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The role of IL-15 in challenging Acquired Immunodeficiency Syndrome

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ABSTRACT

Objective: To determine the functions of in vitro primed Natural Killer (NK) cells in Human Immunodeficiency Virus (HIV-1) infection and the role of IL-2, IL-12 and IL-15 in enhancing the NK survival and activity in terms of viral suppression and of purging of HIV provirus.
Methods: Peripheral Blood Mononuclear Cells (PBMCs) and CD4+ T lymphocytes cells obtained from eight healthy donors were infected in vitro with HIV-1 and p24 was measured with and without IL-2, IL-12 and IL-15. We studied the effect of NK pulsed in vitro with IL-2, IL-12 and IL-15 on HIV replication by measurement of p-24 and DNA-provirus load when added into the culture of PBMCs and CD4+ T lymphocytes cells infected in vitro. We evaluated the effect of NK cells pulsed with IL-2, IL-12 and IL-15 on HIV replication and DNA-load into the culture of CD4+ T lymphocytes cells and PBMCs by trans-well chamber.
Results: We found high levels of p24 in the supernatants of PBMCs and CD4+ T lymphocytes cells cultured with IL-2, IL-12, and IL-15. We observed a significant reduction of p24 in the culture both of infected PBMCs and CD4+ T lymphocytes cells in which was added NK pulsed with IL-15. We did not obtain the some results with NK pulsed with IL-2 and IL-12. We observed a power effect of NK pulsed with IL-15 on HIV-DNA. The trans-well chamber experiments showed that the effect of NK is both direct and both mediated by realizing of soluble factors.
Conclusions: This study highlights some important effects of IL 15 on NK in HIV patients anyway our results are preliminary and descriptive and others studies will be needed to provide rationale for immune therapies.

1. Introduction

The development of new antiretroviral drugs and therapeutic strategies has permitted to obtain the important goals in interfering with HIV replication and in preventing the progression to Acquired Immunodeficiency Syndrome (AIDS) [1,2]. Despite much progress, highly active antiretroviral therapy (HAART) is associated with several side effects and has been shown to be not effective in completely controlling the viral replication or purging the viral reservoirs or restoring the host immune functions; because these limitations of HAART the immunotherapeutic approaches are an attractive field to develop strategies to fight HIV infection [3]. Many studies have provided the importance of adaptive immune response to contrast both viral infections and the wide avoidance strategies implemented by the same virus [4]. The role of innate immune system in HIV-1 infection has been less studied than the adaptive one but it is increasingly considered to be crucial in controlling persistent infections: it plays an important role in the early effector functions because most of its components are at mucosal sites and is essential for activation and maintenance of adaptive immune functions. For this reason approaches to enhance the innate immune activity can be valuable in therapy for HIV infection [5]. NK cells act both as effectors cells of the innate immune system and as immune regulatory cells bridging the innate and adaptive immune system [6–8]. During HIV-1 infection it is possible to observe different impairments of the innate arm of the immune system [9]. Particularly global NK cell functions are reduced by viral replication and moreover the loss of NK cell function could contribute to both the reduction of viral control by the immune system and the progression to AIDS [10,11]. The innate immune system is the first line of defence to microbial entry and without this system, the adaptive immune system would not have developed [12]. Moreover because of its tissue distribution and rapid activation, the innate immune system could potentially yield a unique role in the prevention of HIV-1 transmission and could provide the time needed for the development of a correct and strong adaptive immune response [13]. The crucial role of NK in the early

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host defense against HIV in part depends on interleukin (IL)-15 [14]. IL-15 is a pleiotropic cytokine that can play an important role in the bridging between the innate and adaptive immune system. In addition, IL-15 plays an obligate role in the proliferation and survival of NK cells. In fact, it is known that IL-15 could regulate the survival of different subsets of NK cells by several steps. Moreover, different studies have found that IL-15 acts on NK cells promoting cell development from its precursors providing a required signal for the survival of mature NK cells [15–18] and activating the cytotoxicity of human NK cell subsets. The recovery of normal functions of NK could be useful to increase HIV-specific antibody-dependent cellular cytotoxicity (ADCC) function and reduce the viral replication [19]. Many in vitro studies in human PBMC and in vivo studies in Simian Immunodeficiency Virus (SIV) infected monkeys have shown that IL-15 is able to increase the function, proliferation and survival of NK cells and has a positive effect on the generation and maintenance of CD8+ T cells [20]. To further extend the current knowledge on the virological interactions between IL-15, HIV and NK cells, we have undertaken the present study with the following goals: to evaluate the effects of NK co-cultured with HIV infected PBMCs and CD4+ T lymphocytes after the stimulation with IL-2 or IL-12 or IL-15, on HIV replication both by measurement of p-24 and HIV DNA.

