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Effects of interleukin-2 therapy on the proliferation and differentiation of CD4/CD25 positive and CD4/CD25 negative cells in HIV+ patients

Abstract

Interleukin-2 has been widely used in HIV-1+ subjects as an immunoactivating agent. In this study, we investigated cytokine production, Ki67 antigen expression and the modulation of the surface phenotype of the CD4/CD25+ subset as compared to the reciprocal CD4/CD25- subset in IL-2-treated HIV+ patients. Our findings suggest that CD4 T cells are heterogeneous in responding

to IL-2, because CD4/CD25+ cells sharply increased their "memory" phenotype, their Ki67 antigen expression and were the main in vivo targets for IL-2-dependent proliferation during therapy, while the percentages of IFN-gamma+ (terminally differentiated) cells remained unchangammaed at the end of therapy. Conversely, the CD4+/CD25- subpopulation showed an expansion of differentiated cells and a sligammaht increase in the proliferation rate. The use of anti-retroviral therapy alone (HAART) reduced the proliferation and increased the differentiation of both CD4 subsets. Our data sugammagammaest that IL-2 has a moderate capacity to activate restingamma T cells in vivo and is probably unable to boost HIV-1 from latency to the replicative state.

Full text

INTRODUCTION

Antiretroviral therapy has been shown to reduce HIV-1 replication, to raise CD4 counts and to produce a progressive, although incomplete, increase in CD4 T cell functions [1-7]. Based on the available data, it is now widely accepted that alternative approaches, supplementing HAART, are necessary to increase immune functions and to reduce viral replication to a maximum extent in HIV-infected individuals [8, 9]. Interleukin-2 has been widely and successfully used in these subjects as an immuno-activating agent and several papers have demonstrated its ability to increase CD4 counts, to produce a selective expansion of CD4 "naive" cells in the peripheral blood and in the lymphoid tissue of treated patients and to correct the dysregulation of the cytokine network that contributes to HIV-induced immunopathogenesis [10-14]. The therapeutic use of IL-2 is associated with a preferential expansion of CD4 cells expressing CD25, the alpha chain of the IL-2 receptor [10, 15]. $CD4^+/CD25^+$ cells express high levels of activation molecules and genes and contain, in untreated infection, HIV molecular forms different from those present in $CD4^+$/CD25 negative cells [16, 17]. It is therefore very important to accurately investigate the effects of IL-2 treatment on cells expressing the IL-2 receptor, and compare them to those exerted on the CD25 negative $CD4^+$ counterpart. The purposes of this study were to determine whether, in untreated HIV subjects, the $CD25^+$ cells presented peculiar capacities of cytokine production, of cellular proliferation and of surface marker expression as compared to the CD25 negative subset, and to assess the changes induced in the two subpopulations after treatment with IL-2 plus HAART or with HAART alone. The results of the present study,

describing the responses of the reciprocal CD4 subsets, may be useful for a better understanding the pathogenesis of HIV infection and the effects of immune-based treatments.

MATERIALS AND METHODS

Patients

The patients included in this study were enrolled to participate in a mono-institutional, randomized study for the evaluation of clinical, immunological and virological effects of highly active anti-retroviral therapy (HAART) plus interleukin-2 (IL-2), *versus* HAART alone [14, 18]. Patients were included if they met the following criteria: documented HIV infection, stage A1, A2, B1, B2 according to 1993 CDC classification [19], naive for anti-retroviral therapy or pretreated only with the combination of two reverse transcriptase inhibitors (RTIs), CD4 counts > 200/mmc and HIV viremia > 500 RNA copies/ml in naive patients or two consecutive HIV viremia values 1 log above the nadir value observed during RTIs therapy in pretreated patients; no previous IL-2 therapy, at least 18 years of age, granulocytes < 1,000/mmc, platelets < 100,000/mmc, hemoglobin < 10 g/dl, GOT, GPT, FAL and gammaGT no more than 3 times the normal values, bilirubin 2 mg/dl or less, creatinine 1.5 mg/dl or less. Abnormal thyroid function and significant cardiac, pulmonary and CNS impairment were considered as additive exclusion criteria.

The study protocol was approved by the Institutional review board and informed consent was obtained from the study participants. Patients were randomized to receive RTIs plus Indinavir or 2RTIs plus indinavir plus IL-2: IL-2 was s.c. administered with the follow-ing schedule: 6 MUI of Proleukin (Chiron, Emeryville, CA, USA) at days 1-5 and 8-12 of a 28 day-cycle for a total of 6 cycles (24 weeks duration overall). IL-2 was administered once a day, and just before injection patients received 1 g of paracetamol as pre-medication. This therapeutic schedule was chosen because of the excellent immunological and clinical results obtained in previous trials where RTIs have been used in association with IL-2 [12, 15]. Toxicity was evaluated according to NCI criteria, as described [15]. In case of toxicity greater than grade 2, IL-2 administration was delayed until toxicity became lower than grade 1. When toxicity grade 2 or greater persisted for more than 2 weeks, a reduction in IL-2 to half was planned.

