocytes after stimulation with a mixture of TLR ligands was different compared to this obtained with R848 or LPS (Fig. 4A and B). Compared to R848 and LPS stimulation, the mixture of TLR ligands induced a significantly higher number of monocytes that were IL-8$^-$ TNF-α^{high} (p-value for LPS = 0.0491 and p-value for R848 = 0.0459). In addition, after stimulation with the mixture, this IL-8$^-$ TNF-α^{high} population was the most represented population. Therefore, this result suggests that immune responses induced by the mixture could be different to those induced either by LPS or R848 stimulation.

Same analysis, but based on the co-expression of MIP-1β and IL-6,



(B)

	Condition 1 vs Condition 2 (p-value)		
Population	LPS *vs* R848	LPS *vs* MIX	R848 *vs* MIX
TNF-α^{high} IL-8$^+$	**0.0482**	**<0.0001**	0.0968
TNF-α^{high} IL-8$^-$	**0.0483**	**0.0491**	**0.0459**
TNF-α^{mid} IL-8$^+$	**0.0494**	**<0.0001**	0.4931
TNF-α^{neg} IL-8$^+$	**0.0481**	**0.0483**	0.2537

Fig. 4. The immune responses induced by a mixture of LPS, R848, and poly(I:C) are different from those generated by stimulation involving the individual usage of those ligands. Whole blood cells from healthy donors (n = 3) were stimulated for 6 h with LPS, R848, Poly(I:C) or a mixture of those three TLR ligands. Then monocytes were computationally isolated and (A) the co-production of IL-8 and TNF-α was analyzed for each stimulation condition. Three expression levels of TNF-α (TNF-α^{neg}, TNF-α^{mid,} and TNF-α^{hight}) and two of IL-8 (IL-8$^+$ and IL-8$^-$) were identified. Thus, dot plots were divided into six sections. For each of them, the average percentage of cells was provided. (B) Table of statistics comparing the percentages of cells present in each section and between the different stimulation conditions. Significant results are shown in bold [*means p-value ≤ 0.05].

was performed (Supplementary Fig. 3). Compared to R848 or LPS stimulations, the mixture of TLR ligands induced in monocytes a significantly lower number of monocytes MIP-1β$^+$ IL-6$^+$ (p-value for LPS = 0.0500 and p-value for R848 = 0.0487). These results also support that immune responses induced by the mixture of TLR ligands could be different to those induced by stimulation with a single TLR ligand.

4. Discussion

We have showed that immune responses induced following the TLR engagement with LPS and R848 were delayed in HIV-infected patients. Hence, despite the higher expression of TLR4 in peripheral blood mononuclear cells (PBMCs) from HIV-NT patients compared to healthy subjects [27], our results suggest that the leukocytes from HIV-infected patients could be slower to respond to the translocation of bacterial products. This could be detrimental given that this translocation is increased in this individual group [37]. Same, although PBMC from infected patients have a higher expression of TLR7/8 than those from healthy subjects [27], we suggest that the immune system of HIV-infected patients could be slower in responding to the detection of viral products, such as those from HIV residual replication or other infectious pathogens.

These results could characterize an exhaustion of immune cells in these patients. Indeed, leukocytes are continually stimulated by TLR ligands, such as those resulting from microbial translocation. The maintenance of the inflammatory environment also enhances the generation of regulatory cells such as regulatory T-cells and myeloid-derived suppressive cells [38]. These cell populations could, in turn, restrict the capacity of leukocytes to produce cytokines. Moreover, repeated LPS stimulation of isolated macrophages or monocytes leads to a reduced production of pro-inflammatory cytokines such as TNF-α [39]. The up-regulation of TLR observed in leucocytes from HIV-infected donors could thus be considered as a compensation mechanism by which the immune system of infected patients seek to make up for the incapacity of leucocytes to quickly produce cytokines [27].

Because the delay was also observed in HIV-ART patients, the replication of HIV may not be the main cause of this impairment. Nevertheless, the control of viral replication led to the recovery of monocyte capacity to produce IL-1α after 6 h of R848 stimulation. Therefore, the antiretroviral treatments have beneficial effects on the kinetics of cytokines production observed in monocytes.

The delay in the production of cytokines by myeloid cells is in accordance with the higher risk of bacterial and viral infections in HIV-NT patients. Our data suggests that even ART HIV-infected patients could remain more vulnerable to infections. Taken into account the inefficiency of the current antiretroviral treatments to fully restore the functions of the innate immune system, new therapeutic strategies should be considered. One approach would be limiting inflammation. This could be achieved by restoring the integrity of gastrointestinal barriers in HIV-infected individual and thus limiting the translocation of microbial products.

Previously, Dutertre and al. demonstrated that, compared to monocytes from healthy subjects, monocytes from HIV-infected patients over-produced TNF-α after 24 h of LPS stimulations [28]. Interestingly, we observed after 6 h of stimulation that the production of TNF-α seemed to be higher (yet not significant) in HIV-infected patients. This observation was exclusively observed with TNF-α. Therefore, we hypothesize that the production of this cytokine could be significantly higher after 24 h of stimulation.

