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EXPRESSION OF CD4 ON HUMAN PERIPHERAL BLOOD NEUTROPHILS

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CD4, the primary receptor for entry of HIV, is known to be expressed on T cells and monocytes/macrophages, normal NK lymphocytes, *in vitro* HHV6-infected CD8+, NK, and γδ T lymphocytes, CD34+ progenitor cells and a subset of eosinophils and basophils. We here report the unconventional expression of CD4 at the surface of peripheral blood neutrophils derived from 4/51 (7.8%) HIV-1 infected and 3/25 (12%) uninfected donors, with similar frequency within the two groups. The percentage of CD4+ neutrophils ranged from 39 to 97% of the total neutrophil population. Both surface and cytoplasmic forms of CD4 were present in neutrophils. Quantitative RNA PCR revealed that neutrophils contain levels of CD4 mRNA comparable to those of peripheral blood mononuclear cells derived from the same donor. The conformation of CD4 expressed at the surface of neutrophils was similar to that of CD4 expressed on T lymphocytes as determined by the binding of monoclonal antibodies specific for conformational epitopes and the binding of recombinant HIV-1 gp120. Thus, our data provide evidence that neutrophils express endogenous CD4 and bind HIV. Owing to their abundance in peripheral blood, CD4+ neutrophils may influence significantly the bio-distribution of HIV delivering it to sites of inflammation or to additional tissue reservoirs.

INTRODUCTION

CD4 was originally identified as a T lymphocyte marker and specifically associated to the subset of helper T lymphocytes. In addition to its function as a ligand of MHC Class II molecules[1], CD4 was defined as the primary receptor of HIV viruses[2]. Binding of HIV envelope to CD4 is the first event which allows its binding to other coreceptors, namely the chemokine receptors CCR5 and CXCR4, followed by membrane fusion and entry[3,4]. CD4 is also expressed on monocytes/macrophages[5]. In addition, further studies have reported the expression of CD4 on cells other than helper T lymphocytes and monocytes, including eosinophils[6], CD34+ progenitor cells[7,8], *in vitro* HHV-6-infected CD8+[9], NK[10], and γδ T lymphocytes[11], mast cells/basophils[12] and normal NK lymphocytes[13]. To our knowledge the presence of CD4 on peripheral blood neutrophils has not yet been documented.

Traditionally, flow cytometry-based phenotyping is performed either on separated peripheral blood mononuclear cells or, in routine diagnostic investigation, on whole blood lymphocytes selected by morphologic gating, therefore information regarding neutrophils is not gathered nor recorded. In our Clinic we have set up whole blood based flow cytometry protocols which analyze all leukocyte populations.

By this mean we identified an HIV-infected patient (Pt#1) whose neutrophils were unexpectedly stained by a CD4-specific mAb. We therefore: (i) attempted a preliminary evaluation of the frequency of individuals whose neutrophils display CD4, (ii) confirmed the specificity by staining with mAb directed towards different conformational epitopes of CD4, (iii) tested whether CD4+ neutrophils could bind soluble HIV-1 envelope protein gp120 and (iv) tested the endogenous origin of CD4 in neutrophils.

MATERIALS AND METHODS

Patients.

Fifty-one HIV-infected individuals were tested among patients with chronic HIV infection regularly followed at our Infectious Diseases Clinic. Three out of the four HIV-infected patients who displayed CD4+ neutrophils had significant levels of HIV replication (> 10000 HIV RNA copies/ml) in the presence of antiretroviral treatment. One of these three patients (Pt#1) was resistant to several classes of anti-retroviral drugs; the fourth patient had plasma viremia below the detection level (< 80 copies/ml). All four patients had CD4 cell counts >500. Control individuals were healthy laboratory workers.

Cellular purifications

Whole blood was drawn by venipuncture in EDTA-containing Vacutainer (BD Biosciences Labware) tubes and immediately processed for flow cytometry staining and cellular purification. PBMC were obtained (floating ring) by gradient density centrifugation (Lyphoprep Nycomed, Oslo, Norway); purified neutrophils were prepared by recovering the white blood cell layer over the red blood cells (RBC) after the gradient centrifugation and allowing a 30 min sedimentation on a 4% dextran solution at room temperature followed by lysis of contaminating RBC with NaCl solution (1.2%+0.2%). Extensive washing of both PBMC and neutrophils was performed with 1X phosphate buffered saline (PBS). Purity of the neutrophil preparation was controlled by flow cytometry (CD3neg, $CD14^{dim}$+ and CD66b+) and found to be ≥98.0%. Purified populations were used either for flow cytometry, immunofluorescence, or dry-pelletted ($1x10^6$ cells/tube) and frozen at –80 °C for CD4 mRNA quantification. For the HIV-infected Pt#1 besides PBMC and neutrophils also CD14+ monocytes and CD8-depleted lymphocytes (CD4-enriched) were prepared by sequential magnetic bead (Dynabeads,

Oxoid S.p.A., Garbagnate M.se, Italy) purification following the manufacturer's directions. Each cell population was dry-pelletted ($1x10^6$ cells) and frozen at –80 °C for HIV-1 DNA load quantitation.

