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XX CICLO

**"Transcriptional regulation by thymosin alpha-1
in peripheral blood monuclear cell from
HIV positive patients"**

Nome e Cognome

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INTRODUCTION

THYMOSIN α 1: history and biology

The isolation from the thymus gland of the thymosins, a family of biologically active molecules with hormone-like properties, was first described in 1966 by AL Goldstein and A White. Since that time, significant progress has been made in understanding the role of the thymosins in immunity and in the nature of the growth factors, cytokines and chemokines that they modulate. The thymosins include a family of biochemically and functionally distinct polypeptides with clinically important physiological properties. In the early 1970s, preclinical studies establishing the immunorestorative effects of a partially purified thymosin preparation termed thymosin fraction 5 (TF5) provided the scientific foundation for the first clinical trials with TF5 in 1974. TF5 was effective in stimulating the immune system in children with DiGeorge syndrome and other thymic dysplasias. These trials led to further interest in the active components in TF5 and to the chemical characterization of the biologically active thymosins. Several of these molecules are showing significant clinical promise in cancer, infectious diseases and wound healing (Goldstein *et al* 2004).

TF5 consists of a family of at least 40 mostly small acidic polypeptides, with molecular weights ranging from 1000 to 15,000 Dalton. Several of these polypeptides contribute individually to the biological activity of the parent compound: polypeptide beta 1, thymosin alpha 1 (T α 1), prothymosin alpha 1, parathymosin and thymosin beta 4 have been characterized chemically and biologically (Low *et al.*, 1979).

Thymosin α 1 is a 28 amino acid biologically active protein cleaved from positions 2–29 of a precursor protein known as prothymosin. This is a nuclear protein present in all mammalian cells with unknown biological activity and is believed to be strongly associated with cell

proliferation, transcription, processing and export of m-RNA from the nucleus (Tao *et al.*, 1999).

Thymosin α_1 has been widely studied both *in vivo* and *in vitro* and is currently known as lymphokine or thymic hormone since it is produced by lymphocytes and thymic epithelium.

Like many cytokines, Thymosin α_1 is an immune modulating pleiotropic peptide.

The activities associated with T α_1 include:

- 1) The influence in the maturation of T cells through induction of differentiation markers;
- 2) Induction of various cytokines secretion, including MIF, INF- γ , IL-2 and its receptor (CD25) on natural killer cells (NK), T lymphocytes and blood mononuclear cells (Svedersky *et al.*, 1982; Sztein *et al.*, 1986; Oates *et al.*, 1989);
- 3) It was shown that T α_1 stimulate maturation of stem cell CD34⁺ in CD4⁺ CD3⁺ cells in an experimental model *in vitro* co-culture with thymic epithelium, inducing the synthesis of IL-7 (Knutsen *et al.* 1999), indicating the role of T α_1 in the maturation and differentiation of hematopoietic cells.

Therapeutic application of T α_1

T α_1 has shown a variety of effects on cells and pathways of the immune system, its central role in modulating dendritic cell (DC) function has only recently been appreciated. As DCs have the ability to sense infection and tissue stress and to translate collectively this information into an appropriate immune response, an action on DCs would predict a central role for T α_1 in inducing different forms of immunity and tolerance. It has been shown the capacity of T α_1 of activating infected dendritic cells through Toll-like receptor signalling, thus influencing the inflammation balance, and increasing the expression of tumour, viral, and

major histocompatibility complex (MHC) I antigens (Giuliani *et al.* 2000; Romani *et al.* 2007).

In vitro studies suggest that T α 1 may act via pathways commonly used by various cytokines. This raises the possibility that T α 1 and cytokines may have synergistic activity through potentiation of cytokine activity by T α 1. The immunopharmacology of T α 1 predicts an important clinical role for T α 1 in the restoration of cellular immune activity when used in combination with cytokines *in vivo* (Naylor *et al.* 2007). In this study patients who experience immune suppression due to the presence of tumour, irradiation, and/or chemotherapy or aging of the host would most benefit from this treatment combination. Thymosin alpha1 is also able to potentiate the action of cytokines reducing the hematological toxicity of cytotoxic drug therapy (cyclophosphamide-, 5-fluorouracil-, dacarbazine- or ifosfamide-based regimens) and this demonstrate the mechanism of action of T α 1 and its role as an immune system enhancer (Billich, 2002).

T α 1 plays an important role as an *in vivo* antitumoral in experimental models of animals and humans, alone or in combination with other commonly used anticancer.

It has been emphasized that combination therapy of T α 1 and low doses of interferon (IFN) or interleukin 2 (IL-2) carries a positive effect in the reconstitution of the immune response inhibiting tumour growth and / or cytostatic drugs (Favalli *et al.*, 1989; Mastino *et al.*, 1991; Mastino *et al.*, 1992; Pica *et al.*, 1998).

In combination therapies, T α 1 markedly enhances the effects of anticancer chemotherapy reducing the toxic effects of these drugs in a Lewis lung carcinoma animal model (Garaci *et al.*, 2000; Garaci *et al.*, 2003), using a combination of thymosin alpha 1 (T α 1) and interferon (IFN) after cyclophosphamide. The efficacy of the combination therapy with T α 1 and either IFN or IL-2 in addition to chemotherapy has confirmed in preclinical models cancer such as melanoma (mouse)(Pica *et al.* 1998) and colorectal carcinoma (rat) (Garaci *et al.* 1993, Rasi

et al. 1994) where T α 1 has shown antitumor effects. In the last decade many studies have also highlighted the importance of using biological response modifiers (BRM) in the treatment of different types of immunological dysregulation, related to age, cancer or infectious diseases (Garaci *et al.* 1997, Garaci *et al.* 2003).

Immunotherapy using T α 1 has been found effective in infectious disease control (Rasi *et al.*, 1996; Garaci *et al.* 1994). The combination of T α 1 and IFN- α was also used in patients with chronic hepatitis B and C including IFN-non responders and patients infected with precore mutants or genotype 1b (Rasi *et al.*, 1996). Further studies demonstrated additional biological activities clarifying the mechanism of action of T α 1, partially explaining the synergism with IFN. Based on these information, two clinical trials are ongoing: a large phase II study on advanced melanoma patients treated with T α 1 at different doses after dacarbazine and a phase III study, on IFN-resistant hepatitis C virus (HCV) patients treated with a triple (IFN, ribavirin, and T α 1) combination (Rasi *et al.*, 2003, Garaci E. *et al.* 2007; Camerini *et al.* 2007). By July 2001, T α 1 was in phase III trials in the USA in combination with PEGylated interferon-alpha and later the same month it was approved in the Philippines as a monotherapy or in combination with interferon, in the treatment of HCV infection.

T α 1 has been launched in Argentina, China, Peru, the Philippines and Singapore for the treatment of chronic HBV infection.

The effects of combination therapy with T α 1 and natural human lymphoblastoid interferon-alpha were also investigated in human immunodeficiency virus infection. The effects of combination therapy with T α 1, interferon-alpha and zidovudine in patients with CD4⁺ lymphocytes ranging from 200 to 500/mm³ was analyzed in a randomized non-blinded study and found that the treatment was well tolerated after 12 months of therapy and was associated with a substantial increase in the number and function of CD4⁺ T cells. *In vitro* this combination treatment resulted in synergistic stimulation of the cytotoxic activity against

natural killer-sensitive target cells of lymphocytes collected from human immunodeficiency virus-infected donors and did not interfere with the antiviral activity of zidovudine. A similar effect was not observed in human immunodeficiency virus patients treated with zidovudine alone or associated with single agents (Garaci *et al.*, 1994).

It was also identified that prothymosin alpha (ProT α) in HVS transformed CD8⁺ T cells was a potent inhibitor of HIV-1 replication following proviral integration. These data suggest that the anti-HIV-1 activity of ProT α is mediated through the modulation of a number of genes that have been reported to suppress HIV-1 replication including the dysregulation of transcription factors and the induction of PKR and Rantes mRNA (Mosoian *et al.*, 2007).

There is also a pilot study of the safety and efficacy of thymosin alpha 1 in augmenting immune reconstitution in HIV-infected patients with low CD4⁺ cells counts taking highly active antiretroviral therapy (Chadwick *et al.*, 2003).

The T α 1 has been used as adjuvant during vaccinations. An example of clinical efficacy has been shown in vaccination with influenza vaccine in the elderly (Ershler *et al.*, 2007).

In addition, there are cases in the literature of vaccination in animal models supported by T α 1 and prothymosin α 1, which has confirmed the ability of peptides to function as thymic adjuvant in immune response (Shmelev *et al.*, 1994; Shiau *et al.*, 2001).

T α 1 is thought to activate and increase T cells at the site of immunization and in the draining lymph nodes, these observations have gained interest in the clinical study of thymic peptides, especially in view of the possibility of considerable strengthening of innate immune response (Naylor and Hadden, 2003).

A previous study using microarray system to analyse the expression of 8300 genes in peripheral blood mononuclear cells (PBMCs) from healthy human donors after 48 hours of exposure to the T α 1, have shown that T α 1 induces an elevated transcriptional response in PBMCs (Garaci *et al.*, 2003). These results could explain the mechanisms through which T α 1

increases the immune response and exerts its action, especially when used in combination therapy, for the treatment of cancer or infectious diseases.

The use of T α 1, as immunotherapeutic treatment in combination with conventional drugs, has shown that it was an important lymphokine for immune restoration without substantial toxic effects for the patient and earning an in-depth study of the mechanisms that govern their activities.

Human immunodeficiency virus type 1 (HIV-1)

The first cases of the acquired immune deficiency syndrome (AIDS) were reported in 1981 but it is now clear that cases of the disease have been occurring unrecognized for at least 4 years before its identification. The disease is characterized by an increased susceptibility to infections caused by opportunistic pathogens or by the occurrence of an aggressive form of Kaposi's sarcoma or B-cell lymphoma, accompanied by a profound decrease in the number of CD4⁺ T cells. As it seemed to be spread by contact with body fluids, it was early suspected to be caused by a new virus. By 1983 the agent now known to be responsible for AIDS, called the human immunodeficiency virus (HIV), was isolated and identified (Barre-Sinoussi, 1996). The initial infection with HIV generally occurs after transfer of body fluids from an infected person to an uninfected one. The virus is carried in infected CD4⁺ T cells, dendritic cells, and macrophages, and as a free virus in blood, semen, vaginal fluid and milk. Most patients who are infected with HIV will eventually develop AIDS, after a period of apparent quiescence of the disease known as clinical latency or the asymptomatic period (**Figure A**).

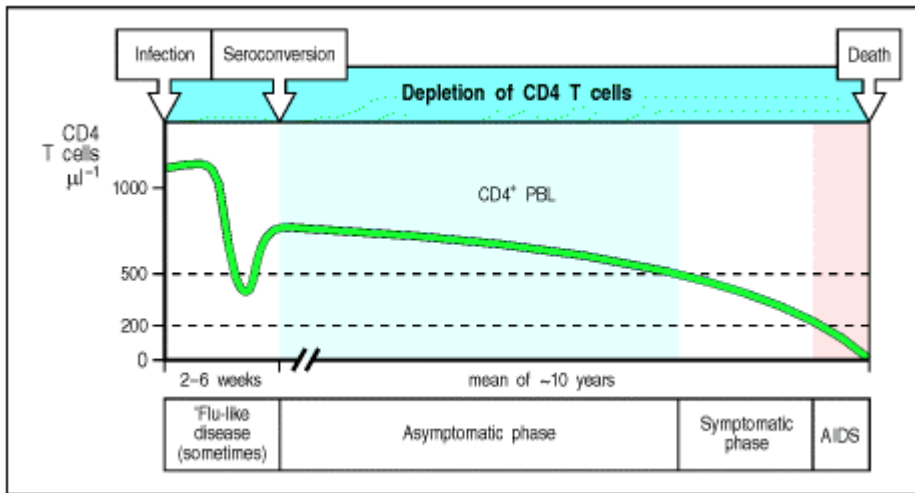


Figure A *The course of HIV infection*

This period is not silent, however, there is persistent replication of the virus and gradual decline in the function and numbers of CD4⁺ T cells. At this point, which can occur anywhere between 2 and 15 years or more after the primary infection, the period of clinical latency ends and opportunistic infections begin to appear. However, it has become increasingly clear that the course of the disease can vary widely. Thus, although most people infected with HIV go on to develop AIDS and ultimately to die of opportunistic infection or cancer, this is not true for all individuals (Baltimore, 1995; McCune, 1995).

Biology of HIV

One of the proteins that enters the cell with the viral genome is the viral reverse transcriptase, which transcribes the viral RNA into a complementary DNA (cDNA) copy. The viral cDNA is then integrated into the host cell genome using viral integrase, which also enters the cell with the viral RNA. The infectious cycle up to the integration of the provirus is shown in **Figure B**. In activated CD4⁺ T cells, viral replication is initiated by transcription of the

provirus, as shown in the next section. However, HIV can, like other retroviruses, establish a latent infection in which the provirus remains quiescent. This seems to occur in memory CD4⁺ T cells and in dormant macrophages, and these cells are thought to be an important reservoir of infection. The entire HIV genome consists of nine genes flanked by long terminal repeat sequences (LTRs), which are required for the integration of the provirus into the host cell DNA and contain binding sites for gene regulatory proteins that control the expression of the viral genes (**Figure C**). Like other retroviruses, HIV has three major genes *gag*, *pol*, and *env*. The *gag* gene encodes the structural proteins of the viral core, *pol* encodes the enzymes involved in viral replication and integration, and *env* encodes the viral envelope glycoproteins. The *gag* and *pol* mRNAs are translated to give polyproteins chains that are cleaved by the viral protease (also encoded by *pol*) into individual functional proteins. The product of the *env* gene, gp160, has to be cleaved by a host cell protease into gp120 and gp41, which are then assembled as trimers into the viral envelope. HIV has six other, smaller, genes encoding proteins that affect viral replication and infectivity in various ways (Kinoshita, 1997, Cullen 1998). The production of infectious virus particles from an integrated HIV provirus is stimulated by a cellular transcription factor that is present in all activated T cells. Activation of CD4⁺ T cells induces the transcription factor NFκB, which binds to promoters not only in the cellular DNA but also in the viral LTR, thereby initiating the transcription of viral RNA by the cellular RNA polymerase. This transcript is spliced in various ways to produce mRNAs for the viral proteins. The Gag and Gag-Pol proteins are translated from unspliced mRNA; Vif, Vpr, Vpu, and Env are translated from singly spliced viral mRNA; Tat, Rev, and Nef are translated from multiply spliced mRNA. At least two of the viral genes, *tat* and *rev*, encode proteins, Tat and Rev respectively, that promote viral replication in activated T cells. Tat is a potent transcriptional regulator, which functions as an elongation factor that enables the transcription of viral RNA by the RNA polymerase II complex. Tat encloses two binding sites, contained in one domain, named the transactivation domain.

