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Dendritic cell recruitment in response to skin antigen tests in HIV-1 infected individuals correlates with the level of T cell infiltration

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Abstract

Objectives—To study whether *in vivo* recruitment of dendritic cells (DCs) in response to antigen administration in the skin is altered during HIV-1 infection.

Design—Skin punch biopsies were collected from HIV-1+ as well as seronegative individuals at 48 hours post intradermal injection of inactivated antigens of mumps virus, *Candida albicans* or purified protein derivate (PPD) from *Mycobacterium tuberculosis*.

Methods—Cryosections were analyzed by *in situ* staining and computerized imaging.

Results—Control skin biopsies showed that there was no difference in the number of skin-resident DCs between seronegative and HIV-1+ individuals. Antigen injection resulted in substantial infiltration of DCs compared to the frequencies found in donor-matched control skin. In HIV-1+ individuals, CD123+/CD303+ plasmacytoid DCs and CD11c+ myeloid DCs, including the CD141+ cross-presenting subset, were recruited at lower levels compared to healthy controls in response to PPD and mumps but not *C. albicans*. The level of DC recruitment correlated with

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Author contributions

FL, E, JA, KL. designed research. FL, EB KJS performed experiments. FL, EB, KJS, AS-S, RAK, JA, KL analyzed data. MXR, CL, GAM, MML, RJW contributed with the clinical samples, FL, EB and KL wrote the paper.

Conflict of Interest

The authors state no conflict of interest.

the frequencies of T cells infiltrating the respective antigen sites. Ki67+ cycling T cells at the injection sites were much more frequent in response to each of the antigens in the HIV-1+ individuals, including those with AIDS, compared to healthy controls.

Conclusions—Multiple DC subsets infiltrate the dermis in response to antigen exposure. There was no obvious depletion or deficiency in mobilization of DCs in response to antigen skin tests during chronic HIV-1 infection. Instead, the levels of antigen-specific memory T cells that accumulate at the antigen site may determine the level of DC infiltration.

Keywords

HIV-1; dendritic cells; plasmacytoid; skin; skin test; delayed-type hypersensitivity reaction; Ki67

Introduction

Dendritic cells (DCs) are the foremost initiators of adaptive immune responses. They prime and condition T cells and are therefore critical for cellular immunity. The major human DC subtypes are the CD11c+ myeloid DCs (MDCs) and CD123+ plasmacytoid DCs (PDCs). Both MDCs and PDCs are susceptible to HIV-1 infection, though they are poor hosts for viral replication [1-6]. The frequency of infected DCs *in vivo* is undetectable or very low compared to CD4+ T cells, at least after initial transmission [7-9]. It is well documented that the numbers of blood MDCs and PDCs are reduced in HIV-1+ individuals [10-14] but whether this is due to depletion resulting from direct infection or immune exhaustion, or anatomical redistribution, is largely unknown [15-17]. The decline in circulating DCs correlates with an increase in HIV-1 viral load, suggesting that there is an inverse relationship between the loss of DCs and control of the disease. However, whether DCs are functionally defective in HIV-1 infected individuals to a degree that would affect the priming or re-activation of antigen-specific immune responses is not understood.

Skin-resident DCs are almost exclusively of the MDC lineage. However, PDCs that do not usually reside in the skin have recently been shown to infiltrate skin lesions caused by herpes and varicella infections, and the level of PDC recruitment to the lesions was shown to correlate with control of infection [18-21]. In addition, we earlier demonstrated that there is a robust infiltration of several distinct DC subsets including PDCs into dermal indurations induced by the tuberculin skin test (TST) [22]. The level of recruitment of functionally different DC subsets to sites of antigen exposure is likely to shape the quality and magnitude of the adaptive response. In this study, we asked if the recruitment of different DC subsets *in vivo* in response to antigen skin testing is affected during HIV-1 infection. We utilized widely employed clinical skin tests in which antigens are injected intradermally for evaluation of previous antigen exposure or integrity of the cellular immune system. These tests can, for example, be based on injection of inactivated mumps virus, *Candida albicans* antigens, or purified protein derivate (PPD) from *Mycobacterium tuberculosis*. In sensitized individuals, a delayed-type hypersensitivity (DTH) reaction appears at the injection site as a consequence of accumulation of T cells, which are presumably antigen-specific. The immune reaction is likely initiated by antigen uptake by skin DCs and subsequent presentation to antigen-specific memory T cells [23, 24]. T cells homing to and accumulating at the antigen site then drive the immune reaction which in turn leads to