Flow cytometry

Whole blood (100 µl/tube) cells were stained with fluorochrome directly conjugated mAb: phycoeritrin (PE)-anti- CD8 (OKT8, mouse (m) IgG2a) (Ortho Diagnostic Systems Inc., Raritan, NJ), Tri-color (TC)-anti- CD4 (clone S3.5, mIgG2) (Caltag Laboratories, Burlingame, CA), FITC-anti- CD66b (clone G10F5, mIgM) (Pharmingen BD), as well as with fluorochrome- and isotype-matched controls. After 30 min incubation at 4°C, cells were fixed and RBC lysed with the Cal-Lyse lysing solution (Caltag). Staining of purified PBMC and neutrophils was performed with the unconjugated OKT4, 13B8-2 and C9F11 mouse mAb followed by FITC-conjugated rabbit anti-mouse IgG (Dako S.p.A.) and fixed with 1% formaldehyde. Only the second step reagent was added to control tubes. Stained samples were acquired with a FACscan cytometer and analyzed by Cell Quest and Lysis softwares (BD Biosciences).

Immunofluorescence

PBMC and neutrophils were fixed for 15 min at 4°C with 4% paraformaldehyde in 125mM PBS and washed with the same buffer. The cells were then treated for 30 min with a solution containing 0,3% Triton X-100, 15% filtered goat serum, 0,45 M NaCl, and 10 mM phosphate buffer, pH 7,4. After washing, the preparations were exposed (90 min at 37°C) to the primary Ab (non conjugated anti-CD4, clone SK3, mIgG1, BD) diluted in the above Triton X-100 and goat serum-containing solution. After an additional thorough wash, the cells were treated with the appropiate rhodamine-labeled goat anti-mouse Ab (1:100 dilution in the Triton X-100-goat serum solution, 60 min, 37°C), washed again, and mounted in glycerol to be examined with a laser scanning confocal microscope (MRC 1024, Bio-Rad House).

Quantitation of CD4 mRNA

Total RNA from PBMC and neutrophils pellets was extracted, quantified, and reverse transcribed for generating cDNA using MultiScribe Reverse Transcriptase from the TaqMan Reverse Transcription Reagents (PE Biosystems). A cDNA amount corresponding to 200ng total RNA was amplified. Quantitative real-time PCR was performed using the SYBR Green PCR Master mix (PE Biosystems) and 300 nM gene specific forward and reverse primers designated using the PrimerExpress software (Applied Biosystems, Foster City, CA); CD4: for 5' - CTG GCC CTT GAA GCG AAA A - 3, rev 5' – CCA CCA GGT TCA CTTCCTGATG-3'; β-actin: for 5' – CCC AAG GCC AAC CGC GAG AAG AT- 3', rev 5' – GTC CCG GCC AGC CAG GTC CAG - 3'. The reaction conditions were 10 min at 95°C (one cycle) and 15 sec at 95°C and 1 min at 60°C (40 cycles). Gene-specific products were continuously measured by means of an ABI PRISM 5700 detection system (Perkin Elmer, Norwalk, CT). Samples were normalized using the housekeeping gene β-actin. Three replicates for each experimental point were performed, and differences were assesed with the two-tailed Student's *t* test.

Cell-associated viral load

Proviral HIV-1 DNA load was measured in purified PBMC, neutrophils, CD14+ monocytes and CD8-depleted lymphocytes of Pt#1 using an in-house Taqman “Real-Time” PCR assay specifically developed to accurately quantitate all clades belonging to the HIV-1 M group (Scarlatti et al., manuscript in preparation). Primers and probe were derived from highly conserved sequences placed in the LTR-gag junction. The cellular content of each sample was evaluated by a second in-house Taqman “Real-time” PCR assay developed with a highly conserved fragment (from nucleotide +3175 to nucleotide +3255) of the single-copy human CCR5 gene. Three replicates for each experimental point were performed in both PCR assays.