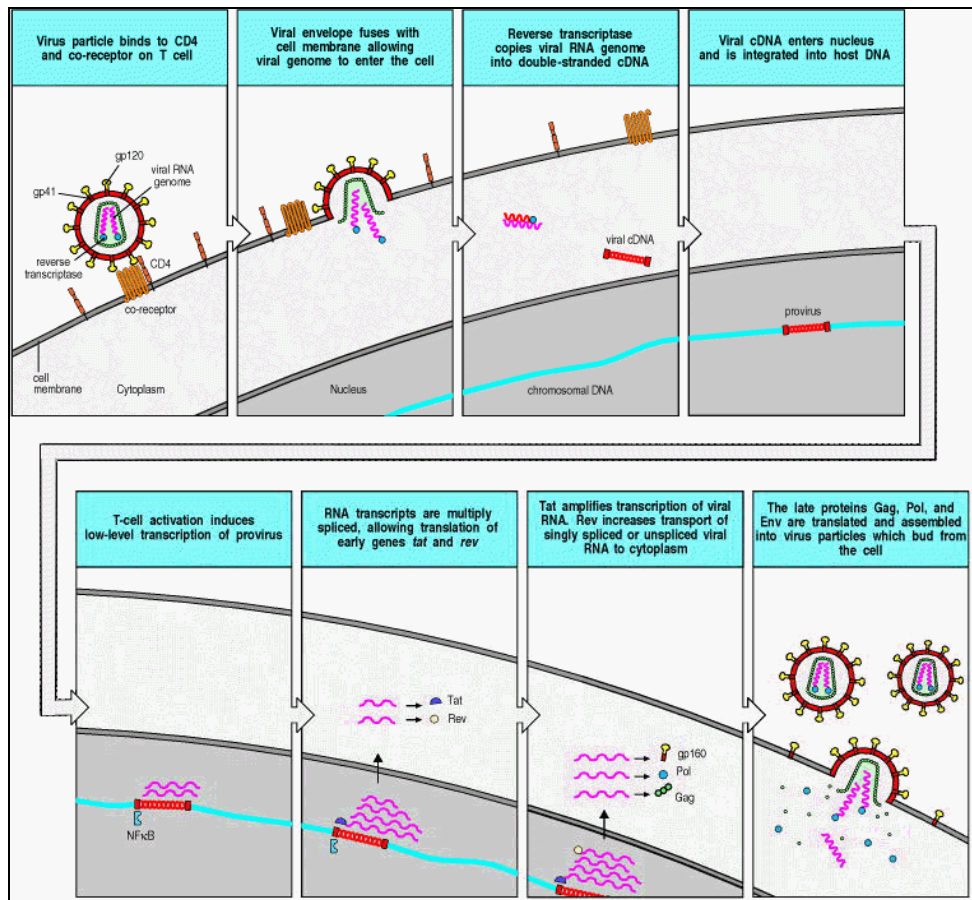


Figure B *The infectious cycle of HIV*

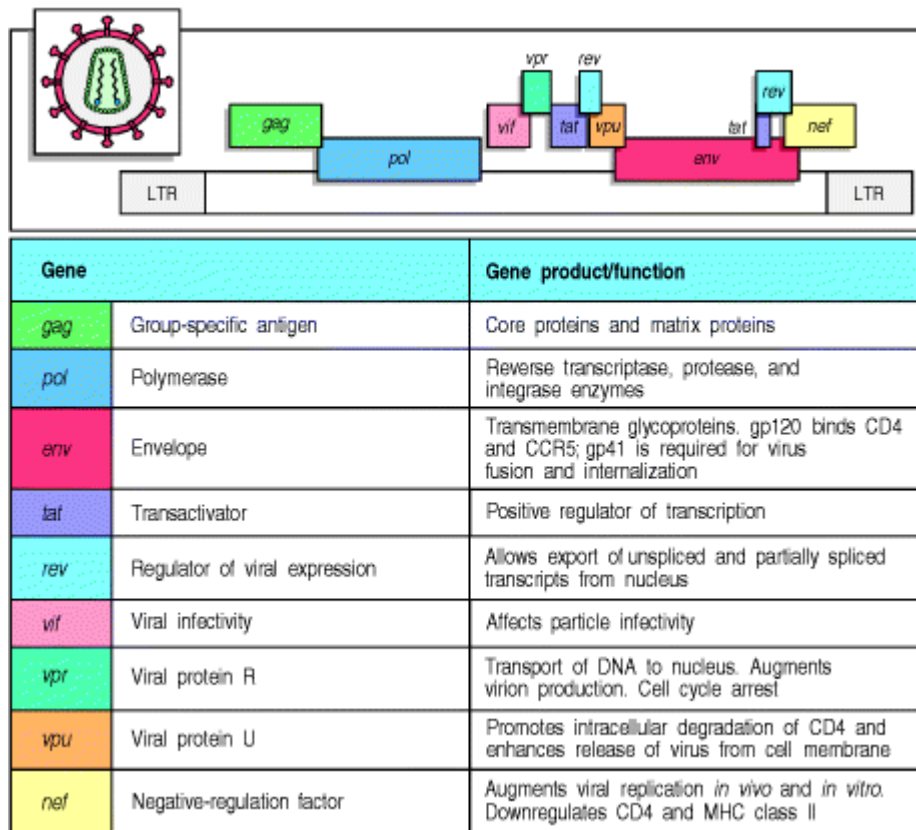


Figure C *The Genome of HIV*

The first of these allows Tat to bind to a host cellular protein, cyclin T1. This binding reaction promotes the interaction of the Tat protein through the second binding site in its transactivation domain to an RNA sequence in the LTR of the virus known as the transcriptional activation region (TAR). The consequence of this interaction greatly enhances the rate of viral genome transcription, by causing the removal of negative elongation factors that block the transcriptional activity of RNA polymerase II. The expression of cyclin T1 is greatly increased in activated compared with quiescent T lymphocytes. This, in conjunction with the increased expression of NF κ B in activated T cells, may explain the ability of HIV to lie dormant in resting T cells and replicate in activated T cells (Cullen, 2000; Cullen, 1998; Emerman and Malim, 1998; Fujinaga *et al.*, 1999; Kinoshita *et al.*, 1997; Subbramanian and Cohen, 1994; Trono, 1995).

HIV infection: CD4+ T cells, dendritic cells, and macrophages

The ability of HIV to enter particular types of cell, known as the cellular tropism of the virus, is determined by the expression of specific receptors for the virus on the surface of those cells. HIV enters cells by means of a complex of two noncovalently associated viral glycoproteins, gp120 and gp41, in the viral envelope. The gp120 portion of the glycoprotein complex binds with high affinity to the cell-surface molecule CD4. This glycoprotein thereby draws the virus to CD4 T cells and to dendritic cells and macrophages, which also express some CD4. Before fusion and entry of the virus, gp120 must also bind to a co-receptor in the membrane of the host cell. Several different molecules may serve as a co-receptor for HIV entry, but in each case they have been identified as chemokine receptors.

Chemokine and HIV-1: interactions and clinical implications.

Chemokines are small peptides that are potent activators and chemoattractants for leukocyte subpopulations and some non-hematopoietic cells. These chemotactic cytokines belong to the chemokine superfamily, which can be divided into 4 groups or families (CXC, CX₃C, CC, and C) based on a cysteine motif (**Table 1**). Their actions are mediated by a family of 7-transmembrane G-protein-coupled receptors (Onuffer and Horuk, 2002). Chemokines are known to function as regulatory molecules in leukocyte maturation, trafficking, homing of lymphocytes and in the development of lymphoid tissues. Besides these functions in the immune system, they also play a critical role in many pathophysiological processes such as allergic responses, infectious and autoimmune diseases, angiogenesis, inflammation, tumor growth and hematopoietic development (Onuffer and Horuk, 2002). Alteration and inappropriate expression of chemokine receptors is thought to be implicated in multiple sclerosis, asthma, some cancers, rheumatoid arthritis and some cardiovascular diseases. In

addition, certain chemokines and their receptors are involved in HIV pathogenesis. The discovery of chemokine receptors as co-receptors for HIV-1 has opened the door for a number of novel anti-viral approaches. Two chemokine receptors, known as CCR5, which is predominantly expressed on dendritic cells, macrophages, and CD4⁺ T cells, and CXCR4, expressed on activated T cells, are the major co-receptors for HIV. After binding of gp120 to the receptor and co-receptor, the gp41 causes fusion of the viral envelope and the plasma membrane of the cell, allowing the viral genome and associated viral proteins to enter the cytoplasm. There are different variants of HIV, and the cell types that they infect are determined to a large degree by the chemokine receptor they bind as co-receptor. The variants of HIV that are associated with primary infections use CCR5, which binds the CC chemokines RANTES, MIP-1 α , and MIP-1 β , as a co-receptor, and require only a low level of CD4 on the cells they infect. These variants of HIV infect dendritic cells, macrophages, and T cells *in vivo*. However, they are often described simply as 'macrophage-tropic' because they infect macrophage but not T-cell lines *in vitro* and the cell tropism of different HIV variants was originally defined by their ability to grow in different cell lines. In contrast, 'lymphocyte-tropic' variants of HIV infect only CD4⁺ T cells *in vivo* and use CXCR4, which binds the CXC chemokine stromal-derived factor-1 (SDF-1), as a co-receptor. The lymphocyte-tropic variants of HIV can grow *in vitro* in T-cell lines, and require high levels of CD4 on the cells that they infect. Infection of CD4⁺ T cells via CCR5 occurs early in the course of infection and continues to occur, with activated CD4⁺ T cells accounting for the major production of HIV throughout infection. Late in infection, in approximately 50% of the cases, the viral phenotype switches to a T-lymphocyte-tropic type that utilizes CXCR4 co-receptors, and this is followed by a rapid decline in CD4⁺ T-cell count and progression to AIDS (Chan and Kim, 1998) and can be blocked by therapeutic antagonists of the CCR5 receptor.

Cytokines and HIV-1: interactions and clinical implications.

Cytokines play an important role in controlling the homeostasis of the immune system (**Table 2**). Infection with HIV results in dysregulation of the cytokine profile *in vivo* and *in vitro*. During the course of HIV-1 infection, secretion of T-helper type 1 (Th1) cytokines, such as interleukin (IL-2), and antiviral interferon (IFN)-gamma, is generally decreased, whereas production of T helper type 2 (Th2) cytokines, such as IL-4, IL-10, proinflammatory cytokines (IL-1, IL-6, IL-8) and tumour necrosis factor (TNF)-alpha, is increased. Such abnormal cytokine production contributes to the pathogenesis of the disease by impairing cell-mediated immunity. A number of cytokines have been shown to modulate *in vitro* HIV-1 infection and replication in both CD4+ T lymphocytes and cells of macrophage lineage. HIV-inductive cytokines include: TNF-alpha, TNF-beta, IL-1 and IL-6, which stimulate HIV-1 replication in T cells and monocyte-derived macrophages (MDM), IL-2, IL-7 and IL-15, which upregulate HIV-1 in T cells, and macrophage-colony stimulating factor, which stimulates HIV-1 in MDM. HIV-suppressive cytokines include: IFN-alpha, IFN-beta and IL-16, which inhibit HIV-1 replication in T cells and MDM, and IL-10 and IL-13, which inhibit HIV-1 in MDM. Bifunctional cytokines such as IFN-gamma, IL-4 and granulocyte-macrophage colony-stimulating factor have been shown to have both inhibitory and stimulatory effects on HIV-1. The beta-chemokines, macrophage-inflammatory protein (MIP)-1alpha, MIP-1beta and RANTES are important inhibitors of macrophage-tropic strains of HIV-1, whereas the alpha-chemokine stromal-derived factor-1 suppresses infection of T-tropic strains of HIV-1 (Kedzierska and Crowe, 2001).

Highly active antiretroviral therapy (HAART)

Considerable progress has been made in the development of substances that interfere with the various stages of the replication cycle of HIV. Current anti-HIV therapeutic strategies were developed by the early 1990s, and they consist of at least three different low molecular weight inhibitors of the HIV enzymes: reverse transcriptase and the HIV protease. Used properly, these compounds are highly active against HIV. The first group of drugs encompasses both nucleoside and nucleotide analogues and the non-nucleoside reverse transcriptase inhibitors. The second group includes protease inhibitors of HIV-1 blocking the maturation of the viral precursor proteins, preventing the generation of viral proteins copies.

Used alone, these medicines determine the onset of mutants that can resist their action and it is for this reason that in therapy nucleoside inhibitors are associated with those of protease, such anti-HIV drug combination regimes have been generally referred to as HAART (for highly active antiretroviral therapy) .

HAART patients show a significant increase in the absolute number of CD4+ lymphocytes and CD8+, a reduction in the expression of molecules and activation of a transient improvement of immune response. The HAART has allowed us to make the transformation of AIDS into a chronic disease since it allows you to sets aside the plasma viremia for a long time, although it has some side effects such as toxicity and development of resistance (Richmann , 2006). There are, at present, 22 compounds which have been formally approved by the US Food and Drug Administration for the treatment of HIV infection (AIDS). According to their point of intervention with the HIV replicative cycle, these compounds can be classified in 5 categories: (1) NRTIs (nucleoside reverse transcriptase inhibitors): azidothymidine, didanosine, zalcitabine, stavudine, lamivudine, abacavir and emtricitabine; (2) NtRTIs (nucleotide reverse transcriptase inhibitors): tenofovir, administered as its oral prodrug form TDF (tenofovir disoproxil fumarate); (3) NNRTIs (non-nucleoside reverse transcriptase inhibitors): nevirapine, delavirdine and efavirenz; (4) PIs (HIV protease

inhibitors): saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, tipranavir and darunavir; and (5) FIs (fusion inhibitors): enfuvirtide. Starting from the drugs which are currently available for the treatment of AIDS, numerous combinations could be envisaged. Drug combinations are, in principle, aimed at obtaining synergism between the compounds, reasonably expected if they act by different mechanisms, while reducing the likelihood for drug resistance development (De Clercq, 2007).