In case of toxicity or intolerance to Indinavir, patients were allowed to switch to Ritonavir or Saquinavir.

Fourteen HIV^+ patients were enrolled in this study, as a part of the above-mentioned randomized trial. Seven were treated with HAART + IL-2 and seven with HAART alone.

Fourteen healthy subjects the same age as the HIV patients were included as controls.

CD4 T cell counts

Peripheral blood samples were obtained in EDTA and evaluated using a whole blood lysing technique [12, 14]. Briefly, 100 mul of blood were added to the appropriate monoclonal antibody combination and incubated for 15 min: after this incubation the samples were lysed and fixed with a commercial preparation (Immunoprep, Coulter, Milan, Italy). The CD3/CD4/CD8/CD45 monoclonal antibody combination (Coulter, Milan, Italy) was used to stain peripheral blood lymphocytes. The cell suspension was then analyzed in an EPICS XL flow cytometer (Beckman-Coulter).

Flow cytometric determination of the proliferation rate of $CD4^+$ cells

Cryopreserved PBMCs were thawed, counted and checked for viability by trypan blue dye exclusion. Cells were then incubated with ECD-labelled anti-CD4, with PC5-labelled anti-CD25 and with PE-labelled CD45R0 monoclonal antibodies (Beckman-Coulter, Milan, Italy). Cells were then fixed and permeabilized by the CytoFix/CytoPerm plus kit (Becton Dickinson, Milan, Italy) and stained with PermWash solution containing an optimal concentration of FITC-labelled anti-Ki67 monoclonal antibody (Dako, Milan, Italy) [20]. Then cells were analyzed, after live gating on the $CD4/CD25^+$ or on the CD4/CD25 negative subset, in an EPICS XL flow cytometer (Beckman-Coulter).

Flow cytometric determination of CD4 intracellular cytokine production

Ficoll-Hypaque purified PMBCs at 2×10^6 cells/ml were mixed with 10 ng/ml PMA (phorbol 12-myristate 13 acetate, Sigma, Milan, Italy), 0.75 mug/ml ionomycin (Sigma, Milan, Italy) and 1 mug/ml Brefeldin A; cells were then aliquoted into 12 x

75 mm plastic tubes and incubated for 5 hours in 5% CO_2 at 37° C; after washing and centrifugation, the cellular pellet was resuspended in 100 mul of staining buffer contain-ing an optimal concentration of ECD-conjugated anti-CD4 and PC5 anti-CD25 monoclonal antibodies. Cells were then fixed by adding 500 mul of CytoFix/CytoPerm solution (Pharmingen, San Diego, CA, USA), permeabilized by adding the Perm/Wash reagent and stained with 2 ml Perm/Wash solution containing an optimal concentration of FITC labelled anti-IL-2 and PE-labelled anti-IFN-gamma monoclonal antibodies [21]. As negative controls, we used stimulated fixed/permeabilized cells stained with non-conjugated, isotype-matched mouse immunoglobulins or unstimulated fixed/permeabilized cells stained with the above-mentioned monoclonal antibodies against intracellular cytokines. The percentage of cells expressing IL-2, IFN-gamma or both cytokines was determined after live gating on $CD4^+/CD25^+$ or on $CD4^+$/CD25 negative populations.

Statistical analysis

Statistical correlations between groups were analyzed by the Mann-Whitney test and paired samples were analyzed with the Wilcoxon rank test.

RESULTS

CD4 T cell counts during treatments

The absolute numbers of $CD4^+$ lymphocytes in the HAART + IL-2 group were 382 ± 100 cells/mmc at t = 0, 865 ± 290 cells/mmc at t = 2w (p ¾ 0.01 *versus* t = 0), 570 ± 115 cells/mmc at t = 4w, 565 ± 160 cells/mmc at t = 24w and 610 ± 205 cells/mmc at t = 48w (p ¾ 0.05 for all t). In the HAART group, the same values were 323 ± 55 cells/mmc at t = 0, 480 ± 70 cells/mmc at t = 2w, 470 ± 160 cells/mmc at t = 4w, 550 ± 125 cells/mmc at t = 24w and 560 ± 190 cells/mmc at t = 48 (p ¾ 0.05 *versus* t = 0).

In healthy controls, CD4 counts were 732 ± 100 cells/mmc.