Reverse-transcriptase (RT) activity

The virion-associated RT activity was measured in culture supernatants by a home-made radioactive assay, as previously described[14].

p24 enzyme linked immunosorbent assay (ELISA)

Content of the HIV-1 p24 core antigen was evaluated by an in-house ELISA, as previously reported [15].

RESULTS

Dual color staining with CD4 and CD8 mAb of whole blood derived from an HIV-infected patient (Pt#1) revealed an unexpected high proportion (51%) of CD4+ neutrophils in their morphologic gate (Fig. 1).

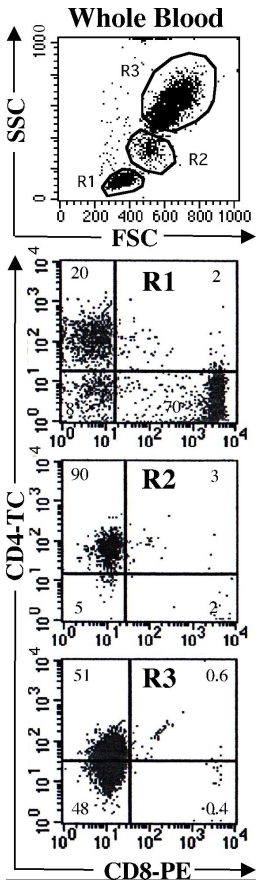

Fig. 1.

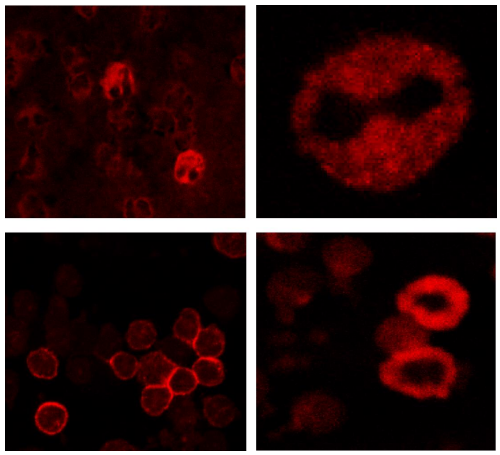

Fig. 2

The first panel displays the morphologic parameters forward scatter (FSC) and side scatter (SSC) of whole blood leukocyte populations. Three morphologic gates are drawn which encompass lymphocytes (R1), monocytes (R2) and granulocytes (R3). The mean fluorescence intensity (MFI) of lymphocytes, monocytes and neutrophils was 166, 37 and

24, respectively. The patient had a normal haematocrit with no signs of eosinophilia or basophilia with >95% of granulocytes being neutrophils.

Immunofluorescence staining with a CD4 mAb on purified neutrophils and PBMC from Pt#1 is shown in Fig. 2. Neutrophils were identifiable by the typical drum-stick morphology of their nucleus. Staining was performed on permeabilized cells and revealed both membrane and intracellular CD4 molecules in neutrophils. The intensity of fluorescence was higher in PBMC compared to neutrophils.

To evaluate the frequency of subjects with CD4+ neutrophils, we screened a group of HIV-infected and uninfected individuals for the presence of cells which double stain with CD4 and a mAb to the antigen CD66b. CD66b is a selective marker of neutrophils[16] which also in our experiments was found consistently negative in lymphocytes and monocytes (not shown). This screening system was selected to exclude from our analysis cells which could contaminate the flow cytometric neutrophil gate. Four out of 51 (7.8%) HIV-infected individuals and three out of 25 (12%) uninfected controls displayed a proportion of CD4+CD66b+ cells ranging from 39 to 97% of the total neutrophil population. Fig. 3 shows the data obtained from two HIV-positive and two HIV-negative individuals who were either negative or positive for the expression of CD4 on CD66b+ neutrophils. The average MFI was 15.3 (±5,5 SD), with the exception of the control individual shown in Fig. 3 which displayed a much higher value (MFI 180). Thus, the expression of CD4 on neutrophils is not restricted to HIV-infected individuals but can also be observed in uninfected individuals. The frequency of individuals displaying CD4+ neutrophils was not significantly different between the two groups as determined by the chi square test ($p=0.868$).

The possibility that the CD4+ cells detected within neutrophils were contaminating monocytes or lymphocytes was ruled out for the following reasons: (1) the proportion of CD4+ cells within the neutrophil gate was too high to be derived from cell populations other than neutrophils, (2) the MFI of CD4+ neutrophils was consistently

different from that of lymphocytes and monocytes, (3) CD4 was detected at the surface of CD66b+ cells, (4) CD4 could be directly visualized in neutrophils by immunofluorescence.