New therapeutic approaches

Currently, a number of compounds that act on new viral targets are being investigated including integrase inhibitors, which were found to be extremely toxic. There are ongoing clinical studies on antagonists of HIV coreceptors, while at an early stage of experimentation are compounds that interact with cellular factors that bind the LTR, and molecules which can act as regulators and inhibitors of cell transcriptional factor NF- κ B and Tat viral protein (De Clercq, 2002). Information was already available on HIV fusion and the earlier event of HIV attachment, the last and first stages of entry, respectively (Gallo, 2002). Identifying and elucidating the mechanisms of action of naturally occurring HIV suppressive factors, led to the discoveries of RANTES, MIP-1 α , and MIP-1 β as HIV inhibitors. In addition, after the discoveries of the chemokine coreceptor usage in HIV, the design of agonists and antagonists of them represent a new avenue of therapy. Their identification help to lead to the discoveries of the HIV coreceptors (CCR5 and CXCR4), a major development in HIV pathophysiology. Other HIV suppressor factors exist and their identification and elucidation of their molecular mechanisms for inhibiting HIV will help to open other new avenues of therapy (Gallo, 2002).

T α 1 and HIV therapy

The possibility to combine antiretroviral chemotherapy with immunotherapy has been suggested years ago. The introduction of the highly active antiretroviral therapy (HAART), able to reduce the viral load in most of the patients, has remarkably reduced the mortality associated to HIV infection. The most relevant immunological consequence of HAART in responders patients is an appreciable quantitative and functional, at least in part, recovery of the CD4⁺ T cell compartment. Treatment of HIV-infected patients with HAART leads to immune reconstitution as shown by increases in CD4⁺ lymphocyte counts, decreased risk of opportunistic infections and improved survival (Palella *et al.*, 1998; Valdez *et al.*, 2001). However, a variable percentage of individuals exhibits a discordant response to HAART. Particularly, some patients show an unsatisfactory increase of CD4⁺ T cells, despite a successful virological response (immunological failure), with risk of serious clinical events. (Saravolatz *et al.*, 1996; Floridia *et al.*, 2002). These patients may benefit from the adjunctive use of immunomodulatory compounds that enhance immune reconstitution. In a previous clinical trials in HIV-infected patients, thymosin α_1 was shown to induce increase in CD4⁺ cell counts, but only when given in combination with either interleukin (IL)-2 or interferon (IFN)- α (Garaci, 1994). These studies were conducted in patients taking zidovudine monotherapy and the efficacy of thymosin α_1 in patients with full viral suppression on combination antiretroviral therapy is unknown. A pilot study (Chadwich *et al.*, 2003) was conducted to determine the safety and efficacy of thymosin α_1 injections in conjunction with combination antiretroviral therapy in patients with advanced HIV infection. It was demonstrated that thymosin α_1 was very well tolerated in this group of patients, which is in agreement with other clinical trials of thymosin α_1 in HIV-infected patients and in patients with hepatitis B and C infection (Lau *et al.*, 2002). The good tolerability contrasts with immunomodulatory cytokines such as IFN- α or IL-2 that have been shown to have substantial toxicity in patients with HIV infection as well as other groups. No benefit of thymosin α_1 in

augmenting CD4⁺ cell count response to HAART was demonstrated. It is conceivable that a higher dose of thymosin α_1 might have a greater effect on CD4⁺ cells, although the dose used for the study was relatively high in comparison to previous studies in patients HIV infection and currently recommended dosage for use in the treatment of hepatitis virus infections. It is also possible that an effect might have become apparent after a longer duration of therapy. In one previous trial, thymosin alpha 1 (given in conjunction with IFN- α) had its greatest effect after 12 months of treatment (Garaci, 1994) in a group of patients with advanced immunodeficiency and it is possible that a greater CD4 response might be obtained in patients with higher initial CD4 counts. HAART has been shown to allow reconstitution of both subsets of T cells (Douek *et al.*, 1998) and there is recent evidence that one mechanism for this effect is through production of *de novo* naive T lymphocytes in the thymus. Immunophenotyping studies were performed to assess the effect of thymosin 1 α in stimulating expansion of naive and memory T cells (Chadwich *et al.*, 2003). Some studies have suggested that impaired T cell restoration in patients with low CD4⁺ cell counts when starting HAART may result from impaired thymic function (Teixeira *et al.*, 2002). The lack of any specific effect of thymosin 1 α in this study, in terms of increases in numbers of naive or memory CD4⁺ or CD8⁺ T cells, did not support a role of thymosin in selectively stimulating these particular lymphocyte subtypes. The most interesting effect of thymosin was the significant increase in signal joint (sj) T cell receptor excision circles (TRECs) levels seen after 12 weeks of treatment. Increase in the *de novo* production of naive T cells by thymopoiesis may be an important component of immune reconstitution in HIV-infected patients taking HAART ,and sjTREC levels have been shown to predict survival in HIV-infected patients as well as the total CD4 count response to HAART after several years (Hatzakis *et al.*, 2002; Pido *et al.*, 2002; Mussini *et al.*, 2002). Further studies are needed to investigate which genes can be regulated by T α 1 in HIV infected individuals in order to

obtain new information on transcriptional activity of T α 1 as adjuvant immunotherapy to HAART.

AIM OF THE STUDY AND EXPERIMENTAL DESIGN

Thymosin alpha-1 (T α 1) has shown a variety of effects on cells and pathways of the immune system. T α 1 has been shown to exert an immunomodulatory activity towards both T cell and NK maturation. Moreover, T α 1 has been shown to act on effector functions of mature lymphocytes, such as cytokine production and cytotoxic activity. However, molecular mechanisms underlying the potent activity of T α 1 have not completely elucidated. Several clinical studies on patients with infectious or other diseases, have already demonstrated the high tolerability and the absolute safety of T α 1 (Garaci *et al.*, 2000). In addition, T α 1 has the ability to activate infected dendritic cells through Toll-like receptor signalling, thus influencing the inflammation balance, and increasing the expression of tumour, viral, and major histocompatibility complex (MHC) I antigens (Giuliani *et al.*, 2000; Romani *et al.*, 2007). However, information regarding a possible use of T α 1 in patients with HIV infection is very limited. There is only one recent study, showing T α 1 utilization in combination with HAART, with encouraging results on T-cell compartment maturation (Chadwick *et al.*, 2003).

A previous study using a microarray to analyse the expression of 8300 genes in PBMCs from healthy human donors after 48 hours of exposure to the T α 1, has shown that T α 1 induced an elevated transcriptional response in lymphoid cells (Garaci *et al.*, 2003), thus giving a novel impetus in understanding the pleiotropic effects exerted by T α 1 and the molecular mechanisms involved in its action. The principal aim of the experiments performed in the framework of this Ph.D. thesis was to investigate which genes belonging to immune response pathways could be regulated by T α 1 in vitro in PBMCs from HIV infected individuals. This, to obtain new information on the effect of T α 1 on the transcriptional activity in these patients and to further understand the impaired immune

response in PBMCs from HIV+ patients and a possible helpfulness of the use T α 1 in combination with antiretroviral therapy for the control of HIV infection. To this purpose, a cohort of HIV+ individuals was enrolled in an open cross-sectional study by the “AIDS Center”, S. Giovanni Hospital, Rome. In *vitro* PBMCs from HIV+ individuals and from healthy donors were isolated. Fresh cells were used for flow cytometry analysis of surface molecule expression (CD4, CD8). Cells were then incubated with T α 1 (50 μ g/ml) for 48h to evaluate gene expression profile using the "Human Autoimmune & Inflammatory Response" microarray systems (SuperArray). Based on the results of microarray analysis, the expression of some selected genes, presumably modulated by T α 1, was quantified using quantitative Real Time PCR.

MATERIALS AND METHODS

Patients and samples

Eleven HIV-1-infected patients with different response to HAART, were enrolled in a open cross-sectional study by Department of Infectious and Tropical Disease, University of Rome “La Sapienza,” or the “AIDS Center”, S. Giovanni Hospital, Rome. The characteristics of the patients are listed in the results (**Table 4**).

The study received the ethics approval by the institutions participating in the study and informed consent was obtained from the patients.

PBMCs from HIV+ individuals and from healthy HIV- donors were isolated from heparinized blood by centrifugation gradient according to standard methods. PBMC isolated were stimulated by T α 1 (50 μ g/ml, SciClone Pharmaceuticals International, USA) for 24 h in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Life Technologies), 2 mM glutamine, 50 IU/mL penicillin, and 50 IU/mL streptomycin (Hyclone), and then readed for others 24h. Cells were then harvested, washed twice in PBS and used for flow cytometry analysis of cellular proteins and RNA preparation to evaluate gene profile expression.

Viral Load

HIV-1 RNA Viral Load (VL) levels were determined using a quantitative ultrasensitive reverse transcription-polymerase chain reaction assay (limit of detection was 50 HIV RNA copies/mL).

Immunostaining and flow cytometry analysis of cellular proteins

For the extracellular protein staining, approximately 5×10^5 cells were resuspended in 50 μ l of PBS with PE conjugated anti-human CD8 and PerCP conjugated anti-human CD4 (BD Biosciences) or irrelevant control antibody and incubated at 4°C for 30 min. After treatment, cells were washed twice in PBS and stored at 4°C. Flow cytometry analysis was performed by a FACScan flow cytometer and Cell Quest™ software.

Extraction of RNA

RNA isolation was performed using Trizol (Life Technologies, Grand Island, NY), according to the manufacturer's instructions. The quantity and the quality of all RNA preparations were assessed by gel electrophoresis and OD₂₆₀/OD₂₈₀ ratios. RNA was used for microarray analysis as well as for quantitative reverse transcription-polymerase chain reaction (Real-time PCR).

Microarray

For these experiments it was used a Oligo GEArray Human Autoimmune & Inflammatory response microarray systems (SuperArray, Bethesda, MD). This Oligo GEArray profiles the expression of 440 genes involved in the autoimmunity and inflammation immune responses. It represents expression of cytokines, chemokines, and their receptors, as well as genes related to cytokine metabolism, production and cytokine-cytokine receptor interaction. Genes involved in the acute phase, inflammatory and humoral immune responses are also represented. Controls are represented by four potentially normalized genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA), glucuronidase beta

(GUSB), and β -actin. Starting from total RNA (3,5 μ g) it was utilized a proprietary and patent-pending linear RNA amplification and labelling procedure to synthesize labelled antisense RNA (aRNA) also known as labeled cRNA target. Total RNA was amplified, and cRNA probes were generated by biotin-16-dUTP (Roche Diagnostics, GmbH) incorporation using the AmpoLabeling-LPR Kit (SuperArray, Frederick, MD), according to the manufacturer's instructions.

The array membranes (SuperArray) were prehybridized in GEAhyb (SuperArray) solution for 2 h at 60°C. Labeled cRNA probes were hybridized overnight at 60°C with continuous agitation. After two wash hybridized cDNA probes were detected by chemiluminescence. Images of the membranes were recorded on x-ray films (Amersham Biosciences, UK ltd) and were digitally recorded on a personal densitometer scanner. Data were further processed with GEMatrix Analyzer software (<http://www.superarray.com>). All signal intensities were PUC18 plasmid (negative control) DNA background subtracted, and normalized to the housekeeping genes.

Relative gene expression levels were calculated as the ratio between the mean values of gene expression in PBMCs from HIV infected in patients or healthy donors treated with Ta1 and the mean values obtained from untreated control .

Changes in gene expression was expressed as a fold increase/decrease. The cut-off to express the fold induction or inhibition of gene expression in comparison with controls was ≤ 1.5 or ≤ 0.6 fold-changes respectively. Genes which suited both above criteria were considered to be induced or suppressed. Scatter plots were made from normalized signals.

Real-time PCR

Real-time PCR was performed on a ABI Prism 7300 instrument (PE Applied Biosystems, Foster City, CA, USA) with gene-specific primers and the SybrGreen protocol to confirm the gene expression changes observed in the microarray analysis. All primers employed were cDNA specific and were purchased from Primm (MI, Italy). Names and primers for all genes quoted in the text are listed in **Table 3**. An amount of 0.75 micrograms of total RNA from each sample was reverse-transcribed in a total volume of 20 µl with high-capacity cDNA Reverse Transcription Kits (Applied Biosystems), according to the manufacturer's instructions.

Amplification of specific PCR products was detected using the SYBR Green PCR Real Master Mix (SuperArray). The RQ-PCR was performed in duplicated in a total reaction volume of 25 µL containing 11,25 µL SYBR Green PCR Master Mix, 150 nM forward and reverse primers, 10,25 µL dH₂O, and 750 ng cDNA as a template. Samples were heated for 10 min at 95°C and were subjected to 45 cycles of PCR amplification, each cycle consisting of 15s at 95°C and 60s at 60°C. Within each experiment, no template control (NTC) and housekeeping genes β-actin (ACTB), used as reference, were run in parallel to verify any contamination and to determine amplification efficiency. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of unspecific products and primer dimers. Quantification was performed using the Ct comparative method, relative gene mRNA levels were calculated as follows: $2^{-[\Delta C_t(T\alpha.1) - \Delta C_t(CTR)]} = 2^{-\Delta\Delta C_t}$, where $\Delta C_t(\text{sample}) = [C_t(\text{gene target}) - C_t(\text{ACTB})]$ represents the difference, in threshold cycle number, between genes and ACTB.

Gene bank number	name		SEQUENCE 5'→3'
NM_002988	CCL18 (PARC)	<i>FORWARD</i> <i>REVERSE</i>	CCAAGCCCCAGCTCACTCT TGCAAGGCCCTTCATGATG
NM_002982	CCL2 (MCP-1)	<i>FORWARD</i> <i>REVERSE</i>	CAAGCAGAAGTGGGTTTCAGGAT TCTTCGGAGTTTGGGTTTGC
NM_002990	CCL22 (MDC)	<i>FORWARD</i> <i>REVERSE</i>	TGCCCTGGGTGAAGATGATT AAGGCCACGGTCATCAGAGT
NM_002983	CCL3 (MIP1alpha)	<i>FORWARD</i> <i>REVERSE</i>	CCATGGCTCTCTGCAACCA GCGGTCGGCGTGTCA
NM_021006	CCL3L1 (LD78 beta)	<i>FORWARD</i> <i>REVERSE</i>	GCTGACACTCGAGCCACAT AGCAGTGGAGACCTGCATGAT
NM_001001435	CCL4L1	<i>FORWARD</i> <i>REVERSE</i>	CACCGCCTGCTGCTTTTC TAATCTACCACAAAGTTGCGAGGAA
NM_002985	CCL5 (Rantes)	<i>FORWARD</i> <i>REVERSE</i>	AGCCTCTCCCACAGGTACCA GGCAGTAGCAATGAGGATGACA
NM_005211	CSF1R	<i>FORWARD</i> <i>REVERSE</i>	CCTGCTGACTGTTGAGACCTTAGA CACTCCCCACGCTGTTGTG
NM_000758	CSF2	<i>FORWARD</i> <i>REVERSE</i>	TTCTGCTTGTCATCCCCTTTG CTCATCTGGCCGGTCTCACT
NM_000760	CSF3R	<i>FORWARD</i> <i>REVERSE</i>	AGGCGATCTGCATACTTTAAGGA AACAAGCACAAAAGGCCATTG
NM_022059	CXCL16	<i>FORWARD</i> <i>REVERSE</i>	GGAGTCTCGCTCTGTATCCA GTTGCAGTGAGCCGAGATCA
NM_002341	LTB	<i>FORWARD</i> <i>REVERSE</i>	CACCCCGATATGGTGGACTT CCATCACGGCCCCAAAG
NM_002415	MIF	<i>FORWARD</i> <i>REVERSE</i>	GCGGGTCTCCTGGTCCTT GCACGTTGGTGTTTACGATGA
NM_002468	MYD88	<i>FORWARD</i> <i>REVERSE</i>	GAGCTGGCGGGCATCAC TCGAAACGCTCAGGCATATG
NM_003264	TLR2	<i>FORWARD</i> <i>REVERSE</i>	GCGGGAAGGATTTTGGGTAA TGGTCTTAAATATGGGAACCTAGGA

Table 3

Primer pairs used in quantitative reverse transcription-polymerase reaction analysis.