Proliferating $CD4^+$ lymphocytes are "memory" cells

We investigated the expression of CD45R0 and of Ki67 after live gating on $CD4^+/CD25^+$ lymphocytes; Figure 1 shows that, in the $CD25^+$ subpopulation, the

great majority of Ki67$^+$ proliferating cells are CD45R0$^+$ "memory" lymphocytes; this figure also shows that the percentage of Ki67$^+$ memory cells is higher in HIV$^+$ patients before treatment (panel B), as compared to healthy control values (panel A). The simultaneous coexpression of CD45R0/Ki67 antigens was also present in the reciprocal CD25 negative subpopulation (data not shown). For this reason, we considered the longitudinal measurements of CD45R0/Ki67$^+$ cells as a marker reflecting CD4 T cell proliferation during the treatments. Figure 2 demonstrates that CD4/CD25$^+$ cells proliferated vigorously during IL-2 administration, while, when IL-2 is stopped (24 weeks), HAART alone reduced the proliferation rate of this subset. The CD4$^+$/CD25 negative subpopulation increased the proliferation rate to a lesser extent, as compared to the CD25$^+$ cells. Figure 3 shows the kinetic analysis of Ki67 expression in the HAART-treated subjects, suggesting that the percentages of Ki67$^+$ cells both in the CD4/CD25$^+$ subset and in the CD4/CD25 negative subsets progressively decreased during therapy, confirming previous observations showing a reduced, HAART-dependent, cell proliferation [22].

Kinetics of change of CD4 intracellular cytokine production

Analysis of cytokine production in CD4$^+$ cells revealed that the composition of this subset is different in HIV$^+$ patients as compared to controls. Figure 4 shows a representative distribution of intracellular cytokines in the CD4/CD25$^+$ cells: HIV infection (panel B) produced an obvious decrease in terminally differentiated cells (IFN-gamma$^+$/IL-2$^-$), as compared to healthy controls (panel A). A longitudinal analysis in HIV-infected subjects demonstrated that IL-2$^-$/IFNgamma$^+$, terminally differentiated CD4$^+$ cells did not increase in the CD25$^+$ subset, while there was a significant increase in the reciprocal CD25 negative population after IL-2 therapy (Figure 5). In the HAART-treated subjects, the kinetic analysis of the percentages of IL2-/IFNgamma$^+$ cells in the CD4/CD25$^+$ subset and in the CD4/CD25 negative population suggested that HAART induced a progressive increase of terminally differentiated CD4 cells in both subsets (Figure 6).

DISCUSSION

Several clinical and laboratory trials have investigated the virological and immunological parameters in IL-2-treated HIV$^+$ patients. However, the activity of this cytokine on the functions of CD4$^+$ cells, identified on the basis of IL-2

receptor expression, has not yet been characterized [8, 21, 23]. The $CD4^+CD25^+$ subset appears to be a unique regulatory T cell having a role in the prevention of autoimmune diseases [24]. Therefore, because IL-2 treatment up-regulates CD25 expression [15, 25], and because IL-2/IL-2 receptor interactions are responsible for the cytokine-dependent activities on T cells [26], it may be particularly important to closely study, *in vivo*, the dynamics of these parameters. We therefore investigated at the single cell level, multiparameter flow cytometry, and the response of CD4/$CD25^+$ and of CD4/CD25 negative T cells to IL-2 treatment in HIV^+ patients.

This approach permitted the analysis of the kinetics of changes in $CD4^+$ cells, functionally defined by their cytokine production profile, by their proliferation rate and by their phenotype. Our findings suggest that CD4 T cells are quite heterogeneous in their response to IL-2; in fact, CD4 cells bearing the IL-2 receptor alpha CD25 sharply increased their "memory" phenotype and their Ki67 antigen expression during therapy and are the main *in vivo* target for IL-2 dependent proliferation; in this subset, the frequency of IFN-gamma positive cells, a profile known to be associated with a population of terminally differentiated cells [6, 27, 28], remained unchanged at the end of therapy. Conversely, IL-2 administration produced in the $CD4^+$/CD25 negative subpopulation an expansion of fully differentiated cells (*i.e.* IFN-$gamma^+$), while the proliferation rate of this subset was only slightly increased. In agreement with previously published data [6, 22], we observed an increase of CD4 T differentiation and a progressive reduction of CD4 proliferation rates in patients treated with HAART alone. Our data suggest that IL-2 may act by inducing the expansion of CD4 cells that are already activated before cytokine administration, while the reciprocal CD25 negative subset is less prone to proliferation after IL-2. These observations raise important questions regarding the possible influences of IL-2 on the latent reservoir of HIV that is contained in resting, CD25 negative, CD4 cells [29-31]; if IL-2 has a moderate capacity to activate resting T cells *in vivo*, it is probably unable to significantly boost HIV-1 from latency to the replicative state, while the transient increase of viral load observed during cytokine administration depends probably upon an amplification of ongoing viral replication [32]. If this hypothesis is correct, it may explain why previous clinical trials using IL-2 were unable to eliminate, after two years of follow-up, the HIV reservoir [18, 33], that is the source of the re-emergent viremia in patients who discontinued HAART [34]. A sustained induction of HIV replication in latently infected CD4 cells has been obtained *in*

