

Phenotypic and Functional Characterization of the Thymus in HIV-infected patients

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ABSTRACT

Background. A key question in understanding immune impairment and immune reconstitution in HIV infection is whether functional defects of thymus are involved in alterations of T-cell homeostasis.

Methods. We evaluated thymic tissue from 4 HIV-infected and from 9 HIV-negative adults who underwent heart surgery. Thymocytes phenotype (CD34, CD1, CD3, CD4, CD8), CD127 and activation/proliferation markers (CD69, CD27, Ki67) were measured, together with the expression of HIV co-receptor (CCR5, CXCR4) and the TCR subsets (TCR $\alpha\beta$, TCR $\gamma\delta$). T-student test was used for statistical analysis.

Results. All thymopoietic stages were detected in both HIV+ and HIV- thymic tissues, but these stages were skewed in HIV-infected compared to uninfected thymuses. Thus, whereas the percentage of immature triple negative cells (CD34+CD3-CD4-CD8-) was comparable in HIV+ and in HIV- patients, a significant increase of double negative (DN) CD3+CD4-CD8- cells (14.7% versus 2.3%; $p=0.01$) and of CD3+CD4-CD8+ single positive (SP) cells (35.5% versus 22.5%) as well as a reduction of double-positive (DP) (CD3+CD4+CD8-; 24.5% versus 31%) and CD3+CD4+CD8- SP cells (21.7% versus 37.7%) were observed in HIV+ compared to HIV- subjects. Proliferation and activation status, as evaluated by the expression of Ki67, CD27 and CD69, was significantly augmented in HIV+ thymuses both in DN/DP ($p<0.05$) and in SP thymocytes ($p<0.05$), with a predominant expression of CD69 in CD4+ SP cells. IL-7R expression in HIV+ thymuses was significantly reduced in CD34+CD3-CD4-CD8- cells ($p=0.01$), but was increased in both DP and SP cells. CCR5 and CXCR4 were comparable in HIV infected and -uninfected thymuses. Nevertheless, whereas CCR5 expression was greater on immature thymocytes in HIV+ patients, this HIV coreceptor was mostly expressed by mature thymocytes in HIV- individuals. Finally $\gamma\delta$ TCR was expressed on a minority of thymocytes subsets in both groups of patients.

Conclusions. The effect of HIV infection on human thymus seems to act by at least two mechanisms: reduction of intrathymic CD4+ precursors and increased activation status of thymocytes at multiple stages of differentiation. Additionally, HIV infection impairs the IL-7/IL-7R circuit in the earlier intrathymic maturation stages (CD34+CD3-CD4-CD8-). The higher activation status of immature thymocytes seen in HIV-infected thymuses could explain the increased expression of CCR5 seen in these cells and the augmented susceptibility of thymus to HIV infection.

BACKGROUND

The thymus is the primary organ of thymopoiesis and is highly active during early life. Clinical and experimental evidence suggests that thymus is one of the important target organs for HIV-1 infection, allowing rapid virus replication with consequent cell death.

Mechanisms by which HIV affects the thymus are multiple and only partially known, as a number of studies addressing mechanisms of CD4+ thymocyte death in the thymus organ have indicated that both direct viral lysis and bystander apoptosis may occur during thymocyte depletion.

Functional thymus has been demonstrated in some HIV-1 infected adults, and, although not univocally, some studies have reported correlations between thymic size, TREC levels and immune reconstitution after initiation of HAART.

The aim of the study is to phenotypically characterize thymic cells isolated from HIV-infected patients, specifically analyzing:

- i. the presence and proportions of thymic progenitors and of different stages of T-cell development (Figure 1);
- ii. the degree of thymic cell proliferation and activation;
- iii. the level of expression of IL-7 receptor, considering the dominant role of IL-7 on proliferation and differentiation of developing thymocytes;
- iv. the distribution of HIV co-receptors (CXCR4 and CCR5), from which derives the dissemination of HIV-1 in the thymus.

METHODS

Patient population. Four HIV-infected and 7 age-matched HIV-negative adult donors undergoing cardiac surgery for coronary disease or cardiac valve disease were enrolled at at the Institute of Infectious and Tropical Diseases, Luigi Sacco Hospital, University of Milan. Thymectomy is a routine procedure during cardiac surgery in adult patients. Adult donors with autoimmune diseases or donors treated with immunosuppressive drugs were excluded. The informed consent of all donors was obtained according to the local Hospital Ethical Committee procedures.