Statistical Analysis

Statistical Analysis were done using SPSS (version 12.0, SPSS). For statistical comparisons of the laboratory data from HIV+ patients and HIV- individuals, the independent samples *t*-test analysis was performed; for statistical comparisons of the laboratory data within each patients group, the paired samples *t*-test analysis was performed; for non-parametric bivariate correlation, Spearman's rho correlation coefficient was calculated. For statistical and graphical reasons, an arbitrary value of 50 HIV RNA copies/mL was assigned to samples under the limit of HIV-RNA detection and the numbers of HIV copies/mL were transformed as decimal logarithms.

RESULTS

Patients characteristics: virological and immunological status.

Eleven HIV-1-infected patients with different response to HAART, were enrolled in a open cross-sectional study on the effect of thymosin alpha 1 in vitro, at the Department of Infectious and Tropical Disease, University of Rome “La Sapienza,” and at the “AIDS Center”, S. Giovanni Hospital, Rome. For comparison, a group of HIV, HBV, HCV negative healthy donors were also enrolled in the study. The characteristics of the patients are shown in **Table 4**. The table list encompasses the age of the patients (average 41), the sex (3 female and 8 male), the HIV-1 RNA viral load (VL) levels (8 patients < 50 copies/ml; 1 patient < 5000 copies/ml; 1 patient = 49915 copies/ml and 1 patient = 135000 copies/ml); the average CD4⁺ cell number was 587 (cells/mm³) and the average CD8⁺ cell number was 1163 (cells/mm³). As a percentage value, the mean CD4⁺ cells were 23,82% and CD8⁺ cells were 46,27%. All patients, except one, were under HAART during this study. The results, reported in **Figure 1**, show a statistically significant ($p < 0,001$) inverse ratio of CD4⁺ and CD8⁺ peripheral blood mononuclear cells in HIV⁺ patients, while the healthy donors showed a normal phenotype. In fact, the percentages of positive cells, as detected by flow cytometry, in fresh PBMCs were: CD4⁺ = $41,94 \pm 3,5$ % and CD8⁺ = $38,52 \pm 2,87$ %, for healthy donors, and CD4⁺ = $23,81 \pm 2,52$ % and CD8⁺ = $46,27 \pm 1,83$ %, for HIV⁺ patients, respectively (mean $\pm$ standard error). As shown in **Figure 2**, in this group of patients VL levels, expressed as Log₁₀ of the RNA copies number per mL, were significantly inversely correlated with levels of CD4⁺ cells per mm³ (Spearman’s rho = -723; $p = 0,018$). Taking into consideration that most of the patients were under therapy and that the viral load was <50 copies/mL, results demonstrate a general, both virological and immunological, positive response to the therapy.

Patient	Age	Sex	HIV-RNA (copies/ml)	CD4 (cells/mm3)	CD8 (cells/mm3)	CD4 %	CD8 %	THERAPY
1	34	M	50	831	1220	32	47	LPV/r+3TC+DDI
2	44	F	50	624	1170	24	45	TDF+TMC114+R
3	52	M	50	513	1642	15	48	ATV200+DDI+3TC
4	46	M	50	571	1088	21	40	3TC+DDI+EFV
5	43	M	1300	511	1022	24	48	ATV150+ABC+3TC
6	43	M	50	829	800	41	38	3TC+AZT+LPV/r
7	44	F	49915	536	839	28	44	no therapy
8	26	M	50	687	1026	24	45	TPV+TDF+3TC
9	49	M	135000	106	791	9	46	LPV/r+3TC+DDI (no adherente)
10	33	M	50	674	2089	20	62	ATV/r+ddi+3TC
11	36	F	50	580	1108	24	46	TPV+TDF+3TC

Table 4

Characteristics of patients.

Evaluation of the effects of thymosin alpha 1 on the transcriptional activity of PBMCs from HIV+ and HIV- individuals, by microarray assay

In order to provide new information on the effects of thymosin alpha 1 (T α 1) in vitro on the transcriptional activity of PBMCs from HIV+ patients and to compare these effects on those exerted on PBMCs of healthy donors, the “Human Autoimmune and Inflammatory response” microarray system (SuperArray) was used. PBMCs from HIV+ or HIV- healthy donors were stimulated with T α 1 (50 μ g/ml) for 48 h and the mRNA from different samples was used to perform the microarray analysis of 440 gene expression. The results were expressed as fold change with respect to corresponding untreated control samples, after subtraction of background and normalization to the housekeeping expression from either counterpart. The cytokine, chemokine and related receptor genes correspond to about 41% of the total genes present in the microarray. Other genes involved in cytokine and cytokine-receptor interaction represent another 15% of the total genes. The genes involved in humoral immune and inflammatory responses correspond to 12% and 32% of the total genes present in the microarray, respectively (**Figure 3, panel a**). The summary of percentage values of the T α 1-regulated genes in PBMCs from HIV+ patients according to their functional gene grouping is reported in **Figure 3, panel b**. Cytokine, chemokine and their receptor genes represented 24 % of the total genes modulated by T α 1, while other genes involved in cytokine-cytokine receptor interaction represented another 20%. The remaining genes modulated by T α 1 were grouped in the humoral immune response sub-group for 18% , and in the inflammatory response subgroup for 38 %.

Conversely, **Figure 3, panel c** shows the distribution of the T α 1-modulated genes in healthy donors. The percentage values of modulated genes were: 29% for cytokine, chemokine and receptor gene subgroup, 14% for the other genes involved in cytokine-cytokine receptor interaction, 16% for the humoral immune response subgroup and 41% for the inflammatory

response subgroup. These results show that the effects on the transcriptional response by thymosin alpha 1 were effective on genes belonging to the same functional groups in PBMCs from HIV+ as well as in HIV- donors.

The number of positively and negatively T α 1-modulated genes in PBMCs from HIV+ and in healthy donors is shown in **Figure 4**. Ninety-four genes were regulated in HIV + patients, of which 64% were up regulated and 36% were down regulated. In HIV- donors the total number of genes regulated were 74, of which 62% were positively modulated and 38% were negatively modulated. These results show that the number of genes modulated by T α 1 was relatively high in HIV+ as well as in HIV- donors, and that the distributions of positively and negatively modulated genes were comparable in the two groups.

The distribution of the 94 mRNAs whose transcription was significantly different in the presence of T α 1 in PBMCs from HIV infected patients, compared to untreated control samples, and selected on the basis for the family group, are listed in **Tables 5-9**. On the other hand, **Tables 10-12** show the gene subgroup distribution of the 74 mRNAs modulated by T α 1 in PBMCs from healthy donors. Each table contains the gene names, a brief description of the function, the gene bank access number of the mRNA sequence, and the fold change. Particularly, the fold change represents the ratio between the mean values of gene expression in PBMCs from HIV+ patients or healthy donors treated with T α 1 and the mean values obtained from corresponding untreated control samples.

The effects of T α 1 treatment on the transcriptional response of PBMCs from HIV+ patient and HIV– donors is described in **Figure 5-9**. The relative gene expression levels is expressed as fold change.

The expression of several genes from cytokine, chemokine and their receptors were modulated (17 positively modulated and 13 negatively modulated). In HIV+ patients, genes as MCP-1(CCL2), MIP-1alpha (CCL3), LD78 beta (CCL3L1), CCL4L11, Rantes (CCL5),

PARC (CCL18), MDC (CCL22), interleukin 8 (IL8), CCR1, CCR7, CCRL2, IL4R, IL6R, IL10RA and IL10RB were positively modulated by T α 1, while eotaxin (CCL11), CXCL1, CMTM1, CCR2, CXCR6, IL5RA IL2RG, IFNA1-2 and LEFTY1- 2 were negatively modulated by the same treatment in vitro (**Figure 5a**). Several of these genes are also involved in the humoral and inflammatory response (**Figure 7a and Figure 8**), together with other genes such as interferon regulatory factor 7 (IRF7), myeloid differentiation primary response gene (MYD88), Allograft inflammatory factor 1 (AIF1), Arachidonate 5-lipoxygenase (ALOX5), Leukotriene A4 hydrolase (LTA4H), Toll receptor 2 (TLR2) and Toll receptor 8 (TLR8). Genes involved in cytokine-cytokine receptor interaction were also modulated (16 positively modulated and 9 negatively modulated); among the others, Colony stimulating factor 1 receptor (CSF1R), Colony stimulating factor 2 (CSF2), Colony stimulating factor 3 receptor (CSF3R), Lymphotoxin beta (LTB), Macrophage migration inhibitory factor (MIF) and CD74 were up regulated, while CD28 and chemokine receptor 1 (XCR1) were down regulated (**Figure 6a**). Within the inflammatory response group a high number of genes were regulated in PBMCs from HIV+ patients (34 positively modulated and 13 negatively modulated) (**Figure 8**). T α 1 induced an elevated transcriptional response in HIV+ as well as in HIV- donors, but only few mRNAs were expressed in both groups. In healthy donors, several different cytokines, chemokines and their receptors were modulated by T α 1 (15 positively modulated and 13 negatively modulated). Among the others, CCL7, CCL17, CCL19, MCP-1, CXCL2 and CXCL10 (**Figure 5b**). A number of these genes are also involved in the humoral immune response (**Figure 7b**) together with other genes such as CD86, IL7R and CD22. Genes involved in cytokine-cytokine receptor interaction were also regulated (8 positively modulated and 6 negatively modulated); among the others, lymphotoxin alpha (LTA), CD86 and IL5 were down regulated (**Figure 6b**). In the inflammatory response pathway, 21 genes were positively modulated and 17 were down regulated (**Figure 9**). These results confirm the ability of T α 1 to regulate, in lymphoid cells,

the transcriptional response of a high number of genes involved in the immune response. Interestingly, the transcriptional response to T α 1 treatment is elevated in HIV+ patients as well as in healthy donors, but the degree of modulation is different between the two groups, suggesting a differential effect owing to the basal condition of cellular activation of the PBMCs from HIV+ patients and healthy donors.

Gene expression confirmation by Real time PCR analysis

In order to confirm the results obtained by the microarray analysis, 15 genes which resulted as positively modulated by T α 1 in PBMCs from HIV+ patients were selected and their expression was quantified by Real-time-PCR analysis. These genes include the following: Eight chemokines (MCP-1 (CCL2), MIP 1alpha (CCL3), Rantes (CCL5), PARC (CCL18), MDC (CCL22), LD78 beta (CCL3L1), CCL4L1 and a small inducible cytokine B6 (CXCL16) and 7 genes involved in the inflammatory response and in cytokine-cytokine receptors interaction, such as Macrophage migration inhibitory factor (MIF), Colony stimulating factor 3 receptor (CSF3R), Colony stimulating factor 2 (CSF2), Colony stimulating factor 1 receptor (CSF1R), Lymphotoxin beta (LTB), Myeloid differentiation primary response gene (MYD88) and Toll receptor 2 (TLR2). Quantification was performed using the Ct comparative method and expressed as fold change $\text{Log}_2 (2^{-\Delta\Delta C_t})$. For statistical comparisons of the data within each group of individuals, the paired samples *t*-test analysis was performed. **Figure 10a** shows the results of the Real time PCR analysis for the quantitative evaluation of the expression of genes for chemokines in PBMCs from HIV+ patients after in vitro treatment with thymosin alpha 1. The results of the microarray assay for the gene expression of 4 chemokines were completely confirmed by statistical analysis of Real time PCR results: Rantes (mean fold change= 1,55; p=0,022), PARC (mean fold change= 0,67; p= 0,014), MDC (mean fold change= 0,91; p= 0,009) and CXCL16 (mean fold

change= 2,1; $p= 0,041$). The positive modulation of CCL4L1 by microarray was confirmed (fold change = 0,88), but the difference compared to the untreated control samples was not statistically significant ($p>0.050$). Conversely, expression of MIP-1 alpha transcripts was found unchanged (fold change = 0,05) and transcripts for two genes were negatively modulated by T α 1 by Real time analysis. These results didn't confirm those obtained with the microarray assay. However, the values were not statistically significant (MCP-1, fold change = -0,54, and LD78 beta, fold change= -1,04 , $p>0.050$).