vitro using a combination of cytokines such as IL-2, IL-6 and TNF-alpha [30]. The future use of this approach to treat patients appears unlikely, mostly because of the marked toxic side effects of multiple cytokine administration. Furthermore, it has already been shown that IL-2 administration induced high-level production of circulating cytokines implicated as direct modulators of virus expression, while the viral reservoir is only slightly affected by current IL-2 protocols [18, 30, 33, 35]. An alternative approach to obtain high level stimulation of the immune system and to increase the immunological effects of IL-2 in HIV^+ patients has been attempted by the use of the anti-CD3 monoclonal antibody [36]; theoretically, this type of treatment may expose a large number of CD4 T cells to the effects of IL-2 by preactivating the CD25 negative, resting CD4 cells and it may finally produce a sustained induction of HIV-1 replication in latently infected T cells. However, relevant statements included in that study indicated that high level activation may overwhelm the ability of co-administered HAART to control viral replication and that, while IL-2 preferentially stimulates CD4 T cells, the targets of anti-CD3 stimulation are $CD8^+$ T cells. High level stimulation may therefore result in a negative outcome and is probably not an appropriate strategy to reduce the HIV reservoir [36]. Overall, the data available in the literature suggest that immune activating regimens have the potential to improve the immune response and to control viral replication in immunocompromised hosts, but their biological and clinical effects are often complex and difficult to dissect. The results of this study add new data on the immunological consequences of immuno-activating regimens and may be used to improve the efficacy of cytokine-based clinical trials.

CONCLUSION

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Figures

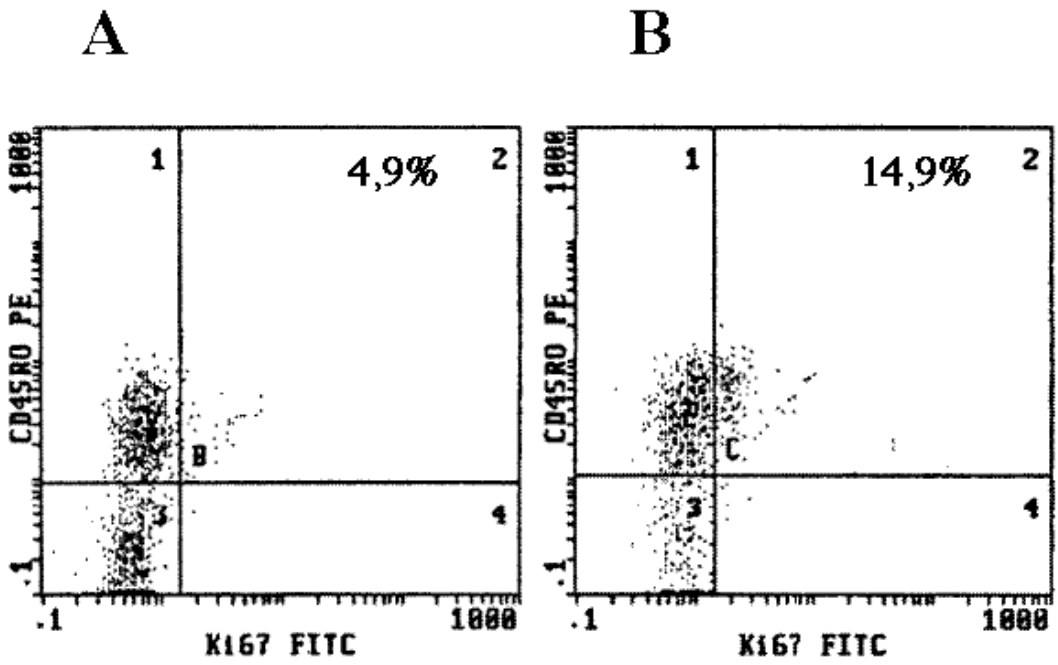


Figure 1
Flow cytometric analysis of CD45R0 and Ki67 expression in a representative healthy control (panel A) and in an HIV$^+$ patient before treatment (panel B).

The percentage of cells co-expressing CD45R0 and Ki67 (quadrant 2) was defined within a CD4$^+$/CD25$^+$ gate.