Interestingly, TLR4 agonists (including LPS) have been shown to suppress *in vitro* the HIV-1 expression in macrophages [40]. Similarly, TLR8 agonists (including R848) have been shown to suppress HIV replication in cultured monocytes [41]. Although the immune responses induced by these two TLR ligands seem to be less reactive in chronic HIV-infected patients compared to healthy subjects, they could be sufficient to limit the replication of HIV. Thus, the microbial translocation could restrict the replication and dissemination of HIV. However, other TLR ligands, such as those targeting TLR2, have been shown to enhance *in vitro* the HIV-1 expression in macrophages [40]. In conclusion, according to the translocated microbial products, the TLR engagement could enhance or restrict the replication and the propagation of HIV. Further understanding of the physiology of immune responses induced by TLR engagement following the translocation of the microbial products could unveil novel targets for immuno-modulatory therapy. This is even truer that responses induced following TLR engagements by microbial products can reactivate the latent virus present in the reservoir [42,43].

Because LPS or R848 induce a strong and fast production of pro-inflammatory cytokines, these molecules (or derived molecules) are currently tested as adjuvants for different vaccines [44-47]. As shown, the responses induced by those molecules can be different in individuals with different immune states. Therefore, the induced safety profiles could also be different. For this reason, the usage of adjuvants to enhance the immune responses during the vaccination processes must be tested for each individual group having risk factors, as patients with chronic viral infections.

Our data showed that the immune responses induced by a mixture of TLR ligands were different to those induced by a unique TLR ligand. These data highlight the complexity of TLR-dependent immune mechanisms induced by the transmucosal passage of microbial products. Indeed, each individual has his own microbiota. Therefore, even if a specific pathogen induces same damages to mucosal barriers in different individuals, the translocated microbial products could be different. This could in turn trigger different inflammatory mechanisms; meaning different immune responses. Out of note, it will be interesting to better understand how do leukocytes from HIV-infected patients react after stimulation with a mix of TLR ligands compared to single TLR. Indeed, because the bacterial translocation is enhanced in these patients, this question should be deeper study.

Unfortunately, due to material availability and technical considerations, it was not possible for us to generate new data allowing understanding how do leukocytes from HIV-infected patients react after stimulation with a mix of TLR ligands, compared to single TLR ligand. Indeed, the collection of fresh samples from non-treated HIV-infected subjects is nowadays difficult, as the number of patients contracting the HIV infection in Europe is low. In addition, the number of HIV-infected patients providing blood samples right after their diagnostics is also very low. This can be explained by the fact that patients are often shocked by this news, and also because patients are scared about the potential spread of this news (social pressure). Moreover, due to the replacement of our CyTOF-1 mass cytometer by a Helios mass cytometer (which is an improved version and do not have the same sensibility to detect marker expressions), it became impossible for us to perform these experiments, as it is not possible to compare data from different versions of mass cytometers.

Our study raised several questions. As expected, R848 and LPS induced production of cytokines in monocytes and dendritic cells. Nevertheless, no cytokine production was observed in neutrophils, even after LPS stimulation. This result was surprising because neutrophils express TLR4 [48]. Additionally, the capacity of neutrophils to quickly produce TNF-α, IL-1α, IL-6, and IL-8 after stimulation with LPS has already been described [48]. However, the stimulation of neutrophils was not directly made on whole blood but on sorted neutrophils. Thus, red blood cells or other leukocytes populations could interfere with the ability of neutrophils to respond to LPS stimulation. Moreover, as neutrophils have a short half-life, the sorting could have also affected their functions.

The stimulation with Poly(I:C) had no effects on leukocytes from both HIV-infected and healthy individuals. However, cytokines production in PBMCs stimulated with this TLR ligand was observed [49]. To reconcile these apparent discordant results, we hypothesize that red

blood cells or PMN could interfere in the TLR3-dependent responses. Indeed, our stimulations were performed on whole blood and not on PBMCs.

Because we focused our study on innate immunity, we did not include the CD4 and CD8 markers in our mass cytometry antibody panels. Thus, it was not possible to differentiate $CD8^+$ T-cells to $CD4^+$ T-cells. Moreover, our mass cytometry panel did not include other markers allowing the identification of T-cell subsets, such as CCR7, CD25, CD27, or FoxP3. The absence of these markers represents a real limitation of this study. Indeed, we worked on the whole $CD3^+$ T-cell populations, and not on subsets of this cell population. Thus, even if we did not observe significant productions of cytokines in the whole $CD3^+$ T-cells, we cannot exclude that a specific subset of T-cells (with a low abundance in the blood) significantly produced cytokines after LPS, R848, or Poly(I:C) stimulations.

We mainly focused our analysis on innate immune responses and, therefore, we did not observe T-cells responses. However, as there are strong links between innate and adaptive immune responses, we can hypothesize that T-cells immune responses are also impacted by the delay. This hypothesis is also supported by the delay of cytokine production observed in cDCs. Finally, although the number of samples per condition was small in our analysis, our data is the first of its kind to be obtained using CyTOF technology, which is a suitable tool to study multiparametric conditions. These data show the complexity to study the TLR-dependent immune responses, yet could pave the way for future functional studies.

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Conflicts of interest

The authors declared that they have no conflicts of interests.

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