The expression of the same genes was analyzed in PBMCs from healthy donors treated with T α 1, using Real time PCR (**Figure 10b**). Three genes that were found to be positively modulated by the microarray assay were not confirmed by Real time PCR: MCP-1 (fold change = -1,14), Rantes (fold change= -0,43) and CCL4L1 (fold change = -1,8), but the fold change was not statistically significant ($p>0.050$). On the other hand, CXCL16 down modulation by microarray analysis, was confirmed by Real time PCR (fold change = -2,3), even if the difference with the untreated control was not statistically significant ($p>0.050$). Regarding to other analysed genes that showed no significant modulation by microarray, some of them were down modulated by Real time PCR: however, the difference with the untreated control samples was not statistically significant ($p>0.050$). These genes were: MIP1alpha (fold change = -0,85), PARC (fold change = -0,39), LD78 beta (fold change = -0,85) and MDC (fold change = -2,25). **Figure 11a** shows the Real time PCR results for gene expression of genes involved in the inflammatory response and in the cytokine-cytokine receptors interaction. In PBMCs from HIV+ patients treated with thymosin alpha 1, CSF2 and TLR2 expression was not regulated (respectively fold change = 0,058 and 0,06). The others selected genes that were positively modulated by microarray and were significantly up regulated also by Real time analysis were the following: CSF1R (fold change = 1,47; $p= 0,039$), CSF3R (fold change= 2,84; $p= 0,005$), LTB (fold change = 2,5; $p=0,003$), MYD88 (fold change = 1,03; $p= 0,001$) and MIF (fold change = 3,07; $p= 0,044$). In PBMCs from

healthy donors treated with T α 1, the same genes were analysed by Real time PCR (**Figure 11 b**). All genes were found to be not significantly modulated by T α 1 ($p > 0.050$) except for the TLR2 ($p = 0.002$). The Real time PCR analysis for CSF3R expression (fold change = -1,88) confirmed the result of the microarray assay. Conversely, the Real time PCR analysis for CSF1R gene expression (fold change = -1,14) did not confirm the result of the microarray. Two genes analysed that were not significantly modulated by microarray, were found to be up regulated by Real time PCR; they are: CSF2 (fold change = 0,28) and MYD88 (fold change = 0,15). Three other genes were found to be negatively modulated by Real time PCR, i.e. LTB (fold change = -1,89), MIF (fold change = -2,19). and TLR2 (fold change = -0,46; $p = 0.002$), although they were not modulated by microarray assay. The above reported results indicate that T α 1 specifically modulated the expression of genes belonging to the chemokine family or involved in the inflammatory response and in cytokine-cytokine receptors interaction, in PBMCs from HIV+ patients.

Figure 12a, reports the values of some gene expression, normalized for the housekeeping gene and expressed as $\text{Log}_2(2^{-\Delta C_t})$, in HIV+ and HIV- individuals. For statistical comparisons of the laboratory data from HIV+ patients and HIV- individuals, the independent samples *t*-test analysis was performed. Levels of expression of these genes in the untreated samples were not significantly different between the HIV+ and the HIV- groups. After treatment with T α 1, CXCL16, CCL4L1, CSF3R, LTB and PARC genes were positively modulated in PBMCs from HIV+ patients, with a significant difference in comparison with the gene expression in the PBMCs from healthy donors. CXCL16 (untreated samples: HIV+= -2,03 , healthy donors= -2,04; T α 1 treated samples: HIV+= 0,06 , healthy donors= -4,33 , $p = 0.021$), CCL4L1 (untreated samples: HIV+= -0,48 , healthy donors= -1,96; T α 1 treated samples: HIV+= 0,4 , healthy donors= -3,75 , $p < 0.001$), CSF3R (untreated samples: HIV+= -2,1 , healthy donors= -1,22; T α 1 treated samples: HIV+= 0,74 , healthy donors= -3,10 , $p = 0.021$),

LTB (untreated samples: HIV+= -2,01 , healthy donors= -1,3; T α 1 treated samples: HIV+= 0,47, healthy donors= -3,19 , p=0,019) and PARC (untreated samples: HIV+= 0,8 , healthy donors= 1,47; T α 1 treated samples: HIV+= 1,47, healthy donors= 0,27 , p=0,017). As a show in **Figure 12b** the expression of MIF (untreated samples: HIV+ = - 6,96 , healthy donors= - 0,53 , p= 0,016; T α 1 treated samples: HIV+ = -3,9 , healthy donors= -2,73) and MDC (untreated samples: HIV+= -0,22, healthy donors= 2,21 , p= 0,023; T α 1 treated samples: HIV+= -0,69, healthy donors= -0,03) was significantly different in untreated PBMCs from healthy donors compared to untreated PBMCs from HIV+ patients. Interestingly, treatment with T α 1 minimized the difference in expression of this gene between HIV+ and HIV- individuals. **Figure 12b** reports also the analysis of the expression of TLR2. The expression of this gene was not modulated by T α 1 in PBMCs from HIV+, while it was negatively modulated in healthy donors (untreated samples: HIV+= -0,61 , healthy donors= -0,52; T α 1 treated samples: HIV+= -0,55 , healthy donors=0,98 p=0,003) with a significant difference after treatment between the two groups. These results indicate a differential transcriptional regulation induced by thymosin alpha-1 in PBMCs from HIV+ patients and healthy donors.

DISCUSSION AND CONCLUSIONS

The importance of the thymus in regenerating a functional immune system has been clearly shown after cancer chemotherapy, bone marrow transplantation and combination antiretroviral therapy in HIV infection. In fact, the production of new T cells by the thymus is necessary for the reconstitution of a functional immune system (reviewed in Fang et al., 2008). It is known that the thymic peptide T α 1 has the ability to promote the maturation of CD4⁺/CD8⁺ thymocytes in CD4⁺ and CD8⁺ T cells and to stimulate the cell maturation in bone marrow by IL-7 production. Thus, using T α 1 in combination with antiretroviral drugs and other cytokines or growth factors to improve the immune reconstitution induced by antiviral therapy, seems an attractive strategy for the treatment of HIV infection.

In this Ph.D. thesis the effects of T α 1 in vitro on the transcriptional activity of PBMCs from HIV⁺ patients in comparison with a group of healthy donors, have been studied. The technical approach to perform this study started with the use of a microarray assay. In fact, this technique provides a powerful methodology for studying biologic systems on genomic scale. A previous study using a microarray system to analyse the expression of 8300 genes in PBMCs from healthy human donors after 48 hours of exposure to the T α 1 in vitro, showed that T α 1 induced an elevated and multiple transcriptional response in lymphoid cells (Garaci *et al.*, 2003). Those findings provided new information to explain the mechanisms through which T α 1 increases the immune response and exerts its action, especially when used in combination therapy, for the treatment of cancer or infectious diseases. Aim of the present Ph.D. thesis was to extend that observation by investigating which genes belonging to immune response pathways could be regulated by T α 1 in PBMCs from HIV infected individuals. This was considered to be useful not only to get new basic information on molecular mechanisms underlying impairment of immune cell functions during HIV

infection and the immune regulatory activity of T α 1, but also to obtain new information to further understand the potential helpfulness of using T α 1 in combination with antiretroviral therapy. To this aim, a well characterized cohort of HIV+ individuals were enrolled in an open cross-sectional study. In this group of patients, the levels of viral load inversely correlated with the number of CD4+ cells per mm³ of blood and taking into consideration that most of the patients were under therapy and that the viral load was <50 copies/mL, it is possible to assume that these patients are representative for the general positive response of the patients to the most updated antiretroviral regimens during HIV infection.

To provide new information on the regulation of the transcriptional activity by T α 1 on PBMCs from HIV+ patients, a targetted microarray systems for the analysis of the expression of selected 440 genes was used. The targetted gene list included cytokine, chemokine and related receptors, other genes involved in cytokine and cytokine-receptor interaction, genes involved in the humoral immune response and in the inflammatory response. The results obtained by the microarray analysis confirmed the ability of T α 1 to regulate, in lymphoid cells, the transcriptional response of a wide number of genes involved in immune regulation and response. Actually, the number of genes modulated by T α 1 was relatively very high in HIV+ as well as in HIV- donors, with a distribution of positively and negatively modulated genes that was comparable in the two groups. Interestingly, a general aspect of the results is that, in spite of a comparable, elevated number of identical genes for which the transcriptional response was modulated both in HIV+ patients and in healthy donors, the sort of the modulation was different between the two groups. This finding suggests that: i) T α 1 exerts its regulatory action on a quite wide, but still specifically selected, number of target genes, ii) being difficult at the moment to imagine how T α 1 could simultaneously regulate the transcription of such an elevated number of genes, it seems plausible that its activity could be exerted indirectly, acting as a transcription factor regulator, rather than directly acting as a

transcription factor by itself and iii) that the differential response was probably due to the dissimilar basal state of cellular activation of the PBMCs from HIV+ patients and healthy donors. Moreover, the phenotype analysis showed a clear inverse ratio of CD4+ and CD8+ cells in HIV+ patients, while the healthy donors showed a normal phenotype. Hence, the different transcriptional response by T α 1 in the two groups could be due to the different distribution of the target cellular subpopulations. In fact, some differential effects of T α 1 on the transcriptional response of PBMCs from HIV+ patients could be attributable to the capability of T α 1 to exert a preferential regulatory activity on the expression of specific genes on CD8+ target cells, rather on CD4+ target cells. Further studies are necessary to address this issue. Nevertheless, regarding to this point it is worthy to remind that a major component of anti-HIV immune response is the innate immunity and, particularly, the CD8(+) cell antiviral non-cytotoxic response (CNAR), mediated by a novel CD8(+) cell antiviral factor (CAF) (Levy J.A, 2003). It has not been established if the CNAR response is mediated by a single potent factor, unknown at the moment, or if it is the result of a mix of antiviral factor produced by CD8+ T cells. In this contest the role of the production of anti-HIV chemokines has been hypothesized. The stimulation of the production of chemokines by T α 1 in PBMCs from HIV+ patients indicates for a dual role of this peptide in the simultaneously immune modulator and antiviral action. The phenotype of HIV+ PBMCs showed an higher percentage of CD8+ cell. Thus, the different modulation of chemokines in the patients and healthy donors, might be due to the preferential cell target of T α 1.

Interestingly, among the others, several genes of cytokines, chemokines and related receptors were found to be modulated by T α 1 in PBMCs from HIV+ patients. Chemokines are small peptides whose actions are mediated by a family of 7-transmembrane G-protein-coupled receptors (Onuffer and Horuk, 2002). These peptides are known to function as regulatory molecules in leukocyte maturation, trafficking, homing of lymphocytes and in the

development of lymphoid tissues, as well as mediators of the inflammatory response of monocytes/macrophage cellular subset. Besides these functions in the immune system, they also play a critical role in many pathophysiological processes such as allergic responses, infectious and autoimmune diseases, angiogenesis, inflammation, tumour growth and hematopoietic development (Onuffer and Horuk, 2002). In addition, certain chemokines and their receptors are also involved in HIV pathogenesis. Identifying and elucidating the mechanisms of action of naturally occurring HIV suppressive factors, led to the discoveries of RANTES, MIP-1 α , and MIP-1 β as HIV inhibitors. The discovery of chemokine receptors as co-receptors for HIV-1 has opened the door for a number of novel anti-viral approaches. Two chemokine receptors, known as CCR5, which is predominantly expressed on dendritic cells, macrophages, and CD4 T cells, and CXCR4, expressed on activated T cells, are the major co-receptors for HIV. The 'macrophage-tropic' variants of HIV that are associated with primary infections use CCR5, which binds RANTES, MIP-1 α , and MIP-1 β , as a co-receptor, and require only a low level of CD4 on the cells they infect. These variants of HIV infect dendritic cells, macrophages, and T cells *in vivo*. The 'lymphocyte-tropic' variants of HIV infect only CD4 T cells *in vivo* and use CXCR4, which binds the stromal-derived factor-1 (SDF-1), as a co-receptor. Infection of CD4 T cells via CCR5 occurs early in the course of infection and continues to occur, with activated CD4 T cells accounting for the major production of HIV throughout infection. Late in infection, in approximately 50% of the cases, the viral phenotype switches to a T-lymphocyte-tropic type that utilizes CXCR4 co-receptors, and this is followed by a rapid decline in CD4 T-cell count and progression to AIDS (Chan *et al.*, 1998). Thus, the fact that T α 1 showed to exert a strong effect on the modulation of numerous genes encoding for chemokines in PBMCs from HIV+ patients, as well as from HIV- individuals, could open new perspectives for future studies. For these reason, in particular, MCP-1 (CCL2), MIP 1alpha (CCL3), Rantes (CCL5) , PARC (CCL18), MDC (CCL22) , LD78 beta (CCL3L1), CCL4L1 and small inducible cytokine B6 (CXCL16) genes,

were selected as genes whose expression was quantitatively evaluated using Real Time PCR to confirm the microarray analysis. Actually, expression of Rantes, PARC, MDC and CXCL16 showed a significant increase after T α 1 treatment in PBMCs from HIV+ patients. The positive modulation of CCL4L1 by microarray was confirmed, although the difference with the untreated control was not statistically significant. Differently, the positive modulation of MIP 1alpha, LD78beta and MCP-1 was not confirmed by Real Time analysis. Other than the potent HIV inhibitor Rantes, which expression has been found to be induced by T α 1 in PBMC from HIV+ patients, other chemokines have been demonstrated to be endowed with an antiviral activity toward HIV infection. LD78 beta (CCL3L1), a natural isoform of MIP 1alpha, is a potent inhibitor of HIV through the interaction of CCR5 (Nibbs *et al*, 1999; Aquaro *et al*, 2001) and CCL4L1 showed the ability to inhibit HIV infection but different authors are discordant in the effectiveness of this chemokine (Howard *et al*, 2005; Shao *et al*, 2007). Interestingly, T α 1 induced also the expression of MDC, CXCL16 and PARC. MDC has been described in 1997 as an inhibitor of HIV (Pal *et al*, 1997). More recently, it has been reported that the production of HIV-suppressive chemokines MDC and MIP 1alpha is associated with better outcome in children receiving antiretroviral regimens in settings where drug-resistant mutations are prevalent (Lambert *et al*, 2007). CXCL16 is a transmembrane chemokine ligand of the HIV-coreceptor Bonzo (also known as STRL33 and TYMSTR) (Matloubian *et al*, 2000). CXCL16 may function in promoting interactions between DCs and CD8 T cells and in guiding T cell movements in the splenic red pulp. CXCL16 was also found in the thymic medulla and in some nonlymphoid tissues, indicating roles in thymocyte development and effector T cell trafficking. CCL18/PARC primarily targets lymphocytes and immature DC, although its agonistic receptor remains unknown so far, and is mainly expressed by a broad range of monocytes/macrophages and DC. CCL18 is constitutively present at high levels in human plasma and likely contributes to the

physiological homing of lymphocytes and DC and to the generation of primary immune responses (Schutyser *et al.*, 2005).

Among other new therapeutic approaches, there are ongoing clinical studies on antagonists of HIV coreceptors (De Clercq, 2002). Moreover, the possibility that other biological HIV suppressor factors exist and that their identification and elucidation of their molecular mechanisms for inhibiting HIV will be an interesting strategy for future therapies, has been hypothesized by years (Gallo, 2002). Thus, results reported in this Ph.D on the regulatory effects of T α 1 on chemokine transcription could furnish a rational basis for a reasonable use of T α 1 in HIV infection.