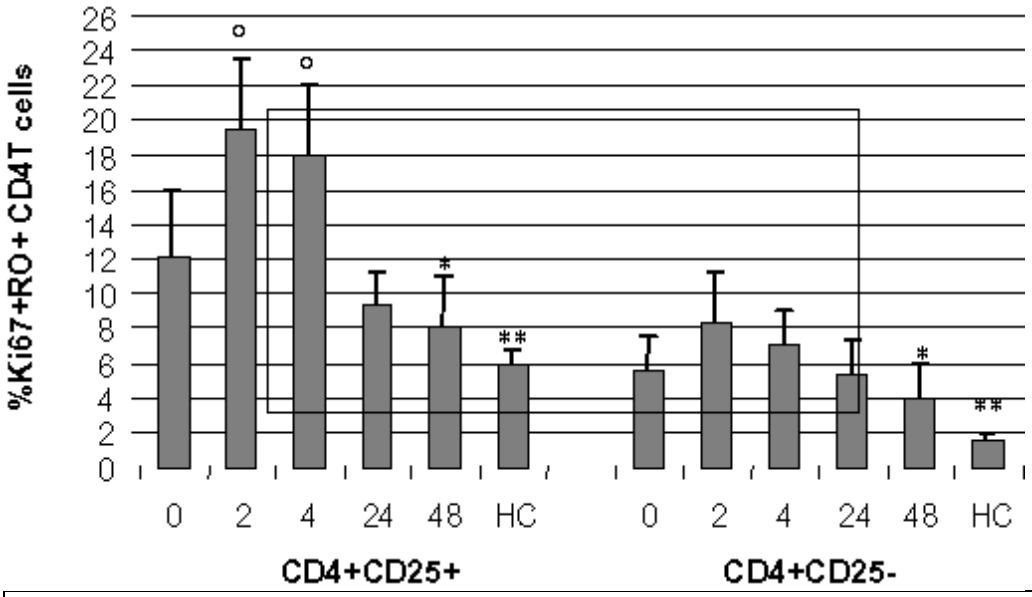


Figure 2

Longitudinal analysis (0, 2, 4, 24 and 48 weeks) of Ki67/CD45R0 expression in the $CD4^+/CD25^+$ and $CD4^+/CD25^-$ subsets of HIV^+ patients treated with IL-2.

* $p < 0.05$ *versus* t = 0, ° $p < 0.01$ *versus* t = 0, ** $p < 0.05$ in HC (healthy controls) *versus* t = 0 in HIV^+ patients.

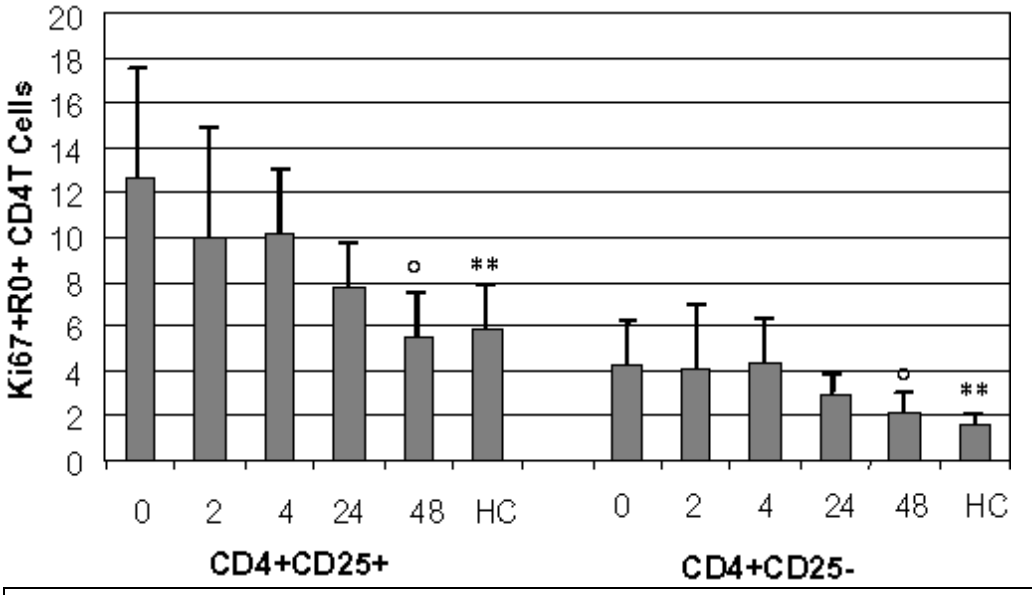


Figure 3

Longitudinal analysis (0, 2, 4, 24 and 48 weeks) of Ki67/CD45R0 expression in the $CD4^+/CD25^+$ and $CD4^+/CD25^-$ subsets of HIV^+ patients treated with HAART.