2. Methods

2.1. Cell culture conditions

PBMCs were obtained from eight healthy donors and separated by Ficoll–Hypaque gradient centrifugation. Untouched CD4+ T lymphocytes cells were obtained from human PBMCs using the human CD4+ T-cell isolation kit II (Miltenyi Biotec, Italy) and the purity of isolated CD3+ CD4+ cells was >97%. Natural Killer cells were isolated via a magnetic cell sorter by using NK cell isolation kit II (Miltenyi Biotec, Italy) according to the manufacturer's protocol. The purity of isolated CD3–CD56+ NK cells was >95%.

PBMCs and isolated CD4+ T lymphocytes cells were stimulated with 3 μg/mL phytohaemoagglutinin (PHA, Sigma) for 3 days and incubated with 1000× $TCID_{50}$ of HIV-1 89.6 (X4R5) (obtained from AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) for 3 h at 37 °C; after three washes to remove the unbound virus, infected PBMCs and CD4+ T lymphocyte cells were resuspended at 2×10^6 cells/mL in complete T-cell medium, consisting of RPMI 1640 medium (EuroClone, Italy) with 10% heat-inactivated foetal bovine serum (FBS, EuroClone, Italy), 2 mM glutamine (Sigma, Italy), 100 μg/ml Streptomycin, 100 U/ml penicillin (Sigma, Italy); infected cells were cultured for 72 h with medium (control), recombinant human interleukin-2 (IL-2; 20 U/ml), interleukin-15 (IL-15; 20 ng/ml) or interleukin 12 (IL-12; 20 ng/ml) (PeproTech EC, UK) alone or in presence of NK cells previously cultured for 3 days in the same conditions. CD4+ T lymphocytes cells (2×10^6) were cultured with NK cells at ratios 4:1 and 2:1 in 24 wells plate; PBMCs (2×10^6) were cultured with NK cells at ratios 10:1 and 4:1 in 24 wells plate. All culture conditions were carried out in triplicate. On day 3 all the medium was replaced by fresh culture medium containing or not the cytokines; after 24 h supernatants were collected and tested for HIV-1 p24 antigen by ELISA method (Innogenetics, Belgium). PBMCs and CD4+ cells were collected for further assays [21].

2.2. Trans-well culture

For each experiment regarding CD4+ T lymphocytes cells a further condition was evaluated: NK cells were physically separated from infected CD4+ T lymphocytes cells by a trans-well membrane (0.45 mm) 24-well insert (Costar, Cambridge, MA, USA). CD4+ cells (2×10^5) were placed into the bottom chamber; NK cells (5×10^5 or 1×10^6) on the top. Supernatant and cells were collected as described above [22].

2.3. Quantification of HIV-DNA

Before processing the cultured PBMCs and CD4+ cells for HIV DNA quantification, the NK cells were removed by using anti CD56 antibody coupled to magnetic beads according to manufacturer's instructions (Miltenyi Biotech, Italy). DNA was extracted from PBMCs and CD4+ T lymphocytes cells using the QIAamp DNA Blood Mini kit (Qiagen, Italy); in each extraction were included PBMCs and CD4+ T lymphocytes cells not infected as negative controls.

The HIV-1 DNA load was measured by a real-time PCR assay using the ABI Prism 7500 real time PCR System (Applied Biosystem, Foster City, CA) for PCR amplification, acquisition and data analysis. HIV-1 DNA copy number was determined using *gag* primers and FAM-MGB probe and normalized to the amount of cellular DNA by quantification of RNAseP copies (RNAseP Control Reagents, Applied Biosystem). All samples and HIV-1 negative controls were run in triplicate and the normalized value of HIV-1 DNA load was expressed like number of HIV copies/10^6 cells. The 8E5 cell line, a T lymphoblastoid cell line that contains a single defective genome copy of HIV per cell, was used to generate a standard curve for HIV-1 DNA and RNAseP P quantification. HIV-1 *gag* and RNAseP standard curves had slopes between −3.35 and the coefficients of correlation was >0.987. The limit of detection was 2 copies/10^6 cells. PCR HIV DNA standard curve from 1 to 50 copies was included in each experiment as control (AIDS Research and Reference Reagent Program, DAIDS, NIAID, NIH: PCR Panel 001 from Dr. Shirley Kwok and Dr. Cindy Christopherson, Roche Molecular Systems) [23].

