Cytokine 111 (2018) 97–105

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

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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ARTICLE INFO

Keywords:
HIV infection
TLR stimulations
Cytokines
Mass cytometry

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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https://doi.org/10.1016/j.cyto.2018.08.018
Received 20 April 2018; Received in revised form 14 August 2018; Accepted 16 August 2018

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