* $p < 0.05$ *versus* t = 0, ** $p < 0.05$ in HC (healthy controls) *versus* t = 0 in HIV^+ patients.

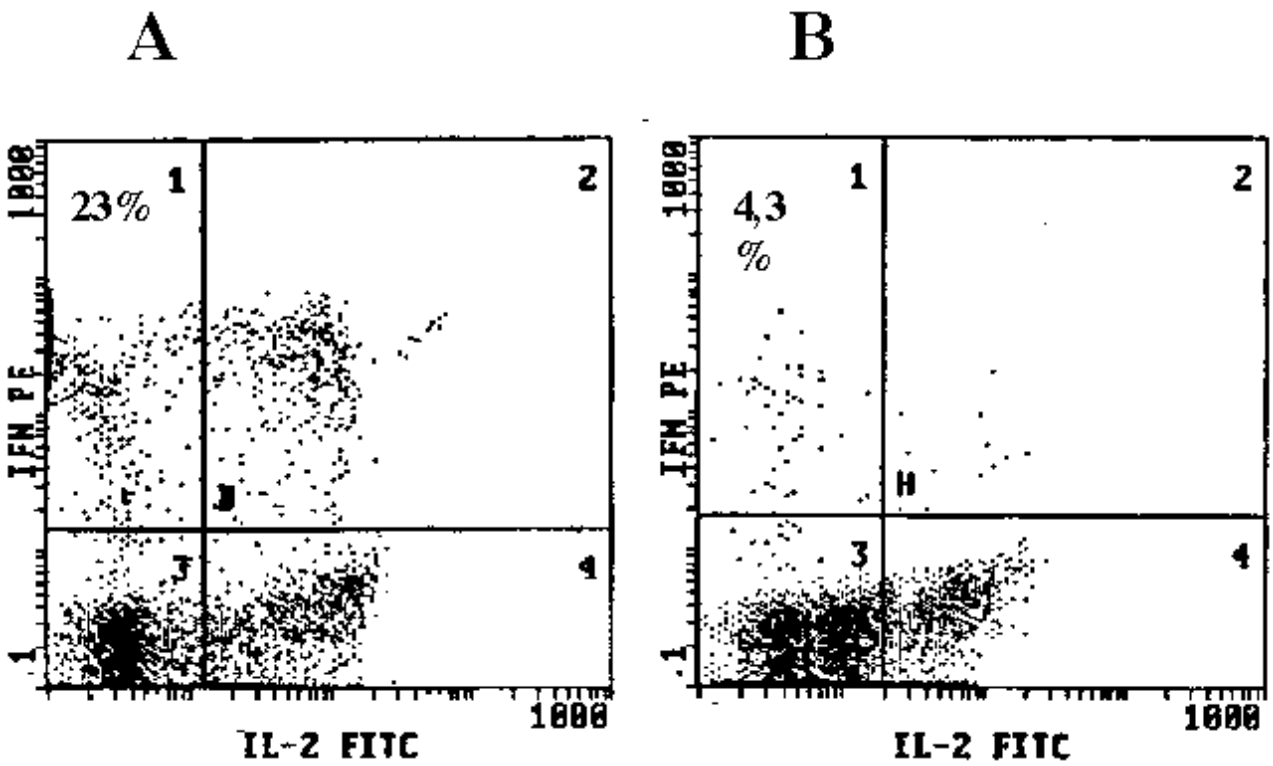


Figure 4
Flow cytometric analysis of cytokine synthesis in a representative healthy control (panel A) and in an HIV^+ patient before treatment (panel B).

The percentage of terminally differentiated cells (IFN-gamma$^+$/IL-2$^-$; quadrant 1) was defined within a $CD4^+/CD25^+$ gate.