Other seven genes involved in the inflammatory response and in cytokine-cytokine receptors interaction, i.e. Macrophage migration inhibitory factor (MIF), Colony stimulating factor 3 receptor (CSF3R), Colony stimulating factor 2 (CSF2), Colony stimulating factor 1 receptor (CSF1R), Lymphotoxin beta (LTB), Myeloid differentiation primary response gene (MYD88) and Toll receptor 2 (TLR2) were selected to confirm microarray analysis by Real Time PCR. In PBMCs from HIV+ patients treated with T α 1, CSF2 and TLR2 were not confirmed for positive modulation. The others selected genes were positively modulated in microarray and were significantly up regulated also by Real time analysis.

CSFR1 is the receptor of the M-CSF. Has been reported that the HIV protein Nef negatively regulates cell surface expression of CSFR1, thereby inhibits M-CSF functions in monocytes/macrophages (Suzu *et al.*, 2005; Hiyoshi *et al.*, 2008). Nef has been shown to have a positive role in viral replication and pathogenesis, Nef interacts with host cell signal transduction proteins to provide for long term survival of infected T cells and for destruction of non-infected T cells. Nef also advances the endocytosis and degradation of cell surface proteins, including CD4 and MHC proteins (Peterlin and Trono, 2003). This action possibly impairs cytotoxic T cell function, thereby helping the virus to evade the host immune

response (Schwartz, *et al.*, 1996). The positive modulation of the CSFR1 by T α 1 in PBMCs from HIV+ patients seems to counteract the effect of this viral protein.

Even if the positive induction of the receptor TLR2 by T α 1 showed by microarray analysis were not confirmed by Real time PCR in PBMCs from HIV+ patients, the positive modulation of the adaptor molecules MYD88 demonstrates the ability of T α 1 to act on the innate response pathway also in lymphoid cells affected by the HIV infection, in addition to its capability of modulating inflammation and tolerance during fungal infections and of sensing antiviral response against MCMV, as already demonstrated (Romani *et al.*, 2004; Romani *et al.*, 2007; Bozza *et al.*, 2007).

After treatment with T α 1, CXCL16, CCL4L1, CSF3R, LTB and PARC genes were positively modulated in PBMCs from HIV+ patients, with significant differences in comparison with the gene expression in the PBMCs from healthy donors. In the two groups of individuals the basal level of expression for these genes in untreated samples has shown similar values. After T α 1 treatment, the expression of all these genes was negatively modulated in PBMCs from healthy donors. Conversely, the expression of the same genes was up regulated by T α 1 in PBMC from HIV+ patients. These results clearly demonstrate the differential transcriptional regulation induced by thymosin alpha-1 in PBMCs from HIV+ patients and healthy donors. The discordant response to T α 1 showed by the PBMCs from HIV+ individuals or from normal donors could be explained by the different cellular status, dependent on the pathological state. Recently, it was better demonstrated the ability of T α 1 to regulate inversely the immune response depending upon the fisiopathological state of the target cell. Thus, T α 1 is able to negatively control inflammation during immunopathological pro-inflammatory infections and diseases, and to enhance the immune-reconstitution during infections and cancer in immunosuppressive conditions, exerting an interesting, until now not-completely understood, role as homeostasis regulator.

In the last decade, many studies have highlighted the importance of using biological compounds in the treatment of different types of immunological dysregulation, related to age, cancer or infectious diseases (Garaci *et al.*, 1997; Garaci *et al.*, 2003). The use of different compounds of biological origin in combination with conventional therapies has been explored, especially in diseases accompanied with the immuno-compromising of the host. In vitro and in vivo studies demonstrated that T α 1 may act via pathway commonly used by various cytokines, and that the combination of T α 1 and cytokines may have synergistic activity. Immunotherapy using T α 1 has been found effective in infectious disease control, in particular in patients with HBV, HCV e HIV infection (Rasi *et al.*, 2003; Hugle and Cerny, 2003; Garaci *et al.* 1994; Ramachandran *et al.*, 1996; Garaci *et al.* 1998; D. Chadwick *et al.*, 2003). However, information regarding a possible use of T α 1 in patients with HIV infection is very limited.

The possibility to combine antiretroviral chemotherapy with immunotherapy has been suggested years ago, and the association with antiretroviral therapy with cytokines and adjuvants is still proposed to improve the antiviral response during HIV infection (Matthew *et al.*, 2008). In a forefront clinical trial in HIV-infected patients under mono-antiretroviral therapy with zidovudine, thymosin α 1 was shown to induce increase in CD4⁺ cell counts, but only when given in combination with interferon (IFN)- α (Garaci, 1994). A more recent pilot study was conducted to determine the safety and efficacy of thymosin α 1 injections in conjunction with combination antiretroviral therapy in patients with advanced HIV infection (Chadwich *et al.*, 2003). Thymosin α 1 was very well tolerated, but no benefit in augmenting CD4⁺ cell count response to combined antiretroviral therapy (ART) was demonstrated. In fact, the most interesting effect of thymosin was the significant increase in signal joint (sj) T cell receptor excision circles (TRECs) levels seen after 12 weeks of treatment. Increase in the de novo production of naive T cells by thymopoiesis may be an important component of immune reconstitution in HIV-infected patients taking ART, and sjTREC levels have been

shown to predict survival in HIV-infected patients as well as the total CD4 count response to HAART after several years (Hatzakis *et al.*, 2002; Pido Mussini *et al.*, 2002).

The new finding from this study, could contribute in explaining some of the mechanisms through which T α 1 promotes the immune response and exerts its action for the treatment of cancer or infection diseases, especially when used in combination therapy.

The modern ART, able to reduce the viral load in most of the patients, has remarkably reduced the mortality associated to HIV infection. Treatment of HIV-infected patients with ART leads to immune reconstitution as shown by increases in CD4+ lymphocyte counts, decreased risk of opportunistic infections and improved survival (Palella *et al.*, 1998; Valdez *et al.*, 2001). However, a variable percentage of individuals exhibits a discordant response to ART. Particularly, some patients show an unsatisfactory increase of CD4+ T cells, despite a successful virological response (immunological failure), with risk of serious clinical events (Saravolatz *et al.*, 1996; Floridia *et al.*, 2002). These patients may benefit from the adjunctive use of immunomodulatory compounds that enhance immune reconstitution. Taking in account the impossibility to eradicate the virus from the reservoir, the ART therapy is able at the moment to inhibit the virus replication and promote the recovery of T cell compartment. The application of thymosin alpha 1 that could be able to act at the same time as immune adjuvant and simultaneously opposite the infection would represent an interesting additional approach to HIV infection.

BIBLIOGRAPHY

Al-Harthi L., Marchetti G., Steffens CM., Poulin J., Sèkaly R., Landay A.

Detection of T cell receptor circles (TRECs) as biomarkers for *de novo* T cell synthesis using a quantitative polymerase chain reaction-enzyme linked immunosorbent assay (PCR-ELISA).
J Immunol Methods 237 (1-2): 187– 97, 2000.

Andrake M.D. and Skalka A.M.

Retroviral integrase, putting the pieces together.
J. Biol. Chem. 271 (33): 19633-19636, 1996.

Aquaro S., Menten P., Struyf S., Proost P., Van Damme J., De Clercq E., Schols D.

The LD78beta isoform of MIP-1alpha is the most potent CC-chemokine in inhibiting CCR5-dependent human immunodeficiency virus type 1 replication in human macrophages.
J Virol. 75(9): 4402-4406, 2001.

Barré-Sinoussi F.

HIV as the cause of AIDS .
Lancet. 348 (9019): 31-35, 1996.

Baltimore D.

The enigma of HIV infection
Cell. 82 (2): 175-176, 1995.

Berger E.A., Murphy P.M., Farber J.M.

Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease.
Annu. Rev. Immunol. 17 : 657-700, 1999.

Billich A.

Thymosin alpha 1. Sci.Clone Pharmaceuticals.

Curr. Opin. Investig. Drugs 3 (5): 698-707, 2002.

Bozza S, Gaziano R, Bonifazi P, Zelante T, Pitzurra L, Montagnoli C, Moretti S, Castronari R, Sinibaldi P, Rasi G, Garaci E, Bistoni F, Romani L.

Thymosin alpha1 activates the TLR9/MyD88/IRF7-dependent murine cytomegalovirus sensing for induction of anti-viral responses in vivo.

Int Immunol. 19 (11): 1261-1270, 2007.

Camerini R., Ciano A., De Rosa A., Rizzetto M.

Studies of therapy with thymosin alpha1 in combination with pegylated interferon alpha2a and ribavirin in nonresponder patients with chronic hepatitis C.

Ann N Y Acad Sci. 1112: 368-74, 2007

Chan D.C. and Kim P.S.

HIV entry and its inhibition.

Cell 93 (95): 681-684, 1998.

Chadwick D., Pido-Lopez J., Pires A., Imami N., Gotch F., Villacian J.S., Ravindran S. and Paton N.I.

A pilot study of the safety and efficacy of thymosin alpha 1 in augmenting immune reconstitution in HIV-infected patients with low CD4 counts taking highly active antiretroviral therapy.

Clin. Exp. Immunology 134 (3): 477-481, 2003.

Connor R.I., Sheridan K.E., Ceradini D., Choe S. and Landau N.R.

Change in coreceptor use correlates with disease progression in HIV-1—infected individuals

J. Exp. Med. 185 (4): 621-628, 1997.

Cullen B.R.

Connections between the processing and nuclear export of mRNA: evidence for an export license?

Proc. Natl. Acad. Sci. USA. 97 (1): 4-6, 2000.

Cullen B.R.

HIV-1 auxiliary proteins: making connections in a dying cell.

Cell. 93 (5): 685-692, 1998.

De Clercq E.

New development in anti-HIV chemotherapy.

Biochim Biophys Acta.; 1587 (2-3): 258-75, 2002.

De Clercq E.

Anti-HIV drugs.

Verh K Acad Geneeskd Belg.; 69 (2): 81-104, 2007.

Douek D.C., McFarland R.D., Keiser P.H., Gage P.H., Massey J.M., Haynes B.F., Polis

M.A., Haase A.T., feinberg M.B., Sullivan J.L., Jamieson B.D., Picker L.J., Koup R.A.

Changes in thymic function with age and during the treatment of HIV infection.

Nature 396 (6712): 690-5, 1998.

Emerman M. and Malim M.H.

HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology.

Science. 280 (5371): 1880-1884, 1998.

Ershler W.B., Gravenstein S., Geloo Z.S.

Thymosin alpha 1 as an adjunct to influenza vaccination in the elderly: rationale and trial summaries.

Ann N Y Acad Sci. Sep;1112:375-84 , 2007.

Favalli C., Mastino A., Jezzi T., Grelli S., Goldestein A.L., Garaci E.

Synergistic effect of thymosin alpha 1 and alpha beta-interferon on NK activity in tumor-bearing mice.

Int. J. Immunopharmacol. 11 (5): 443-450, 1989.

Floridia M., Fragola V., Galluzzo C.M., Giannini G., Pirillo M.F., Andreotti M., Tomino C., Vella S.

HIV-related morbidity and mortality in patients starting protease inhibitors in very advanced HIV disease (CD4 count of <50 cells/microL): an analysis of 338 clinical events from a randomized clinical trial.

HIV Med. 3 (2): 75-84, 2002.

Fujinaga K., Taube R., Wimmer J., Cujec T.P., B.M. Peterlin.

Interactions between human cyclin T, Tat, and the transactivation response element (TAR) are disrupted by a cysteine to tyrosine substitution found in mouse cyclin T.

Proc. Natl. Acad. Sci. USA. 96 (4): 1285-1290, 1999.

Gallo R.C.

Human restroviruses after 20 years: a perspective from the past and prospects for their future control.

Immunol. Rev. 185: 236-265, 2002.

Garaci E, Pica F, Mastino A, Palamara AT, Belardelli F, Favalli C.

Antitumor effect of thymosin alpha 1/interleukin-2 or thymosin alpha 1/interferon alpha,beta following cyclophosphamide in mice injected with highly metastatic Friend erythroleukemia cells.

J Immunother Emphasis Tumor Immunol. Jan;13 (1):7-17, 1993.

Garaci E., Rocchi G., Perroni L.,D'Agostini C., Soscia F., Grelli S.,Mastino A.,Favalli C.

Combination treatment with zidovudine, thymosin alpha 1 and interferon-alpha in human immunodeficiency virus infection

Int J Clin Lab Res. Jun ; 24 (1):23-8, 1994.

Garaci E., Pica F., Rasi G., Favalli C.

Thymosin alpha 1 in the treatment of cancer: from basic research to clinical application.

Int. Journal of Immunopharmacology 22 (12): 1067-76, 2000.

Garaci E., Pica F., Sinibaldi-Vallebona P., Piermarchi P., Mastino A., Matteucci C., Rasi G.

Thymosin alpha(1) in combination with cytokines and chemotherapy for the treatment of cancer.

International Immunopharmacology 3 (8): 1-6, 2003.

Garaci E., Favalli C., Pica F., Sinibaldi Vallebona P., Teresa Palamara A., Matteucci C., Pierimarchi P., Serafino A., Mastino A., Bistoni F., Romani L., Rasi G.

Thimosyn alpha 1: from bench to bedside.

Ann N Y Acad Sci. 1112: 225-34, 2007.

Giuliani C., Napolitano G., Mastino A., Di Vincenzo S., D'Agostini C., Grelli S., Bucci I., Singer D., Kohn L.D., Monaco F., Garaci E., Favalli C.

Thymosin- α 1 regulates MHC class I expression in FRTL-5 cells at transcriptional level.
Eur J Immunol. 30 (3): 778-86, 2000.

Goldstein A.L. and Badamchian M.

Thymosins: chemistry and biological properties in health and disease
Expert opinion on biological therapy 4 (4): 559-573, 2004.

Grouard G. and Clark E.A.

Role of dendritic and follicular dendritic cells in HIV infection and pathogenesis.
Curr. Opin. Immunol. 9 (4): 563-567, 1997.

Hatzakis A., Touloumi G., Karanickolas R., Karafoulidou A., Mandalaki T.,

Anastassopoulou C., Zhang L., Goedert J.J., Ho D.D., Kostrikis LG.

Effect of recent thymic emigrants on progression of HIV-1 disease.
Lancet 355 (9204): 599– 604, 2000.

Hügler T., Cerny A.

Current therapy and new molecular approaches to antiviral treatment and prevention of hepatitis C.

Rev Med Virol. 13 (6): 361-71, 2003 Review.

Howard O.M., Turpin J.A., Goldman R., Modi W.S.

Functional redundancy of the human CCL4 and CCL4L1 chemokine genes.

Biochem Biophys Res Commun. 320 (3): 927-31, 2004.