inflammation, causing a visible skin induration in sensitized individuals. To study infiltration of DCs and T cells in response to antigen injection, skin punch biopsies were collected at 48 hours from such indurations in cohorts of healthy individuals as well as HIV-1+ individuals. We found a large infiltration of several DC subsets. This was accompanied by infiltration of T cells, which were found in clusters with DCs in the dermis. The frequencies of DCs at the antigen-injected sites were significantly lower in HIV-1+ individuals compared to healthy controls in response to PPD and mumps antigens but not to *C. albicans*. The levels of DC infiltration reflected the frequencies of T cells at the respective sites. Hence, there seemed to be sufficient numbers of DCs available to be mobilized when the antigen response/inflammatory milieu was adequate. The level of DC infiltration in this cognate antigen exposure model may therefore instead be determined by the levels of responding antigen-specific memory T cells that home to the antigen site. A depleted or deficient T cell compartment could therefore lead to compromised DC recruitment and insufficient antigen presentation to T cells.

Materials and Methods

Study Cohorts

Written informed consent was obtained from all study subjects. The Institutional Review Boards of Ethics at the respective research institutes approved this study. The cohort that received the TST was recruited in Khayelitsha Township, Cape Town, South Africa. The criteria for study enrollment were described earlier [25]. Healthy seronegative and untreated HIV-1+ asymptomatic individuals, with CD4 counts of mean $\pm$ SEM 579 $\pm$ 82/ μ L blood, were matched by age, sex and size of the TST induration (Table 1). A group of individuals with AIDS, with CD4 counts of 150 $\pm$ 8.9/ μ L blood, was included for comparison. TST was performed according to international standards and considered positive at ≥ 10 mm. All study subjects except two of the AIDS patients, displayed positive TST reactions (18.6 $\pm$ 2.3 mm). Punch biopsies were taken as described [26] from the PPD injection site and a saline injected site on the opposite arm at 48 hours post injection and snap frozen.

Other study cohorts were recruited by the University Hospital of Cleveland, OH, USA. They received mumps-skin test (MSTA) and *C. albicans*-skin test (CASTA), and consisted of seronegative healthy individuals, asymptomatic untreated HIV-1+ subjects with CD4 counts of 690 $\pm$ 72/ μ L and HIV-1 RNA copies of 1667 $\pm$ 1036/ μ L, and individuals with AIDS with CD4 counts of 27 $\pm$ 13/ μ L and HIV-1 RNA copies of $>750\ 000$ / μ L. All donors had positive indurations as defined by ≥ 5 mm. The indurations in the seronegative controls were 14.8 $\pm$ 3.2 mm for MSTA and 10 $\pm$ 2.8 mm for CASTA, while HIV-1+ asymptomatic subjects had indurations of 9.7 $\pm$ 1.7 mm and 13 $\pm$ 1 mm, respectively. AIDS patients had 9 $\pm$ 3 mm. As above, punch biopsies were collected 48 hours after injection.

Immunohistochemical staining and quantitative image analysis

Sections from skin biopsies (8 μ m) were stained as described [15, 27] with anti-CD11c, CD123 and Ki67 (BD Biosciences, San José, CA), CD3, CD45RO, CD45RA (Dako, Glostrup, Denmark), CD303 (BDCA-2), DC-SIGN, Langerin (R&D Systems, Minneapolis, MN), CD141 (BDCA-3) (Miltenyi Biotech, Auburn, CA), DNGR-1 (Dr. Reis e Sousa,

London Research Institute) followed by the anti-mouse, anti-goat (Dako) or anti-rabbit (Vector Laboratories, Burlingame, CA) biotinylated secondary antibodies (Abs). The peroxidase system, Vectastain Elite ABC kit together with substrate diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA) was used to label the secondary antibodies. Cell nuclei were counterstained with Mayer's Haematoxylin (Histolab Products, Gothenburg, Sweden). A blinded analysis of the sections was performed by two separate investigators with a Leica DMR-X microscope (Leica Microsystems GmbH, Wetzlar, Germany) coupled to computerized image analysis (Leica Qwin 5501W, Leica Imaging Systems) as described [22]. Quantification of all markers was performed in the dermis except for Langerin, which was done in the epidermis. Melanin-rich cells at the interface of the epidermis and the dermis were excluded from the analyses.