2.4. Statistical analyses

Levels of p24 Ag and HIV DNA copies were reported as mean and different stimulation conditions were compared by *t* test. All analyses were performed using the SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effects of IL-2, IL-12 and IL-15 on HIV replication in vitro infected CD4+ T and PBMCs cells

The HIV-1 infected CD4+ T lymphocytes and PBMCs were cultured for 48 h with medium alone, IL-2, IL-12 and IL-15; after complete removing and replacement of medium with the appropriate cytokines p24 viral antigen production was detected after the 24 h. The p24 production from different culture conditions was compared to the medium condition.

The mean production of p24 by infected CD4+ T lymphocyte cells in presence of only medium was 92 pg/ml. We observed that IL-2 and IL-12 determined a mean increase of HIV production in CD4+ T lymphocytes by 17.99- and 6.92-fold, respectively (1655.1 and 636.8 pg/ml, respectively); the p24 production increased of 15.36-fold (1413.5 pg/ml), respect to the medium, when the HIV-1 infected CD4+ T lymphocytes were cultured in presence of IL-15. As expected the co-culture of CD4+ T lymphocytes with the IL-15 induced a HIV replication increase comparable with the IL-2 one (Fig. 1a).

In presence of only medium infected PBMCs produced 2450.4 pg/ml of P24 antigen. The mean increase of viral replication in PBMCs was 2.1-fold when cultured with IL-2, and 2.9-fold when cultured with IL-12 (5135.1 and 7306.6 pg/ml).