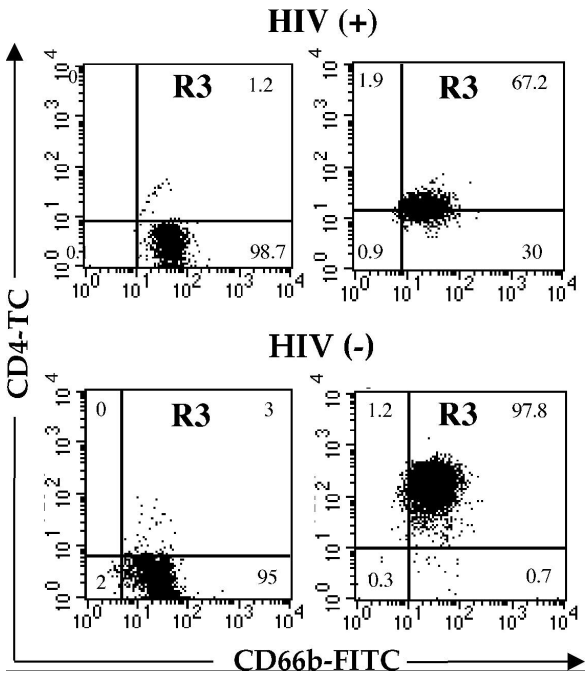

Fig. 3

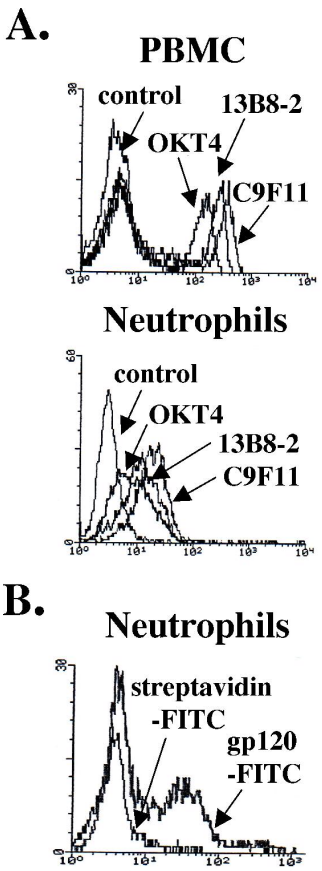

Fig. 4

To rule out that binding of the CD4-specific mAb was due to a CD4-mimicking epitope present on an unrelated molecule we tested three mAb specific for different CD4 conformational determinants: OKT4 recognizes domain 3 and 4[17], 13B8 binds the CDR3-like region of domain 1[18] and C9F11 recognizes domain 1 and 2 (S. Burastero, unpublished observations). All three mAb, tested on neutrophils and PBMC derived from the same HIV-uninfected donor who had CD4+ CD66b+ neutrophils (Ctrl #1), bound both PBMC and neutrophils (Fig. 4A). Also in this case the MFI was higher for

PBMC compared to neutrophils. The patterns of mAb binding to the two cell populations were very similar with C9F11 and 13B8 being more reactive than OKT4. Thus, the molecular conformation of CD4 expressed at the surface of neutrophils appears to be identical to that of CD4 expressed on PBMC. Furthermore, CD4 on neutrophils was cleaved by the enzyme pronase which is known to specifically cleave CD4, CD8 and CD28 on the surface of lymphocytes [19] (data not shown). We then tested the ability of purified neutrophils from Ctrl#1 to bind the HIV-1 envelope surface unit, gp120. The experiment was performed with a fluorescein-isotiocyanate (FITC)-labelled recombinant HIV-1 gp120 derived from the T cell line adapted strain HIV-1_{LAI}. To control for non-specific binding we used FITC-conjugated avidin. As shown in Fig 4B, there was a bimodal pattern of staining with 37% of cells which stained positive (MFI = 83) and a second population which displayed non-specific staining overlapping that of streptavidin-FITC. The high proportion of neutrophils that bound HIV-1 gp120 ruled out potential binding by lymphocytes or monocytes contaminating the neutrophil preparation.