Fluorescent co-staining

Staining procedures were performed as above, except the secondary biotinylated Abs, were detected by Alexa488- or Alexa647-conjugated streptavidin (Molecular Probes, Eugene, OR), and anti-CD4 (BD) and anti-CD8 (BD) were also included. Sections were mounted using SlowFade® Gold Antifade Reagent with DAPI (Life Technologies).

Statistical analyses

Data were analyzed by Student's t test except Ki67 and DNGR-1, which was assessed with Mann Whitney test using GraphPad Prism software (San Diego, CA), and considered significant at *p 0.05, **p 0.01 and ***p 0.001.

Results

Reduced DC accumulation in tuberculin skin test indurations in HIV-1+ individuals

In this study, we used the antigen skin test model to evaluate whether HIV-1+ individuals show impaired DC recruitment *in vivo* in response to antigen exposure and inflammation compared to healthy individuals. As expected, in healthy individuals there was a clear infiltration of cells expressing HLA-DR as found by an increase in the percentage of stained area out of the total dermal sample area in biopsies from the TST indurations compared to the donor-matched saline injected control sites (Figure 1). In addition, expression of the MDC marker CD11c was found to be significantly higher in the TST reaction than in control sites (Figure 1A-B, $p < 0.0001$). In controls, the majority of CD11c⁺ cells were typically present in clusters in the dermis, while the CD11c⁺ cells in the TST reaction were scattered throughout the dermis. PDCs, defined by CD123 and CD303 (BDCA-2) expression, were rare or absent in the controls, but consistently present in clusters in the dermis of the TST sites (Figure 1B, $p = 0.030$ and $p = 0.0002$, for CD123 and CD303 respectively). CD141 (BDCA-3), recently reported to define a distinct MDC subset with superior cross-presenting function [28], was expressed at very low levels in the controls but was detected in all the TST biopsies (Figure 1A-B, $p = 0.002$). Also, DNGR-1, a receptor that contributes to the cross-presenting capacity of this subset [28], was significantly more prevalent at the TST sites compared to control sites (Figure 1B, $p = 0.024$). The C-type lectin receptors (CLRs) DC-SIGN and Langerin, were also significantly more highly expressed in the TST reaction compared to the control (Figure 1, $p = 0.005$ and $p = 0.015$, respectively). All these markers

were almost exclusively expressed in the dermis except for Langerin, which was expressed on Langerhans cells in the epidermis.

There was no difference in the percentage of area expressing DC markers in the saline-injected control sites between the seronegative and the HIV-1+ asymptomatic individuals. Thus, the well-documented reduction in numbers of blood DCs during HIV-1 infection was not apparent among skin-resident DCs. In contrast, the levels of CD11c ($p=0.002$), CD141 ($p=0.039$), and CD303 ($p=0.029$) expression in the TST reaction were significantly lower in the HIV-1+ asymptomatic individuals compared to healthy controls (Figure 1A-B). In addition, there was a noticeably lower accumulation of the rest of the DC markers in the TST reaction of the HIV-1+ individuals. Also, except for DC-SIGN, all DC markers were expressed at even lower levels at the TST sites of the AIDS patients.

Level of T cell accumulation at the TST reaction site correlates with DC infiltration

In the TST indurations, CD3+ T cells were found exclusively in the dermis, in cluster formations with DC markers such as CD11c and CD123 (Figure 2A-B). There was an increased infiltration of CD3+ T cells in the TST reactions compared to the saline injected sites of both the seronegative and the HIV-1+ individuals (Figure 2C), but the infiltration was significantly higher in the seronegative controls ($p=0.0039$). There was a significant correlation between the frequencies of CD3+ T cells at the TST site and the levels of infiltrating CD123+ CD303+ PDCs (Figure 2D) and CD11c+ MDCs plus a trend for the CD141+ MDCs (Figure 2E). The healthy controls showed the highest levels of both CD3+ T cell and DC infiltration, followed by the asymptomatic HIV-1+ individuals. The patients with AIDS had the lowest degree of cell infiltration. Both CD4+ and CD8+ T cells were found to be recruited to the TST sites (Figure 2F-G). In contrast to the cell numbers, Ki67 expression in the T cells, indicating cell cycling, was almost exclusively found in the HIV-1+ individuals, especially those with AIDS (Figure 2H-J).