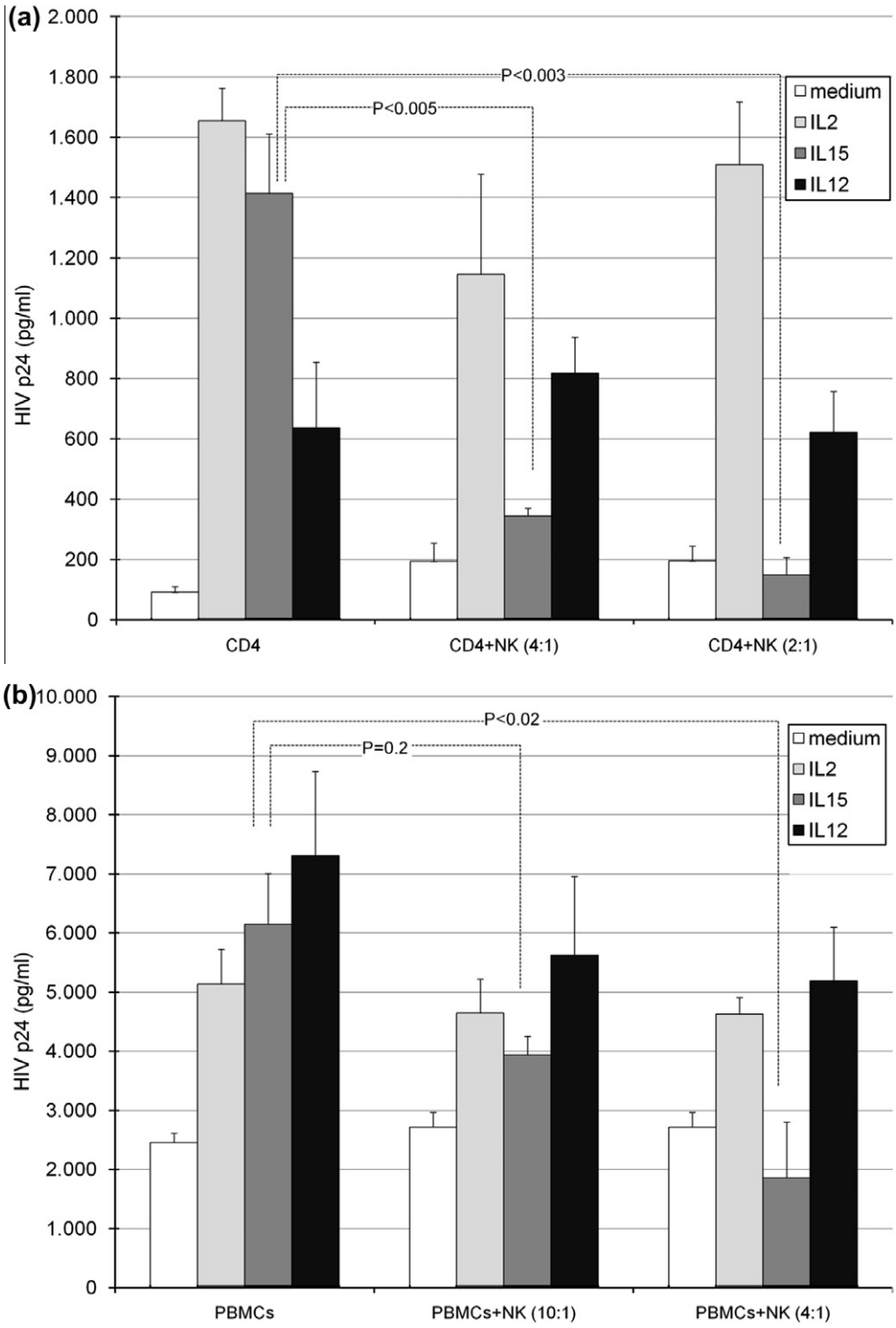

Fig. 1. HIV-p24 antigen (pg/mol) from in vitro infected CD4+ T cells (a) and PBMCs (b) cultured with different ratio of Natural Killer cells pulsed with IL-2 IL-12 or IL15. The bars represent the mean values of eight independent experiments conducted in triplicate and error bars represent the standard errors.

A similar increment of viral replication was observed when HIV-1 infected PBMCs were cultured with IL-15. (6149.1 pg/ml; 2.5-fold) (Fig. 1b).

3.2. Effects of NK pulsed with IL-2, IL-12 and IL-15 on $HIV_{89.6}$ replication in vitro infected CD4+ T and PBMCs cells

We assessed the ability of NK cells pulsed with IL-2, IL-12 and IL-15 in interfering with the $HIV_{89.6}$ replication in acutely infected CD4+ T lymphocyte cells and in PBMCs. IL-2, IL-12 or IL-15 pulsed NK cells were added to $HIV_{89.6}$ infected CD4+ T lymphocyte cells and PBMCs at two target-to-effector (T:E) ratio of 4:1 and 2:1.

When CD4+ infected cells were cultured with NK cells pulsed with IL-2, IL-12 we did not observe a significant change in the production of p24 Ag. In contrast, the addition of IL-15-pulsed NK cells at the target-to-effector (T:E) ratio of 4:1 and 2:1 to the HIV-1 infected CD4+ T cells reduced the levels of viral replication by 75.6% (344.3 pg/ml; $p < 0.005$) and 89.4% (149.5 pg/ml; 0.003) vs. IL-15 pulsed infected CD4+ T cell cultures (1413.5 pg/ml) (Fig. 1a).

The culture of IL-2- or IL-12 stimulated NK cells with HIV infected PBMCs was not able to induce a significant reduction in p24 Ag production respect to the IL-2 or IL-12 culture of HIV infected PBMCs.

When HIV infected PBMCs were cultured with IL-15 stimulated NK cells at the target-to-effector (T:E) ratio of 4:1 and 10:1 of viral replication was reduced by 70% (1861.2; $p < 0.03$) and 36% (3963.8 pg/ml; $p = 0.2$) vs. IL-15 pulsed infected PBMC cell cultures (6149.1 pg/ml) (Fig. 1b).