Tissue collection and cell preparation. Immediately after surgery, thymus tissue was separated from the fibrotic capsule and the visible blood-vessels, mechanically disaggregated using a scalpel and scissors followed by pipetting a few times with phosphate buffered saline (PBS). A single-cell suspension from the thymus was eventually obtained by gently passing the collected PBS and the tissue fragments through a sterile 70 mm stainless-steel sieve. Cell suspensions were centrifuged and fatty debris was discarded. Freshly isolated cells from each sample were filtered through a 50 mm sterile nylon mesh and examined by means of flow cytometry. All steps were performed in asepsis and using sterile PBS containing 0.1% bovine serum albumin.

Flow cytometry. Flow cytometry was performed using a FACScan flow cytometer (FC500 Beckman Coulter Miami, FL). Cells were analysed immediately after immunostaining using forward-scatter and side-scatter signals to establish the thymocyte gate and exclude dead cells, debris and cell clumps. Fluorescence signals were collected in log mode. A minimum of 50.000 cells of interest were acquired for each sample and data analysed using CXP Analysis software.

Immunostaining was performed using conjugated monoclonal antibodies (mAbs) to: CD1a, CD3, CD4, CD8, CD27, CD34, CD69, CD127 (IL-7 receptor α -chain), CCR5, CXCR4, Ki67, TCR $\alpha\beta$ and TCR $\gamma\delta$ (Becton Dickinson Biosciences, San Jose, CA and Beckman Coulter, Miami, FL). The mAbs were conjugated with the fluorescent dyes fluorescein-isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-texas-red (ECD), phycoerythrin-cyanin-7 (PC7), phycoerythrin-cyanin-5 (PC5) and appropriately combined to assess the cell subset of interest in thymus samples in five-colour fluorescence assays. Irrelevant isotypic mAbs were used as negative control.

Statistical analysis. Comparisons between the different groups were made using a two-tailed T-test. Statistical analysis was performed using the SPSS statistical package (SPSS Inc. Chicago, Illinois, USA).

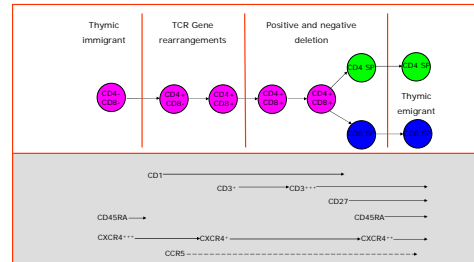


Figure 1. Simplified model of T-cell development (modified from Ho Tsang Fang, AIDS 2008)

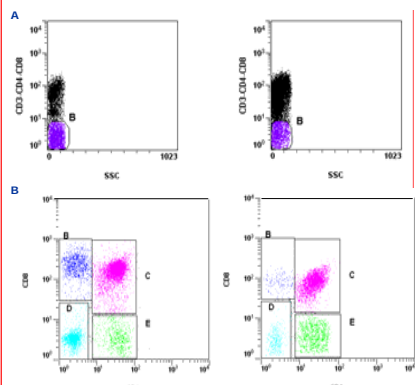


Figure 2. Panel A. The percentage of immature triple negative cells (CD34+CD3-CD4-CD8-, in purple) was comparable in HIV+ and in HIV- patients (34.9% versus 29%, respectively). CD3+CD4+CD8- are represented in black.
Panel B. A significant increase of double negative (DN) CD3+CD4-CD8- cells (14.7% versus 2.3%; $p=0.01$) and of CD3+CD4-CD8+ single positive (SP) cells (35.5% versus 22.5%) was observed in HIV+ versus HIV- patients. Conversely, a trend in a reduction of double-positive (DP) (CD3+CD4+CD8-; 24.5% versus 31%) and CD3+CD4-CD8- SP cells (21.7% versus 37.7%) were observed in HIV+ compared to HIV- subjects.

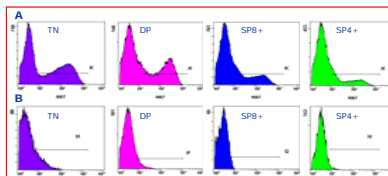


Figure 3. Proliferation was evaluated by the expression of Ki67 on triple negative cells (TN), double positive cells (DP) and single positive cells, CD3+CD8+ (SP8) and CD3+CD4+ (SP4), in HIV-positive patients (panel A) and HIV-negative controls (panel B). Ki 67 was augmented in HIV+ compared to HIV negative patients either in immature cells, triple negative cells (40% versus 17.2%, $p=0.05$) and double positive cells (33% versus 11.45%, $p=0.05$), either in single positive cells, CD3+ CD8+ (22.8% versus 1.15%, $p<0.01$) and CD3+CD4+ (4.8% versus 0.45%).