Differential recruitment of DCs in response to mumps and *C. albicans* antigen injection

Since significantly lower frequencies of both DCs and T cells infiltrated the TST site in HIV-1+ individuals compared to healthy controls, we asked if this was due to cell depletion and/or due to a general immune defect including a reduced ability of the cells to migrate towards antigen exposure/inflammation. To investigate whether the recruitment was antigen specific or general to DTH reactions, we established new cohorts that received clinical skin antigen tests based on inactivated sterilized mumps virus or *C. albicans* (Table 1). As for TST, both of these skin tests stimulate DTH reactions and most adults respond with indurations since they have been vaccinated against or infected with mumps virus, and are constantly exposed to *C. albicans* as a commensal fungus. All donors included in the study had positive indurations.

MDC subsets expressing CD11c and/or CD141 as well as PDCs expressing CD303 accumulated at sites injected with mumps or *C. albicans* in healthy controls as well as in HIV-1+ individuals. As these cohorts contained fewer individuals, fewer comparisons reached statistically significant differences. However, there appeared to be a trend that the levels of infiltrating DCs were higher in the healthy controls compared to the HIV-1+

subjects in response to mumps (Figure 3A-C). Conversely, the DC recruitment in response to *C. albicans* was higher in the HIV-1+ individuals compared to the controls. Since there was a differential pattern of DC infiltration to mumps versus *C. albicans*, we speculated that the magnitude of memory T cells specific for the respective antigens might determine the level of DC recruitment. High *C. albicans*-specific T cell responses have been reported in HIV-1+ patients compared to seronegative controls [29-31]. In line with these data, the mean induration sizes after *C. albicans* antigen test were larger in the HIV-1+ groups and we found significantly higher levels of infiltrating CD3+ T cells in the indurations of the HIV-1+ individuals compared to seronegative controls (Figure 3D) ($p=0.001$). The frequencies of T cells at the *C. albicans* site were also higher than in the donor-matched mumps site in the HIV-1+ asymptomatic individuals, which was not the case in the controls. Thus, as found for TSTs, the CD3 data aligned with the levels of infiltrating DCs in the *C. albicans* skin test sites. The level of T cell accumulation at the antigen site, which is presumably dictated by the pre-existing pool of antigen-specific memory cells in the donors, may therefore regulate the level of inflammation that in turn controls the level of DC recruitment. Intrinsic differences in the characteristics of the antigens in the tests may also play a role in the recruitment of DCs. However, the tests are designed to only stimulate cellular immune responses in sensitized individuals which is why the indurations should be greatly dependent on memory T cells. Consistent with the data in the TST group, the frequencies of Ki67+ cells in mumps indurations and in particular *C. albicans* indurations were higher in the HIV-1+ individuals (Figure 3E). This is in accordance with that HIV-1 infection is associated with chronic immune activation including increased level of cycling T cells in peripheral blood [32]. CD45RO was expressed at higher levels than CD45RA at the sites (Figure 3F-G). Although these are crude marker for phenotyping T cells, this suggests that the majority of infiltrating T cells are cycling memory T cells.

Discussion

Skin testing is a widely employed and readily available method of clinically assessing the cellular immune response. A positive skin-test reaction indicates previous antigenic exposure, T cell competence and an intact inflammatory response. In accordance with the fact that the skin test formulations exclusively induce immune-activation in individuals with pre-existing immunity, we have earlier reported that there is no infiltration of DCs after injection of PPD in non-sensitized individuals who do not display positive TST indurations [22]. The efficiency with which skin test antigen is presented to T cells and with which the DTH reaction develops are likely strongly enhanced by the level of recruitment of DCs to the skin and continuous antigen presentation [23, 24, 33]. Thus, although the initial DCs that take up antigen after skin test injection, and migrate and present to T cells in draining lymph nodes, may be limited, the subsequent wave of activated T cells homing to the antigen site will cause inflammation which is likely what drives the recruitment of the burst of DCs found at the test site in sensitized individuals. Based on the data presented in this study, we conclude that the level of DC infiltration correlates with the magnitude of T cells infiltrating the site, and hypothesize that the level of DC recruitment is determined by the frequencies of antigen-specific T cells in the donor. An immune-compromised T cell compartment could

therefore lead to insufficient DC mobilization, which may further attenuate the cellular responses by limiting antigen-presenting capacity at the site.