3.3. Effects of NK pulsed with IL-2, IL-12 and IL-15 on HIV-DNA from infected in vitro CD4 T cells and PBMCs cells

Infected CD4+ T lymphocytes cultured with IL-15 gave the highest quantity of HIV DNA in comparison to the IL-2 and IL-12 conditions (21.3, 11.0 and 2.7 copies/10^6 cells, respectively). IL-15 pulsed NK cells reduced the HIV DNA load by 93.4% ($p < 0.005$) and 83.1% ($p < 0.01$) in infected CD4+ T lymphocytes when co-cultured at the 2:1 and 4:1 target to effector ratio, respectively (Fig. 2a).

When infected PBMCs were cultured with IL-15 pulsed NK the HIV DNA load was reduced by 82% ($p < 0.05$) and 41% ($p < 0.11$) fold at the 4:1 and 10:1 target to effector ratio, respectively (Fig. 2b).

3.4. Measurement of IL-15 pulsed NK cells to release HIV- inhibitory factors affecting CD4+ T cells

Finally, for CD4+ cells experiments was performed also the trans-wells condition in order to evaluate if the ability of IL-15 pulsed NK cells to affect the HIV replication in CD4+ T lymphocytes was subdued to a physical contact cell to cell or to the release of cytokines. The trans well experiments were conducted with a (T:E) ratio of 2:1; in trans well condition the level of virus replication measured by HIV p24 quantification in supernatants was reduced by 75.8% ($p < 0.03$) vs. IL-15 pulsed infected CD4+ T lymphocyte cultures. Also the HIV DNA copies per cell million was diminished by 66.8% (from 21.3 to 7.2 copies/10^6 CD4+ T lymphocyte cells) ($p < 0.03$) (Fig. 3).

4. Discussion

The introduction of HAART have produced substantial clinical benefits to individuals with HIV infection, as shown by the findings of a significant reductions of opportunistic infections (OIs), rate of hospitalizations and HIV-associated mortality. However actually is not possible the eradication of the infection [24]. The persistence of the virus, during the course of HIV infection, depends on the long term survival of a pool of infected cells, called resting CD4+ T lymphocytes that they constitute the ''reservoir'' of the HIV [25,26]. Early after the acute infection, we can assist to the establishment of this latent reservoir, that is composed of infected memory CD4+ T lymphocytes [27,28]. The reservoirs are just present at the beginning of the HAART and they persist also in presence of several years of undetectable viraemia. The long half-life of these set of cells together with their possibility of self-renewal by proliferation explain also their persistence during HAART [29,30].

A large number of studies have produced evidences about the benefit of immunotherapy in chronic viral infections and malignancies. Adoptive T-cell transfer has shown clinical benefit in the prophylaxis and treatment of viral infections that develop in paediatric patients after allogeneic transplant and in post-transplant lymphoproliferative disease associated with the Epstein-Barr virus [31]. Developing adoptive T-cell therapies for other malignancies presents additional challenges. In this study we have focused our attention on the ability of NK cells stimulated by different interleukins to interfere with HIV replication. The importance of an efficient NK cell response for the promotion of a favorable outcome to viral infections has been demonstrated in both mice and humans [32,33,10], and the rapid activation of NK cells after infection is a hallmark of their potency as innate immune system effectors [34].

Of the multiple cytokines now known to be involved in NK-cell development and activity, IL-15 is emerging as a key modulator. IL-15, a homeostatic NK cell cytokine, can upregulate the expression of the NK activating receptors and concomitantly increase the NK lytic activity. In fact IL-15 has an essential role in NK-cell development, survival and differentiation. IL-15 activates NK-cell proliferation, cytotoxicity and cytokine and chemokine secretion [35–40]. IL-15 binds to the IL-15 receptor, composed of the specific IL-15Rα chain expressed on lymphoid, myeloid, non-hematopoietic cells including macrophages and monocytes, NK cells and CD8+ T cells, the IL-2Rβ chain and the γc chain [41–45]. Trans-presentation of IL-15 has been shown to be a major signaling mechanism although this may not be true for all cell types [46]. When IL-15 binds its receptor, JAK kinase, STAT3, STAT5, and STAT6 transcription factors are activated to elicit downstream signaling events. High affinity IL-15 receptors have been observed on monocytes, NK cells, and stromal cells from bone marrow. During infection, dendritic cells respond to inflammatory cytokines, leading to the production of IL-15 and IL-15Rα [47,48]. Expression of IL-15 and IL-15Rα on activated myeloid cells has thus been thought to contribute to NK cell responses against pathogens.