Hiyoshi M., Suzu S., Yoshidomi Y., Hassan R., Harada H., Sakashita N., Akari H.,

Motoyoshi K., Okada S.

Interaction between Hck and HIV-1 Nef negatively regulates cell surface expression of M-CSF receptor.

Blood.111(1) 243-20, 2008.

Kedzierska K. and Crowe S.M.

Cytokines and HIV-1: interactions and clinical implications.

Antivir. Chem. Chemother 12 (3):133-50, 2001.

Kinoshita S., Su L., Amano M., Timmerman L.A., Kaneshima H and Nolan G.P..

The T cell activation factor NF-ATc positively regulates HIV-1 replication and gene expression in T cells

Immunity 6 (3). 6: 235-244, 1997.

Knutsen A.P., Freeman J.J., Mueller K.R., Roodman S.T., Bouhasin J.D.

Thymosin- α 1 stimulates maturation of CD34⁺ stem cells into CD3⁺CD4⁺ cells in an in vitro thymic epithelia organ coculture model.

Int. Journal of Immunopharmacology 21 (1):15-26,1999.

Lambert JS, Machado ES, Watson DC, Sill AM, Lim JK, Charurat M, Cunha SM, Afonso AO, Oliviera RH, Tanuri A, DeVico AL.

Production of the HIV-suppressive chemokines CCL3/MIP-1 α and CCL22/MDC is associated with more effective antiretroviral therapy in HIV-infected children.

Pediatr Infect Dis J. Oct;26(10):935-44. 2007

Lau G.K., Nanji A., Hou J., Fong D.Y., Au W.S., Yuen S.T., Lin M., Kung H.F., Lam S.K.

Thymosin-alpha1 and famciclovir combination therapy activates T-cell response in patients with chronic hepatitis B virus infection in immune-tolerant phase.

J Viral Hepat. 9 (4): 280-7, 2002.

Littman D.R.

Chemokine receptors: keys to AIDS pathogenesis?

Cell. 93 (5): 677-680,1998.

Liu R., Paxton W.A., Choe S., Ceradini D., Martin S.R., Horuk R., Macdonald M.E., Stuhlmann H., Koup R.A., Landau N.R.

Homozygous defect in HIV 1 coreceptor accounts for resistance of some multiply exposed individuals to HIV 1 infection

Cell. 86 (3): 367-377, 1996.

Low T.L., Thurman G.B., Mc Adoo M., Mc Clure J., Rossio J.L., Naylor P.H. and Goldstein A.L.

The chemistry and biology of Thymosin. I. Isolation, characterization, and biological activities alpha1 and polypeptide beta1 from calf thymus.

J. Bioch. Chem. 254(3): 981-986, 1979.

Morrow M.P.and Weiner D.B.

Cytokine as adjuvants for improving anti-HIV responses

AIDS, 22 : 33-338,2008.

McCune J.M.

Viral latency in HIV disease.

Cell. 82 (2): 183-188, 1995.

Matloubian M. David A. Engel S. Ryan J.E., Cyster J.G.

A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo.

Nat Immunol. 1 (4):298-304, 2000.

Mastino A., Favalli C., Grelli S., Innocenti F., Garaci E.

Thymosin alpha 1 potentiates interleukin 2-induced cytotoxic activity in mice.

Cell Immunol. 133(1):196-205, 1991.

Mastino A., Favalli C., Grelli S., Rasi G., Pica F., Goldstein A.L., Garaci E.

Combination therapy with thymosin alpha 1 potentiates the anti-tumor activity of interleukin-2 with cyclophosphamide in the treatment of the Lewis lung carcinoma in mice.

*Int J Cancer.*50(3): 493-499, 1992.

Moore J.P., Trkola A., Dragic T.

Co-receptors for HIV-1 entry.

Curr. Opin. Immunol. 9 (4): 551-562, 1997.

Mosoian A., Teixeira A., Burns C.S., Khitrov G., Zhang W., Gusella L., Klotman P., Klotman M.

Influence of prothymosin-alpha on HIV-1 target cells.

Ann N Y Acad Sci. 1112: 269-285, 2007.

Murakami T., Nakajima T., Koyanagi Y., Tachibana K., Fujii N., Tamamura H., Yoshida N., Waki M., Matsumoto A., Yoshie O., Kishimoto T., Yamamoto N., Nagasawa T.

A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection.

J. Exp. Med. 186 (8) : 1389-1393, 1997.

Mussini C., Pinti M., Borghi V., Nasi M., Amorino G., Monterastelli E., Moretti L., Troiano L., Esposito R., Cossarizza A.

Features of 'CD4-exploders', HIV-positive patients with an optimal immune reconstitution after potent antiretroviral therapy.

AIDS 16 (12): 1609-1616, 2002.

Naylor P.H. and Hadden J.W.

T cell targeted immune enhancement yields effective T cell adjuvants.

International Immunopharmacology 3 (8): 1205-1215, 2003.

Naylor P.H., Quadrini K., Garaci E., Rasi G., Hadden JW.

Immunopharmacology of Thymosin α 1 and Cytokine Synergy.

N Y Acad Sci. 1112: 235-244, 2007.

Nibbs R.J., Yang J., Landau N.R., Mao J.H., Graham G.J.

LD78beta, a non-allelic variant of human MIP-1alpha (LD78alpha), has enhanced receptor interactions and potent HIV suppressive activity.

J Biol Chem. 274 (25): 17478-17483, 1999.

Nolan G.P.

Harnessing viral devices as pharmaceuticals: fighting HIV-1's fire with fire.

Cell. 90 (5): 821-824, 1997.

Oates K.K., Sztein M.B., Goldstein A.L.

Mechanism of action of the thymosins: modulation of lymphokines, receptors, and T-cell differentiation antigens.

Immunol Ser. 45: 273-88, 1989.

Onuffer J.J. and Horuk R.

Chemokines, chemokine receptors and small-molecule antagonists: recent developments.

Trends Pharmacol. Sci. 23 (10): 459-467, 2002.

Pal R., Garzino-Demo A., Markham P.D., Burns J., Brown M., Gallo R.C., DeVico A.L.

Inhibition of HIV-1 infection by the beta-chemokine MDC.

Science. 278(5338): 695-698, 1997.

**Palella F.J. Jr, Delaney K.M., Moorman A.C., Loveless M.O., Fuhrer J., Satten G.A.,
Aschman D.J., Holmberg S.D.**

Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection . HIV Outpatient Study Investigators.

N Engl J Med 338 (13): 853– 60, 1998.

Pica F., Frascetti M, Matteucci C., Tuthill C., Rasi G.

High doses of thymosin alpha 1 enhance the anti-tumor efficacy of combination chemo-immunotherapy for murine B16 melanoma.

Anticancer Res. 18(5A):3571-8, 1998.

**Pido-Lopez J., Pires A., Nelson M., O'Moore E., Fisher M., Gazzard B., Aspinall R.,
Gotch F., Imami N.**

Thymic activity in late-stage HIV-1 infected individuals receiving highly active antiretroviral therapy: potential effect of steroid therapy.

HIV Med 3 (1): 56-61, 2002.

Rasi G., Silecchia G., Sinibaldi-Vallebona P., Spaziani E., Pierimarchi P., Sivilia M., Tremiterra S., Garaci E.

Anti-tumor effect of combined treatment with thymosin alpha 1 and interleukin-2 after 5-fluorouracil in liver metastases from colorectal cancer in rats.

Int J Cancer. 57 (5): 701-705, 1994.

Rasi G., Di Virgilio D., Mutchnick M.G., Coltella F., Sinibaldi-Vallebona P., Pierimarchi P., Valli B., Garaci E.

Combination thymosin α 1 and lymphoblastoid interferon treatment in chronic hepatitis C.

Gut Nov 39(5): 679-83, 1996.

Rasi G., Pierimarchi P., Sinibaldi-Vallebona P., Colella F., Garaci E.

Combination therapy in the treatment of chronic viral hepatitis and prevention of hepatocellular carcinoma.

Int Immunopharmacol. 3(8):1169-76, 2003.

Richman D.D.

Antiviral drug resistance.

Antiviral Res. 71(2-3):117-121, 2006.

Romani L., Bistoni F., Gaziano R., Bozza S., Montagnoli C., Perruccio K., Pitzurra L., Bellocchio S., Velardi A., Rasi G., Di Francesco P., Garaci E.

Thymosin alpha 1 activates dendritic cells for antifungal Th1 resistance through toll-like receptor signaling.

Blood. 103 (11): 4232-9, 2004.

Romani L., Bistoni F., Montagnoli C., Gaziano R., Bozza S., Bonifazi P., Zelante T., Moretti S., Rasi G., Garaci E., Puccetti P.

Thymosin alpha1: an endogenous regulator of inflammation, immunity, and tolerance.

Ann. N Y Acad Sci. 1112: 326-38, 2007.

Samson M., Libert F., Doranz B.J., Rucker J., Liesnard C., Farber C.M., Saragosti S., Lapoumeroulie C., Cognaux J., Forceille C., Muyldermans G., Verhofstede C., Burtonboy G., Georges M., Imai T., Rana S., Yi Y.J., Smyth R.J., Collman R.G., Doms R.W., Vassart G., Parmentier M.R.

Resistance to HIV 1 infection in Caucasian individuals bearing mutant alleles of the CCR 5 chemokine receptor gene

Nature. 382 (6593): 722-725, 1996.

Saravolatz L., Neaton J.D., Sacks L., Deyton L., Rhame F., Sherer R.

CD4⁺ T lymphocyte counts and patterns of mortality among patients infected with human immunodeficiency virus who were enrolled in community programs for clinical research on *AIDS*.

Clin Infect Dis; 22 (3): 513-20, 1996.

Schutysse E., Richmond A., Van Damme J.

Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes.

J Leukoc Biol. 78 (1): 14-26, 2005.

Shao W., Tang J., Song W., Wang C., Li Y., Wilson C.M., Kaslow R.A.

CCL3L1 and CCL4L1: variable gene copy number in adolescents with and without human immunodeficiency virus type 1 (HIV-1) infection.

Genes Immun. 8 (3): 224-31, 2007.

Shiau A.L., Chen Y. L., Liao C.Y., Huang Y.S. and Wu C.L.

Prothymosin α_1 enhances protective immune responses induced by oral DNA vaccination against pseudorabies delivered by *Salmonella choleraesuis*.

Vaccine 19 (28-29): 3947-3956, 2001.

Shmelev V.A., Grigor'ev B.V., Mozharova T.I. and Popov S.G.

Thymosin alpha-1 and hybrid proteins consisting of tumor necrosis factor-alpha and thymosin alpha-1 enhance the efficacy of vaccination against the causative agent of plague.

Zh. Mikrobiol. Epidemiol. Immunobiol. 4: 85-89, 1994.

Sjogren M.H.

Thymalfasin: an immune system enhancer for the treatment of liver disease.

J Gastroenterol Hepatol. 19(12):S69-72, 2004.

Subbramanian R.A. and Cohen E.A.

Molecular biology of the human immuno-deficiency virus accessory proteins

J. Virol. 68 (11): 6831-6835, 1994.

Suzu S., Harada H., Matsumoto T., Okada S.

HIV-1 Nef interferes with M-CSF receptor signaling through Hck activation and inhibits M-CSF bioactivities.

Blood. 105 (8):3230-3237, 2005.

Svedersky L.P., Hui A., May L., McKay P., Stebbing N.

Induction and augmentation of mitogen-induced immune interferon production in human peripheral blood lymphocytes by N alpha-desacetylthymosin alpha 1.

Eur J Immunol. 12 (3): 244-7, 1982.

Sztejn M.B., Serrate S.A., Goldstein A.L.

Modulation of interleukin 2 receptor expression on normal human lymphocytes by thymic hormones.

Proc Natl Acad Sci U S A. 83(16): 6107-6111, 1986.

Tao L., Wang R.H., Enkemann S.A., Trumbore M.W., Berger S.L.

Metabolic regulation of protein-bound glutamyl phosphates: insights into the function of prothymosin α .

Journal of Cellular Physiology 178 (2):154-163, 1999.

**Teixeira L., Valdez H., McCune J.M., Koup R.A., Badley A.D., Hellerstein M.K.,
Napolitano L.A., Douek D.C., Mbisa G., Deeks S., Harris J.M. Barbour J.D., Gross B.H.,
Francis I.R., Halvorsen R., Asaad R., Lederman M.M.**

Poor CD4 T cell restoration after suppression of HIV-1 replication may reflect lower thymic function.

Aids 15 (14): 1749-56, 2001.

Trono D.

HIV accessory proteins: leading roles for the supporting cast.

Cell. 82 (2): 189-192, 1995.

Unutmaz D. and Littman D.R.

Expression pattern of HIV-1 coreceptors on T cells: implications for viral transmission and lymphocyte homing.

Proc. Natl. Acad. Sci. USA 94 (5): 1615-1618, 1997.

Valdez H., Chowdhry T.K., Asaad R., Woolley I.J., Davis T., Davidson R., Beinker N., Gripshover B.M., Salata R.A., McComsey G., Weissman S.B., Lederman M.M.

Changing spectrum of mortality due to human immunodeficiency virus: analysis of 260 deaths during 1995–1999.

Clin Infect Dis 32 (10): 1487– 93, 2001.

Wyatt R. and Sodroski J.

The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens.

Science 280 (5371): 1884-1888, 1998.

Yang A.G., Bai X., Huang X.F., Yao C., Chen S.

Phenotypic knockout of HIV type 1 chemokine coreceptor CCR-5 by intrakines as potential therapeutic approach for HIV-1 infection.

Proc. Natl. Acad. Sci. USA 94 (21): 11567-11572, 1997.