Several studies have demonstrated that there is a clear loss of blood PDCs and MDCs during HIV-1 infection. In contrast, there is some controversy regarding functional deficiencies of DCs in HIV-1 infection. In order to study DC function *in vivo* during HIV-1 infection, we used the antigen skin test as a model to evaluate DC mobilization following antigen exposure and inflammation. In saline-injected control skin, we did not observe any difference in the numbers of DCs between HIV-1+ individuals and seronegative individuals. There was thus no clear depletion of skin-resident DCs as described for circulating DCs during HIV-1 infection. This is supported by data from larger cohorts of patients that have shown that HIV-1 disease progression is not associated with a decline in epidermal Langerhans cell numbers [34]. Skin DCs are susceptible to HIV-1 infection [35, 36] but do not likely represent a significant viral reservoir [37, 38]. In the skin test model studied here, the infiltrating DCs are presumably mobilized from the blood into the skin. Although data on blood DC counts in our cohorts were not available, it is likely that the HIV-1+ asymptomatic individuals had a mild reduction of blood DC numbers and the AIDS patients had a quite pronounced depletion according to previous reports [13, 39]. We detected lower numbers of infiltrating DCs in response to PPD and mumps injections in the HIV-1+ individuals, including those with AIDS, compared to the healthy controls. In marked contrast, the DC infiltration in response to *C. albicans* was similar or higher in people with HIV-1, indicating that DC numbers and their mobilization capacity were sufficient at least in the individuals with chronic asymptomatic HIV-1 infection. We interpret the lower infiltration of DCs in response to PPD and mumps than to *C. albicans* in the HIV-1+ subjects to be a consequence of the respective levels of memory T cell responses to these antigens. HIV-1+ individuals have been shown to have higher antigen recall T cell responses [40, 41] and larger skin indurations to *C. albicans* compared to PPD and mumps [42]. The fact that an individual can show different levels of T cell recruitment depending on the type of skin antigen test might explain why DTH anergy has been shown to not necessarily correlate to CD4 count or degree of viremia [43]. The integrity of the memory T cell compartment specific for a given antigen is probably the more important determinant for the ability to respond to a skin test. In our study, even the AIDS patients who had severely reduced CD4 counts were able to mount a DTH response in the *C. albicans* test. As it is a commensal fungus, the host is constantly exposed to *C. albicans* antigens. In HIV-1 infection, the associated compromised integrity of the gut may facilitate translocation of *C. albicans* as has been shown for endotoxin [44]. In fact, fungal cell wall products [45, 46] as well as *C. albicans*-specific antibodies [47] are elevated in the serum of HIV-1+ individuals. This constant exposure to *C. albicans* may maintain the elevated T cell responses and explain the increased levels of infiltrating CD3+ T cells that we observed in response to the *C. albicans* skin test in the HIV-1+ group compared to the controls. In contrast, weaker T cell responses in HIV-1+ individuals have been described for several viral antigens compared to *C. albicans*-responses [30]. Also, it has been shown that PPD-specific T cells are preferentially depleted in HIV-1 infection compared to T cells specific for other antigens, which could contribute to lower T cell accumulation at the TST site [48]. On another note, we cannot rule out that the differential DC and T cell recruitment to the

different skin antigen tests also relates to different kinetics of their respective immune responses. Analyses of biopsies taken at other time points would provide valuable information on this. However, these tests are clinically well-validated with a common evaluation time point at 48 hours which is a time when the DTH indurations peak.

In conclusion, our data, based on limited numbers of study subjects, indicate that there is no obvious depletion or deficiency in mobilization of DCs to antigen skin tests in individuals with chronic HIV-1 infection as long as T cell responses are present. In non-human primate models, DC mobilization from skin to lymph nodes has been shown to be intact in early SIV infection but impaired in AIDS which restricted the numbers of antigen-bearing DCs reaching the lymph nodes and in turn hampered stimulation of antigen-specific T cells [49]. A depleted or deficient T cell compartment, as in AIDS, may compromise the stimulation of DCs to either be recruited to the antigen skin test site or further mobilize to lymph nodes to present antigens to T cells. Therefore, even if DCs are not a cell type that is directly affected by HIV-1 to any great extent, a collapsing immune milieu will render DCs inept, which further intensifies the cellular immune deficiencies.