In HIV infection global NK cell functions are reduced by viral replication and moreover the loss of NK cell function could contribute to both the reduction of viral control by the immune system and the progression to AIDS. Anyway IL-15 can contribute to the establishment of an antiviral state in two ways: first by increasing the killing ability of NK cells and second by stimulating the synthesis and secretion of IFN.

In this study we evaluate the efficacy of IL-15 in inducing NK cells to control viral replication in CD4+ and PBMCs cells. Because the need to stimulate the in vitro replication of HIV in our experiments we have cultured purified CD4+ T lymphocyte cells and PBMCs with the same interleukins that we have used to stimulate the NK cells. IL-2 and IL-15 were able to significantly enhance the HIV replication on in vitro infected purified CD4+ T lymphocyte cells and PBMCs; the effect of these two cytokines was comparable. As expected IL-12 was able to induce a lower virus replication.

When NK cells were stimulated by IL-2 and IL-12 we could not see any significant reduction of HIV replication in CD4+ cells and PBMCs. In our study we observed that only IL-15 treatment produced NK cells able to interfere with HIV replication: when NK cells were stimulated by IL-15 we observed a significant reduction of viral p24 antigen in infected purified CD4+ cells culture media; similar results were obtained when the culture supernatants of infected PBMCs were tested. The reduction of viral replication in purified CD4+ cells and in PBMCs was effector-target ratio dependent. To verify if the reduction of p24 in supernatants was due to a killing of infected cells by stimulated NK cells or an interference with viral protein transcription and/or expression we have tested the HIV DNA load in purified CD4+ cells and PBMCs. We observed that only IL-15 stimulated NK cells were able to reduce the HIV DNA viral load both in purified CD4+ cells and PBMCs. All the tested interleukins were able to induce an increase of HIV DNA load in purified CD4+ cells and PBMCs. In particular, IL-15 was able to induce higher levels of HIV DNA load in CD4+ cells compared to PBMC; interestingly IL-15 induced lower HIV DNA levels in PBMCs culture than IL-2 and IL-12 (Fig. 2b). To explain this result we have to consider that: after HIV infection, PBMCs were cultured for 3 days with the same interleukins that were used to stimulate the NK cells that were subsequently added; during these 3 days