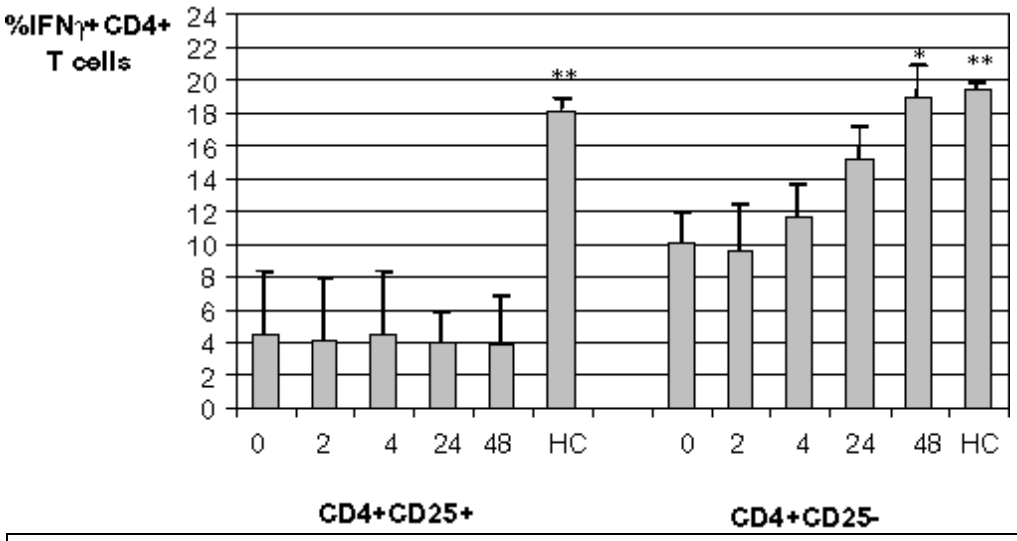


Figure 5

Longitudinal analysis (0, 2, 4, 24 and 48 weeks) of intracellular IFN-gamma expression in the $CD4^+/CD25^+$ and $CD4^+/CD25^-$ subsets of HIV^+ patients treated with IL-2.

* $p < 0.05$ *versus* t = 0, ** $p < 0.05$ in HC (healthy controls) *versus* t = 0 in HIV^+ patients.

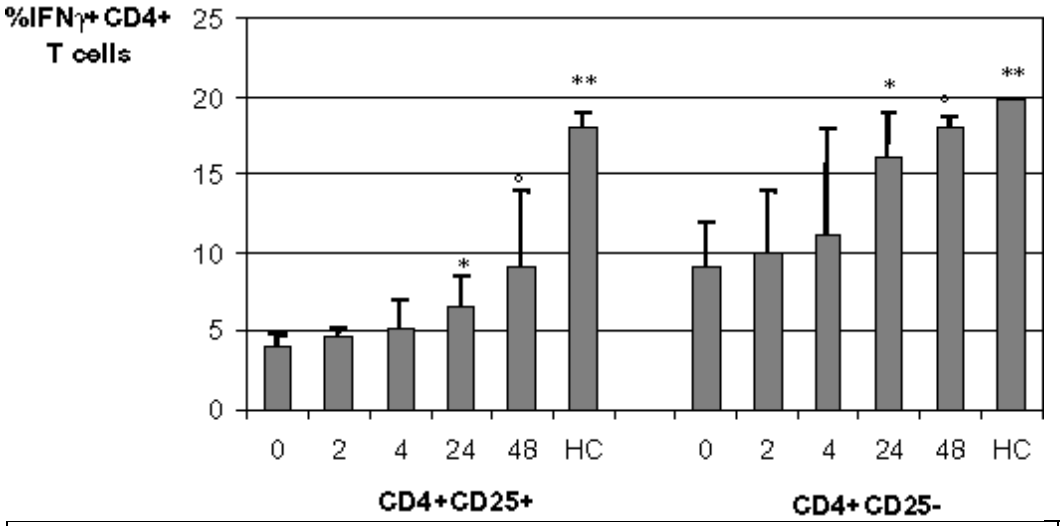


Figure 6

Longitudinal analysis (0, 2, 4, 24 and 48 weeks) of intracellular IFN-gamma expression in the $CD4^+/CD25^+$ and $CD4^+/CD25^-$ subsets of HIV^+ patients treated with HAART.

* $p < 0.05$ *versus* t = 0, ** $p < 0.05$ in HC (healthy controls) *versus* t = 0 in HIV^+ patients.

Authors

L. Caggiari, S. Zanussi, M. D'Andrea, M. T. Bortolin, C. Crepaldi, C. Caffau, P. de Paoli

Microbiology, Immunology and Virology, Centro di Riferimento Oncologico, Aviano, Italy.

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protein metabolism

B. Alvarez, L. S. Quinn, S. Busquets, F. J. López-Soriano, J. M. Argilés
cytokines, myotubes, cachexia, protein turnover.

Receptor-like properties of the 26 kDa transmembrane form of TNF

A. Domonkos, A. Udvardy, L. László, T. Nagy, E. Duda
cytokine, TNF family, receptor, signaling, reverse-signaling.

Local administration of interleukin-1beta for the treatment of lung abscesses induces neutrophil activation and changes in proinflammation cytokine production

A. Simbirtsev, E. Variouchina, V. Konusova, A. Kotov, S. Ketlinsky, A. Salamatov, L. Bisenkov
recombinant human interleukin-1beta, lung inflammation, abscess, neutrophils, cytokines.

Effects of interleukin-2 therapy on the proliferation and differentiation of CD4/CD25 positive and CD4/CD25 negative cells in HIV+ patients

L. Caggiari, S. Zanussi, M. D'Andrea, M. T. Bortolin, C. Crepaldi, C. Caffau, P. de Paoli
cytokines, Ki67, intracellular IFN-gamma.