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Figure 1. PPD injection leads to infiltration of multiple DC subsets

Cryosections of donor-matched skin punch biopsies taken from sites injected with PPD 48 hours earlier. (A) Representative images of DC markers from donor-matched saline and PPD injected sites from healthy as well as asymptomatic HIV-1+ individuals are shown. Specific staining is shown in brown and cell nuclei counterstained with hematoxylin in blue. Note that the dark basal membrane represents melanin-rich cells and not positive staining. Bar graphs (B) show compiled data (mean $\pm$ SEM) of all donors in the cohort. Note that biopsies from the PPD site only were collected from the AIDS cohort. P+ and P- show data

obtained from AIDS patients with positive and negative TST reactions, respectively. Data are from image analysis of dermis only, except for Langerin where only epidermis was analyzed.

Figure 2. Recruitment of CD3+ T cells with increased cell cycling activity to the TST site in HIV-1 infected individuals correlates with level of DC infiltration

Images show immunofluorescent stainings visualizing co-localization of CD11c+ MDCs (A) and CD123+ PDCs (B) with CD3+ T cells in the dermis of the TST site in a healthy individual. (C) The level of infiltrating CD3+ T cells to the TST site was higher in the seronegative controls compared to the HIV-1+ cohorts. Bar graph shows compiled data from all donors (mean $\pm$ SEM). (D-E) Correlation analyses of the levels of DC markers with the level of CD3+ T cells in the TST area of the different study cohorts – healthy controls (open circles), HIV-1+ asymptomatic individuals (grey dots) and subjects with AIDS (black dots). (F-G) Immunofluorescent co-stainings visualizing co-localization of CD3 (green) and CD4 (red, left panel) and CD8 (red, right panel) in the TST reaction of a healthy individual. (H) Bar graph shows the levels of Ki67+ cycling cells (mean $\pm$ SEM) of compiled data from all donors. Note that biopsies from the PPD site only were collected from the AIDS cohort. P+ and P– indicate data obtained from AIDS patients with positive and negative TST reactions, respectively. (I) Representative images of Ki67 (brown) staining, by immunohistochemistry, in the dermis of the TST site of a healthy and an HIV-1+ asymptomatic individual. (J) Image demonstrates that the main proportion of Ki67+ (red) cells was CD3+ (green) T cells.

Figure 3. Recruitment of DCs and T cells in response to injection of mumps and *C. albicans* antigens

The bar graphs (A-G) show compiled data (mean $\pm$ SEM) of the expression levels of the indicated DC and T cell markers at the injection sites receiving mumps (MU – gray bars) and *C. albicans* (CA – black bars) skin tests and in donor-matched control sites (white bars) in the healthy controls, HIV-1 asymptomatic as well as AIDS patients cohorts.

Patient ID	Age (years)	Sex	CD4 T-cell count (cells/ μ l blood)	Plasma viral load (copies/ml plasma)	PPD induration size (mm)
Control group					
D07	43	F	NA	NA	23
D14	41	F	NA	NA	16
D15	47	F	NA	NA	16
D19	33	F	NA	NA	19
D25	21	M	NA	NA	17
D26	29	M	NA	NA	16
D28	23	M	NA	NA	21
D30	22	M	NA	NA	18
HIV-1-positive asymptomatic					
D02	28	F	972	NA	16
D16	37	M	547	NA	17
D22	24	M	466	NA	29
D29	31	M	435	NA	12
D33	32	F	475	NA	22
D35	40	M	578	NA	12
AIDS					
D32	27	F	149	NA	17
D36	27	F	125	NA	22
D38	44	F	164	NA	0
D39	30	F	161	NA	0

Patient ID	Age	Sex	CD4 T cell	Plasma viral load	Mumps induration size	<i>Candida albicans</i> induration size
Control group						
07	37	M	NA	NA	23	10
08	39	F	NA	NA	16	18
10	25	F	NA	NA	12	6
11	40	F	NA	NA	8	6
HIV-1-positive asymptomatic						
01	40	M	590	157	8	15
D2	34	M	650	1193	13	12
09	35	M	830	3650	8	12
AIDS						
05	44	M	40	>750 000		6
12	37	M	14	>750000		12

F, female; M, male; NA, not analyzed.