To prove that neutrophils express endogenous CD4 we tested the presence of CD4 mRNA by "Real-Time" PCR in purified neutrophils and PBMC derived from one donor displaying CD4+ neutrophils (Ctrl#1) and one donor with CD4 negative neutrophils (Ctrl#2) (Fig.5). The data are expressed as the relative fold increase/decrease of CD4 mRNA in neutrophils versus PBMC, used as internal calibrator. Neutrophils displayed levels of CD4 mRNA which were similar to the ones detected in PBMC, consistent with the capacity of these cells to synthesize CD4. Surprisingly, also the individual with CD4 negative neutrophils (Ctrl#2) diplayed similar amounts of CD4 mRNA, suggesting that the regulation of CD4 expression in this cell lineage may be under translational control. Similar levels of CD4 mRNA was found in other two purified neutrophil populations from uninfected individuals.

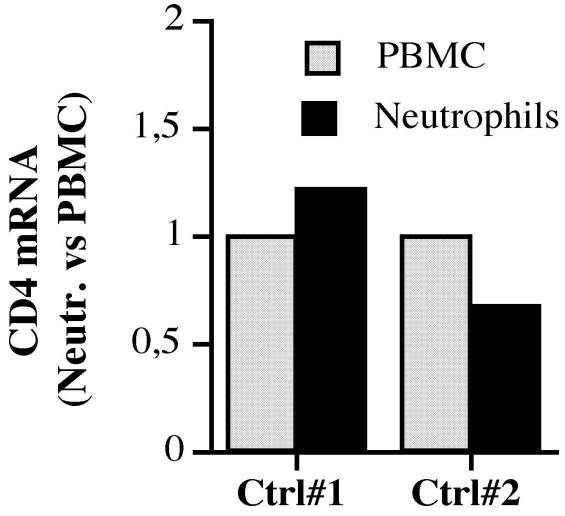

Fig. 5

Among the different chemokine receptors expressed at the surface of neutrophils, CXCR4, one of the HIV entry co-receptors[3,4] is expressed constitutively[20]. Thus, CD4+ neutrophils express the molecular machinery required for entry of HIV viruses which use CXCR4, (X4 viruses)[21]. We tested whether neutrophils derived from Pt#1 were infected *in vivo* by cocultivating them with allogeneic phytohaemagglutinin(PHA)-activated blasts. Unfractionated PBMC, positively selected CD14+ monocytes and CD4+ T cells enriched (CD8-depleted) populations were each cocultured (1:1 ratio) with the same blasts as controls. The HIV structural p24 antigen and reverse transcriptase (RT) activity were measured in culture supernatants. We consistently failed to rescue replicating virus from cocultures containing unfractionated PBMC, monocytes and neutrophils by both RT and p24 assays. In contrast, the CD4 T cell enriched population yielded positive results (771 cpm/µl of RT activity and 7524 pg/ml of p24 Ag at day 12 of culture). We also looked at the cell-associated HIV-1 DNA load in the four cell populations using a quantitative Real-Time PCR and found a detectable amount of HIV DNA in neutrophils and monocytes (59 and 57 genome equivalent x 10^6 cells, respectively). In comparison, the amounts of HIV-1 DNA measured in PBMC and the CD8-depleted population were

ten fold higher. Although the granulocyte preparation used in these experiments was >98.0% pure, we cannot rule out the possibility that the low levels of HIV DNA detected in this cell population was derived by contaminating CD4+ mononuclear cells. Kaneda et al. previously reported the presence of defective HIV-1 provirus in peripheral neutrophils derived from an HIV-infected patient[22]. Thus, the question of whether neutrophils are infected by HIV *in vivo* deserves further investigation.

DISCUSSION

Taken together our data show that: (i) a large fraction of peripheral blood neutrophils of 4/51 HIV-infected individuals and 3/25 uninfected controls express surface CD4, (ii) the molecular conformation of neutrophil-expressed CD4 is very similar to that of CD4 expressed at the surface of lymphocytes, (iii) CD4+ neutrophils bind HIV-1 gp120, (iv) neutrophils express CD4 mRNA consistent with CD4 being directly synthesized by these cells. This is to our knowledge the first report on the expression of CD4 on neutrophils.

Comparable levels of CD4 mRNA in purified neutrophil populations were found in the two donors expressing CD4+ and CD4- neutrophils. The possibility that this could be due to contamining residual lymphocytes or monocytes was ruled out since our neutrophil preparations were consistently 97-98% CD66b positive. Thus 2-3% of contaminating lympho-monocytic cells, which include also CD8+ lymphocytes, cannot account for the amount of CD4 mRNA derived from neutrophils which was similar to that derived from PBMC (which encompass more than 70% CD4+ lympho-monocytes in normal donors). Rather, the presence of CD4 mRNA in neutrophils which do not express CD4 at the surface suggest the existence of mechanisms which act at the translational level. The hematopoietic CD34+ progenitor cell also expresses CD4 at the surface[7,8]. Thus, the CD4 gene within the myelomonocitic lineage, which ultimately gives rise to CD4+ monocytes and CD4- neutrophils, most likely undergoes mechanisms regulating its expression or suppression.