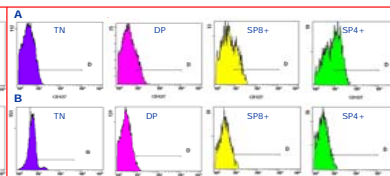


Figure 4. IL-7R α expression (CD127) was evaluated on triple negative cells (TN), double positive cells (DP) and single positive cells, CD3+CD8+ (SP8) and CD3+CD4+ (SP4), in HIV-positive patients (panel A) and HIV-negative controls (panel B). CD127 expression was significantly reduced in CD34+CD3-CD4-CD8- cells of HIV+ thymuses (0.35% in HIV+ versus 2.95% in HIV-controls, $p=0.01$) but a slight increase was observed in both DP (3.3% versus 1.3%) and SP cells, SP8+ (4.55% versus 2.65%) and SP4+ (7.7% versus 4.65%).

Activation status, as evaluated by the expression of CD69 and CD27 on triple negative cells (TN), double positive cells (DP) and single positive cells, CD3+CD8+ (SP8) and CD3+CD4+ (SP4), showed different patterns in HIV+ compared to HIV- subjects. Indeed, the expression of CD69 was significantly augmented in HIV+ patients compared to HIV- subjects in TN cells (6.8% versus 0.8%, $p<0.05$) and a predominant expression of CD69 was observed in SP4+ T cells of HIV-infected thymuses (18.5% versus 3%, $p=0.05$). Moreover, a significant increase of CD27 was observed in CD8+ SP T cells of HIV+ subjects compared to HIV- controls (11.7% versus 1.6%, $p<0.05$)

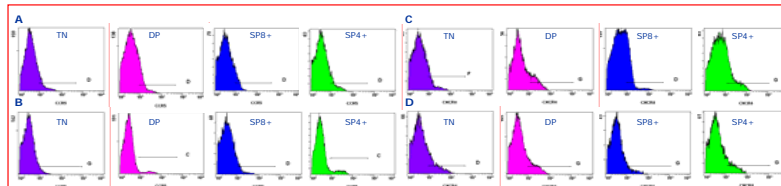


Figure 5. The expression of HIV co-receptors, CCR5 and CXCR4, was analyzed in HIV-infected patients (panel A and C) and in HIV- controls (panel B and D) on triple negative cells (TN), double positive cells (DP) and single positive cells. Although the expression of CXCR4 was more consistent than CCR5 in both groups of patients, the relative expression of these co-receptors was differently modulated in specific thymopoietic stages. Indeed, whereas on TN cells CCR5 expression was relatively greater than CXCR4 in HIV+ patients (CCR5 0.95%, CXCR4 0.35%), we confirmed a more consistent CXCR4 expression on immature TN cells in HIV- subjects (CCR5 3.5%, CXCR4 14.65%) as previously described. The analysis of HIV co-receptors on double positive versus mature single positive cells showed a significant more represented expression of CCR5 in CD4+ SP cells in HIV- subjects (DP: CCR5 1.9% and CXCR4 8.2%, SP4+: CCR5 2.3% and CXCR4 6%, SP8+: CCR5 0.9% and CXCR4 2.55% in HIV+; DP: CCR5 0.7% and CXCR4 14.45%, SP4+ CCR5 2.3% and CXCR4 4.5%, SP8+ CCR5 1.15 and CXCR4 9.8 in HIV-)

CONCLUSIONS

- The effect of HIV infection on human thymus seems to act by at least two mechanisms:
 - reduction of intrathymic CD4+ precursors;
 - increased activation status of thymocytes at multiple stages of differentiation.
- HIV infection impairs the IL-7/IL-7R circuit in the earlier intrathymic maturation stages (CD34+CD3-CD4-CD8-).
- The higher activation status of immature thymocytes seen in HIV-infected thymuses could explain the increased expression of CCR5 seen in these cells and the augmented susceptibility of thymus to HIV infection.
- Enhancing thymic function together with suppression of immune activation will be essential to restoring the function and breadth of the T-cell compartment of the cellular immune system during HIV-1 infection.