Tumor necrosis factor-alpha and nitric oxide in vertically HIV-1-infected children: implications for pathogenesis

J. González-Nicolás, S. Resino, J. L. Jiménez, S. Alvarez, M. Fresno, M. A. Muñoz-Fernández
tumor necrosis factor, nitric oxide, HIV-infection, immunological and virological markers.

Serum levels of angiogenic cytokines in systemic lupus erythematosus and their correlation with disease activity

E. Robak, A. Wozniacka, A. Sysa-Jedrzejowska, H. Stepien, T. Robak
systemic lupus erythematosus, disease activity, angiogenesis, platelets, hepatocyte growth factor, vascular endothelial growth factor, basic fibroblast growth factor, transforming growth factor beta.

Modulation of the allergen-induced human IgE response in Hu-SCID mice: inhibitory effect of human recombinant

IFN-gamma and allergen-derived lipopeptide

C. Duez, H. Gras-Masse, H. Hammad, H. Akoum, A. Didierlaurent, C. André, A.-B. Tonnel, J. Pestel
SCID mouse, IgE, IFN-gamma, lipopeptide, house dust mite.

Cytokine-inducing activity of a proline-rich polypeptide complex (PRP) from ovine colostrum and its active nonapeptide fragment analogs

A. Zablocka, M. Janusz, K. Rybka, I. Wirkus-Romanowska, G. Kupryszewski, J. Lisowski
ovine colostrum, proline-rich polypeptide complex (PRP), nonapeptide (NP) and hexapeptide (HP) from PRP, oligomers of NP and HP, cytokines.

Quantitation of chemokines (MDC, TARC) expression in mucosa from Crohn's disease and ulcerative colitis

F. Jugde, M. Alizadeh, C. Boissier, D. Chantry, L. Siproudhis, S. Corbinais, E. Quelvennec, F. Dyard, J.-P. Campion, M. Gosselin, J.-F. Bretagne, G. Sémana, D. Heresbach
real-time TR-PCR, chemokines, Crohn’s disease, ulcerative colitis.

Activation of caspase-3 by interferon alpha causes interleukin-16 secretion but fails to modulate activation induced cell death

O. Ludwiczek, A. Kaser, R. O. Koch, W. Vogel, W. W. Cruikshank, H. Tilg
interleukin-16, interferon-alpha, caspase-3.

Cytokine deviation induced by intrathymic injection of staphylococcal enterotoxin B (SEB)

P. Goettelfinger, F. Lecerf, S. Berrih-Aknin, M. German-Fattal
SEB, intrathymic injection, anergy, cytokine deviation, quantitative RT-PCR.

Strain-dependent difference in inducible nitric oxide synthesis (iNOS) expression in rat pancreatic islets correlates with interferon regulating factor 1 (IRF-1) and heat shock protein 70 (HSP70) expression

J. Johannesen, F. Pociot, A. E. Karlsen, T. Mandrup-Poulsen, J. Nerup
type 1 diabetes, islets of Langerhans, beta-cells, cytokines, interleukin-1beta, interleukin-1beta receptor type I, nitric oxide, inducible nitric oxide synthase, heat shock protein 70, interferon regulating factor 1.

Neutrophil apoptosis in autoimmune Fas-defective MRL lpr/lpr mice

E. Del Giudice, A. Ciaramella, N. Balestro, D. Neumann, P. G. Romano, M. P. Cesaroni, G. Maurizi, P. Ruggiero, D. Boraschi, P. Bossù
neutrophils, apoptosis, autoimmunity.

A role for interferon-gamma in the hypermetabolic response to murine toxoplasmosis

D. Arsenijevic, F. de Bilbao, P. Giannakopoulos, L. Girardier, S. Samec, D. Richard
toxoplasma, hypermetabolism, reactive oxygen species, lipid peroxidation, UCP1, interferon-gamma.

Protection of mice from malaria after co-administration of recombinant mouse granulocyte-macrophage colony-stimulating factor and methionine-enkephalin

P. Pal Singh, S. Singh
Plasmodium berghei, protection, rmGM-CSF, met-enkephalin.

Semiautomated and simultaneous analysis of the interleukin-10 gene microsatellites IL-10G and IL-10R by fluorescence-based polymerase chain reaction reveals significant differences in allele distributions between Caucasians (Germany) and Africans (Gabon)

D. Kube, D. Schmidt, M. Mörmann, A.C. Uhlemann, J. Tomiuk, H. Tesch, P.G. Kremsner
microsatellites, interleukin-10 promoter, multiplex-PCR, Caucasian, African.

Les éditions JLE

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