Our report raises the question of whether CD4+ neutrophils may have a particular functional phenotype. In this regard, we have investigated other cell surface markers finding that a particular chemokine receptor, CXCR3, was also concomitantly elevated on neutrophils in those individuals with CD4+ neutrophils (P. Biswas et al., unpublished observations). CXCR3, together with CCR5, is a major receptor for inflammatory

chemokines [23,24]. Thus, it is possible that neutrophils expressing unusual surface markers such CD4 and CXCR3 may be endowed with particular migratory patterns towards inflammation sites.

The presence of CD4+ neutrophils in the peripheral blood of healthy uninfected controls indicated that this unusual expression of CD4 is not a consequence of HIV infection but rather pre-exists infection. It will be interesting to determine whether the presence of CD4+ neutrophils may have some influence on the course of HIV infection and on disease progression. Differently from the other unconventional CD4+ cell populations described so far, neutrophils are by far the most abundant among peripheral leukocytes and may influence significantly the spread of HIV. We have provided evidence that CD4+ neutropìhils bind the HIV envelope surface unit gp120. Therefore, CD4+ neutrophils may bind HIV and play an active role in its propagation, similarly to that of DC-SIGN positive dendritic cells which have been shown to promote infection of bystander cells without being themselves infected[25]. In addition, neutrophils constitutively express CXCR4 and may selectively bind X4 viruses, which are generally associated to loss of CD4 T lymphocytes and disease progression, favouring the emergence of these viruses.

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Figures legend

Fig. 1 Detection of CD4 on whole blood leukocyte populations derived from an HIV-infected individual (Pt#1). The first panel displays the morphologic parameters forward scatter (FSC) and side scatter (SSC) of whole blood leukocyte populations. Three morphologic gates are drawn which encompass lymphocytes (R1), monocytes (R2) and granulocytes (R3). The following panels show the fluorescence intensities of each gated population upon dual staining with CD4 and CD8 mAb. The numbers indicate the % of positive cells in each quadrant; the quadrant markers are set on the fluorescence intensities of the isotype-matched control cells within each gate.

Fig. 2 Staining of neutrophils and lymphocytes with a CD4-specific mAb. Immunofluorescence with CD4 mAb on permeabilized purified neutrophils (upper panels, lower and higher magnifications) and PBMC (lower panels, lower and higher magnifications) from Pt#1.

Fig. 3 Detection of CD4 on CD66+ cells. Flow cytometric analyses of whole blood from two representative HIV-infected (first two panels) and two uninfected (last two panels) individuals upon dual staining with CD4 and CD66b mAb. Shown are the fluorescence intensities within morphologic gate R3 (neutrophils). Ten percent of neutrophils from one of the uninfected donors resulted positive also upon staining with another directly conjugated anti-CD4 mAb (anti-CD4-PE, clone SK3, mIgG1, BD Biosciences).

Fig. 4 CD4 epitope expression on neutrophils and PBMC and neutrophil binding of HIV-1 gp 120. A. Histogram plots depicting overlayed

fluorescence intensities of purified PBMC and neutrophils from an HIV-uninfected control after indirect staining with the anti-CD4 mAb OKT4, 13B8-2 and C9F11. The control histogram derives from cells stained with second step reagent only. **B.** The histogram plots show the fluorescence intensities of purified neutrophils stained with fluorochrome (FITC) directly conjugated gp120; as control neutrophils were stained with streptavidin-FITC.

Fig. 5 Comparison of CD4 mRNA content in neutrophils and PBMC. Levels of CD4 mRNA in purified PBMC and neutrophils from uninfected Ctrl#1 (displaying CD4+ neutrophils) and Ctrl#2 (displaying CD4 negative neutrophils) was determined by "Real-time" PCR assay. The amount of CD4 mRNA in autologous PBMC is used as internal calibrator in each donor. Purified PBMC and neutrophils from Ctrl#1 prepared in an independent experiment yielded similar results in terms of CD4 mRNA content (not shown).

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Expression of CD4 on human peripheral blood neutrophils

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