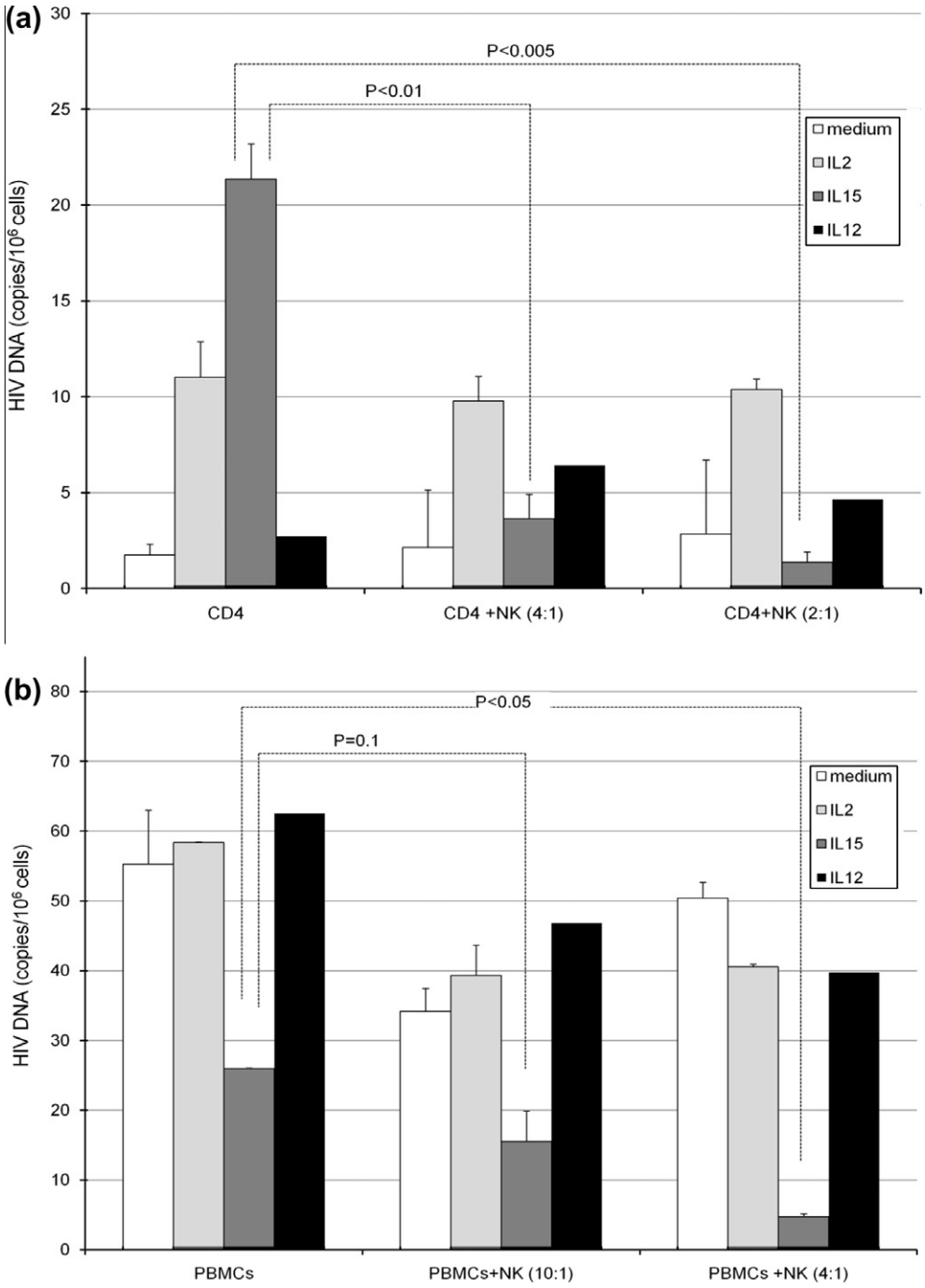

Fig. 2. HIV-DNA (copies/10^6 cells) from in vitro infected CD4+ T cells (a) and PBMCs (b) cultured with different ratio of Natural Killer cells pulsed with IL-2 IL-12 or IL15. The bars represent the mean values of eight independent experiments conducted in triplicate and error bars represent the standard errors.

the IL-15 was able to stimulate the p24 production by infected cells present in PBMC but also acting on normal fraction of NK cells presents in PBMCs that was able to kill a portion of cells that supported the HIV infection; despite the reduction of infected cells in IL-15 PBMCs culture the p24 antigen amount was not different from the IL-2 or IL-12 cultures; to explain these apparently conflicting findings it has to be considered that while the amount of HIV-DNA measured is likely to represent only infected living cells because of the short half-life of cellular DNA following cellular lysis, conversely the p24 measured in the supernatant may be represented not only by p24 produced by living cells with active HIV replication but also by p24 of cytoplasmic origin from already lysed cells.

In a previous ex vivo study we have evaluated the NK cells capacity to kill K562 cells comparing healthy donors and HAART treated patients with or not viremic suppression: HIV infected patients showed a reduced NK activity that was replaced when effector cells were stimulated with IL-15. We also reported in a precedent study that in vitro IL-15 priming induced a significant increase of IFN-γ production in both viremic and aviremic patients. IL-15 stimulated NK cells produced CC-chemokine quantities that are reported to be capable of inhibiting HIV infection and replication [49]. To evaluate in our study if the NK cells stimulated with IL-15 were able to reduce viral p24 antigen in culture media by direct killing of infected cells or by the release of cytokines able to interfere with viral replication we conduced trans-well

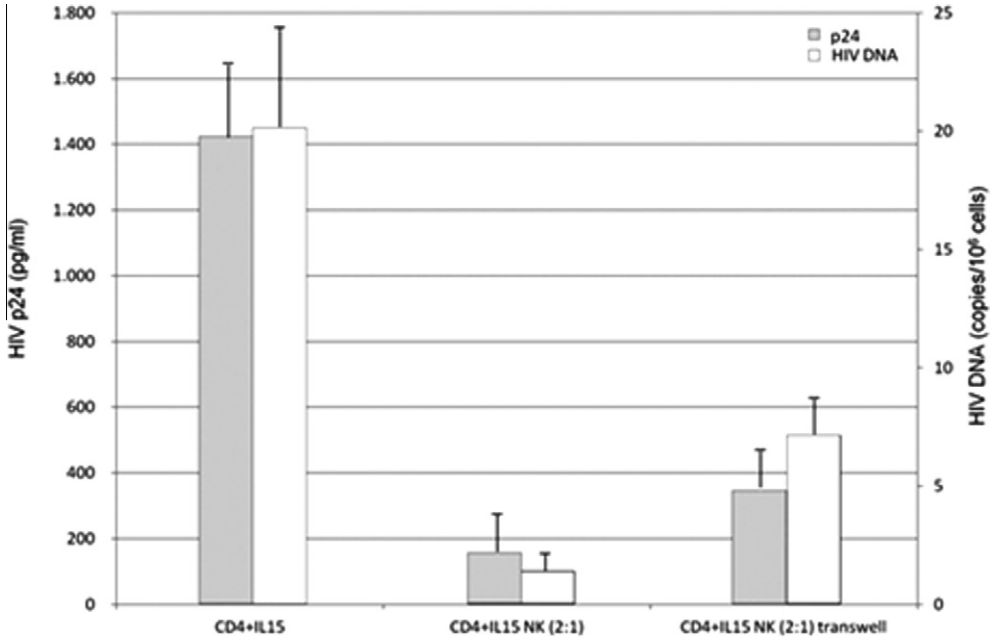

Fig. 3. HIV-p24 antigen (pg/mol) (dotted line) and HIV-DNA (copies/10^6 cells) (dashed line) from in vitro infected CD4+ T cells cultured with Natural Killer cells pulsed with IL15 at ratio 2:1 in the same chamber (with cell contact) or separated by a porous membrane (trans-well condition). The graph dots represent the mean values of eight independent experiments conducted in triplicate and error bars represent the standard errors.

experiments. The trans-well experiments showed that IL-15 stimulated NK cells were able to induce a cytokines mediated process able to reduce p24 and HIV DNA load; although these reductions were significant they were lower than the ones obtained when IL-15 stimulated NK cells were culture in contact with the target cells. We can speculate that the activity of NK stimulated with IL-15 is related both to a direct cytotoxic effect of these cells and to release of soluble factors. As previously reported, the NK ability of controlling HIV-1 replication is also partially accounted by the release of soluble HIV-inhibitory factors by activated NK cells [50]. About the mediators and the cell cell interplay involved in IL-15 stimulated NK cells in the course of HIV infection, in this study we observed that the activity of NK stimulated with IL-15 is related both to a direct cytotoxic effect of these cells and to release of soluble factors. Anyway our results are only descriptive and others studies will be needed to provide how IL-15 exerts its action upon NK cells. On the basis of previous studies we know that IL-15 stimulated NK cells in the course of HIV infection may perform their functions according to multiple pathways. In particular IL-15 stimulates NK cells to produce CC chemokines able of inhibiting HIV infection and replication. This priming effect was observed in aviremic HIV infected patients and viremic subjects with progressive HIV infection. These findings suggest that the ability of IL-15 to up-regulate the release of IFN-γ and CC chemokines by NK cells is an intrinsic biological effect that is independent from the HIV replication [49]. Moreover Ozdemir et al. demonstrated that the overall production of Perforin, Granzyme B and tumor necrosis factor-alpha was significantly higher in IL-15 compared to IL-2 stimulates NK cells [51].

In our in vitro experimental model only IL-15 was able to induce in NK cells a relevant and dose-dependent anti-HIV activity; this effect may be due to the fact that only IL-15 is able to induce in NK cells a substantial increase in both cytotoxic activity and release of cytokines that interfere with HIV replication: previous results from our group showed that in HIV infected patients and in HIV negative donors only IL-15 and not IL-2 and IL-12, was able to increase the expression of CD69 on NK cells and the release of IFNgamma and CC-chemokines [49]. Moreover, Decot et al. demonstrated that only IL-15 and not IL-2 was able to induce a significant increase in NK-cell cytotoxic activity [52].

Our results show that IL-15 has a potent effect on cell-mediated immune responses during HIV infection and plays an important role in modulating immune mechanisms. However we think that ours observations can be defined preliminary and descriptive and other studies will be needed to provide rationale for immune recovery and the optimization of immune therapies.

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