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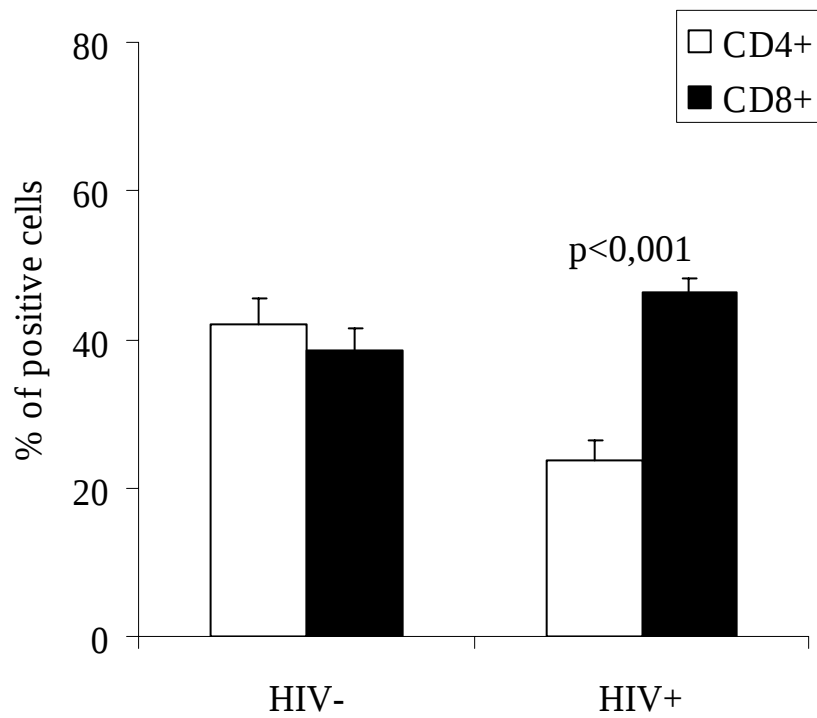


Figure 1

Percentage of positive cells analyzed by flow citometry in fresh PBMCs. These results show a significantly ($p < 0,001$) inverse expression of CD4+ CD8+ cells in HIV+ patients in comparison to the healty donors phenotype.

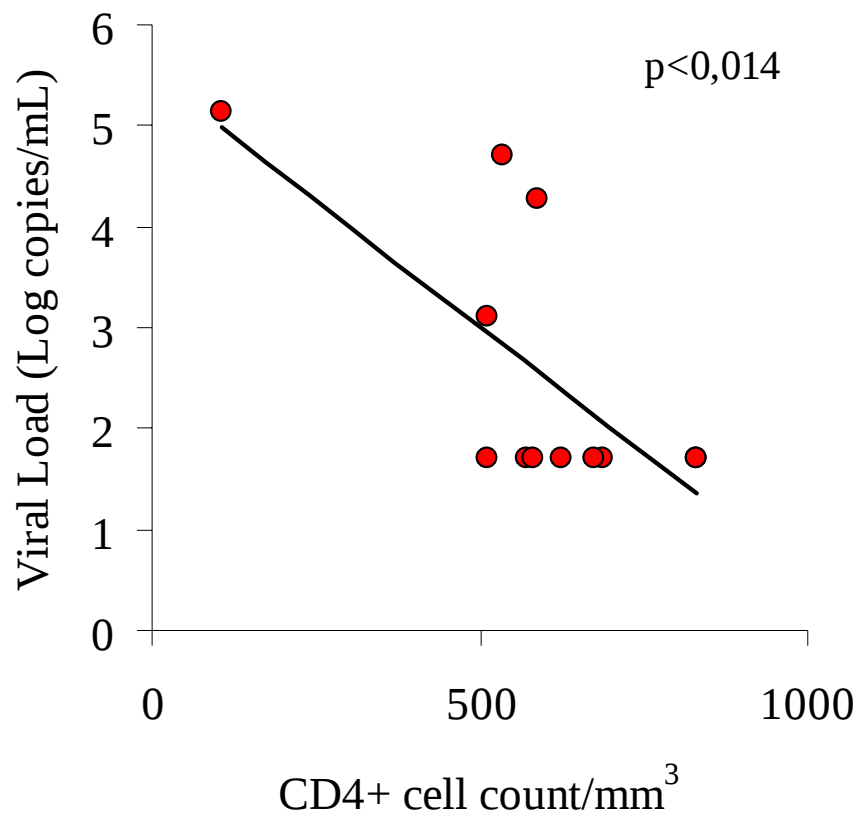


Figure 2

Correlation between the number of CD4+ cells and the Viral Load in HIV+ patients. An increase of CD4+ cells numbers is correlated to a significant decrease of VL (Spearman's rho = -711; $p < 0,014$).

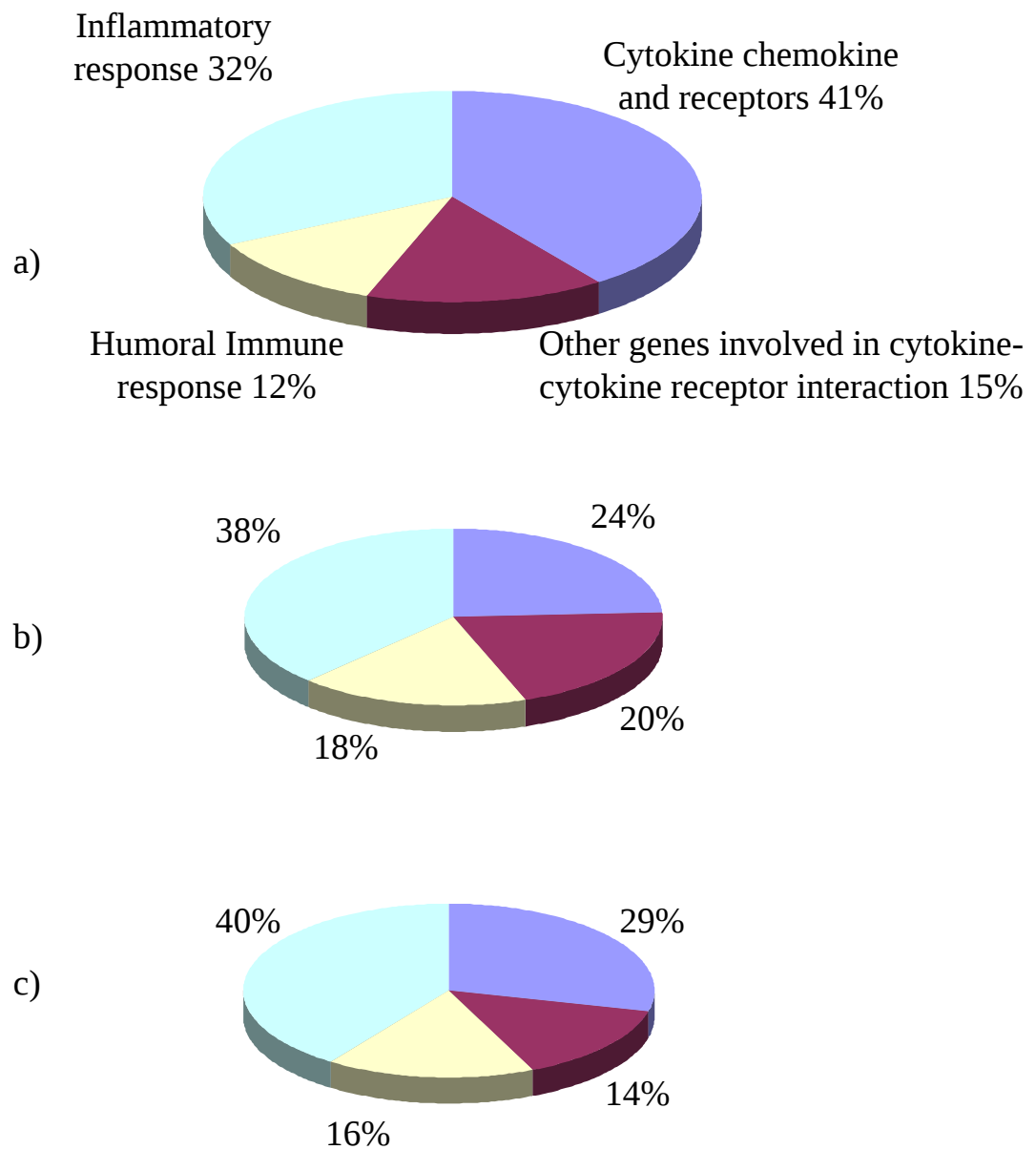


Figure 3

- a) Distribution of functional gene grouping in “Autoimmune and Inflammatory response” microarray systems.
- b) Pie chart summary of $T\alpha 1$ regulated genes in super array experiments in PBMC from HIV+ patients.
- c) Pie chart summary of $T\alpha 1$ regulated genes in super array experiments in PBMC from healthy donors.

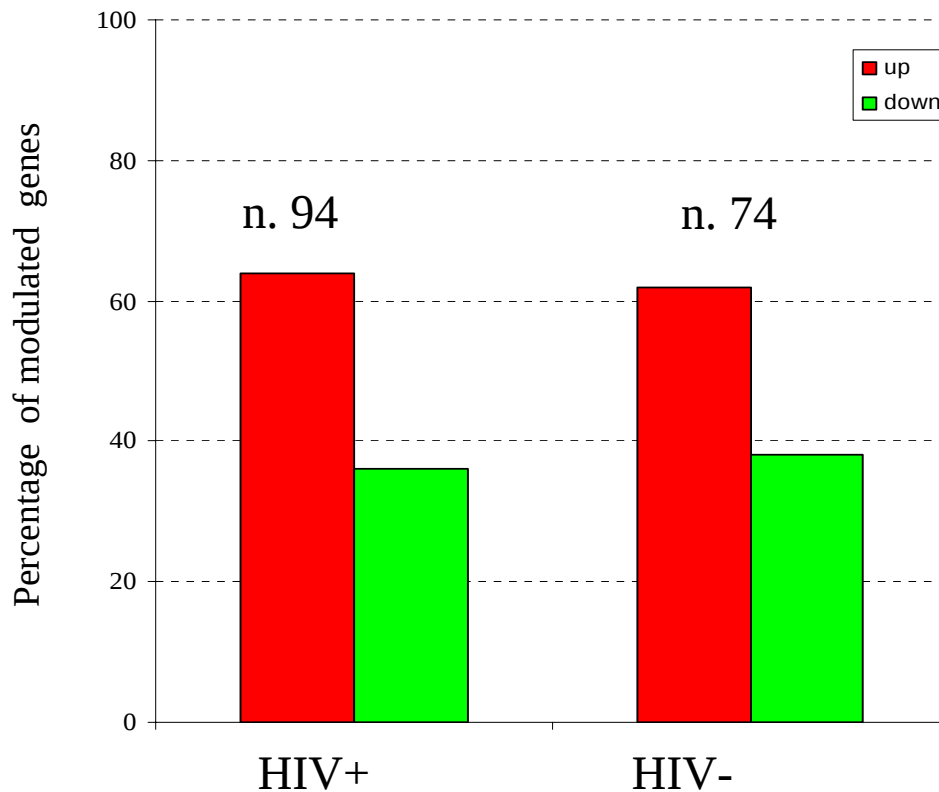


Figure 4

Number of positively and negatively modulated genes by Thymosin alpha 1 in PBMCs from HIV+ patients and healthy donors. 94 genes result regulated in HIV + patients, 64% are up regulated and 36% are down regulated; in HIV- donors the total of regulated genes are 74 whose 62% positively modulated and 38% negatively modulated.

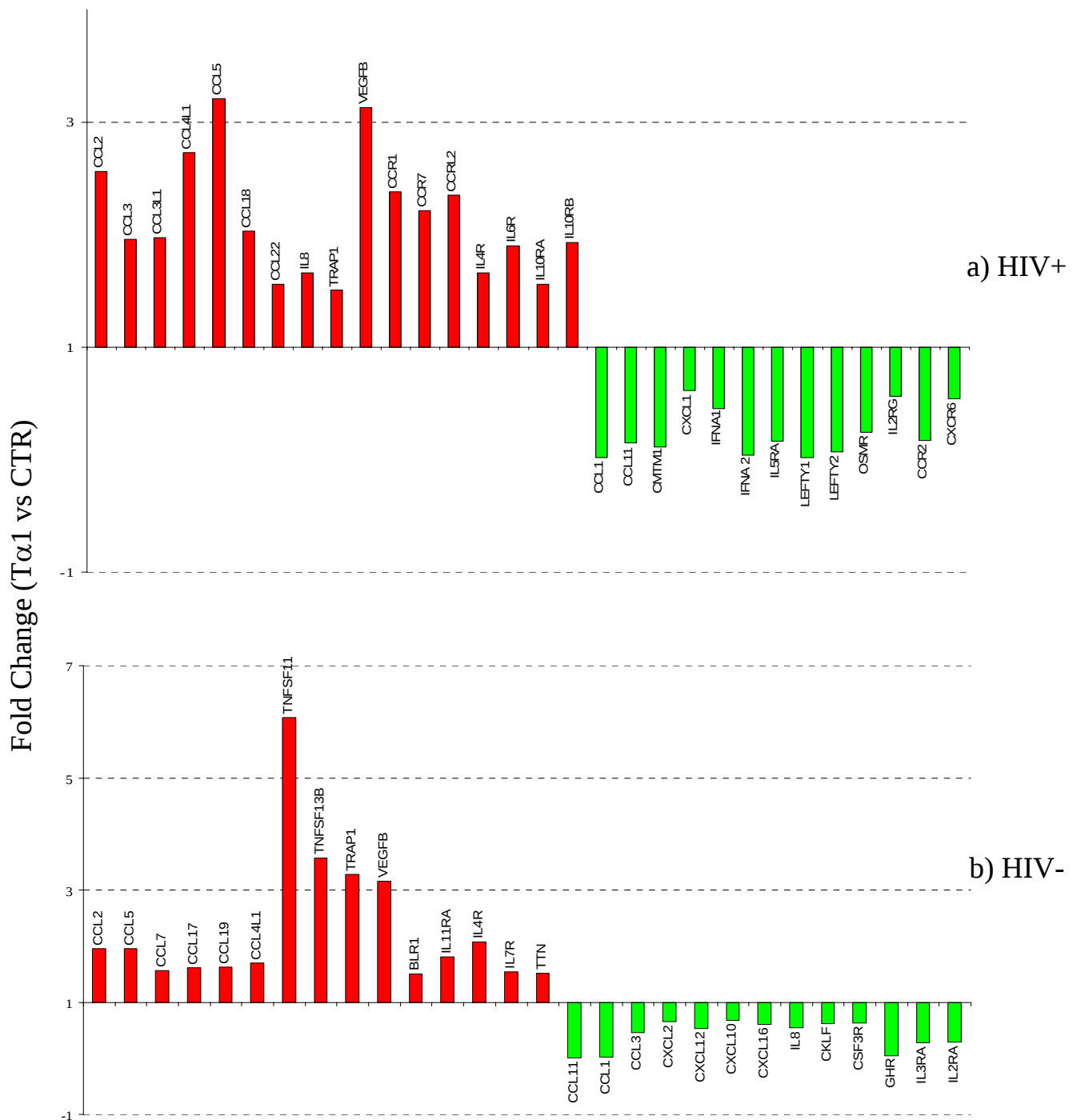


Figure 5

Cytokine, Chemokine and receptors.

Expression of positively modulated genes (red) and negatively modulated genes (green) after treatment with Thymosin alpha 1 in PBMCs from HIV+ patients (a) and HIV- donors (b).

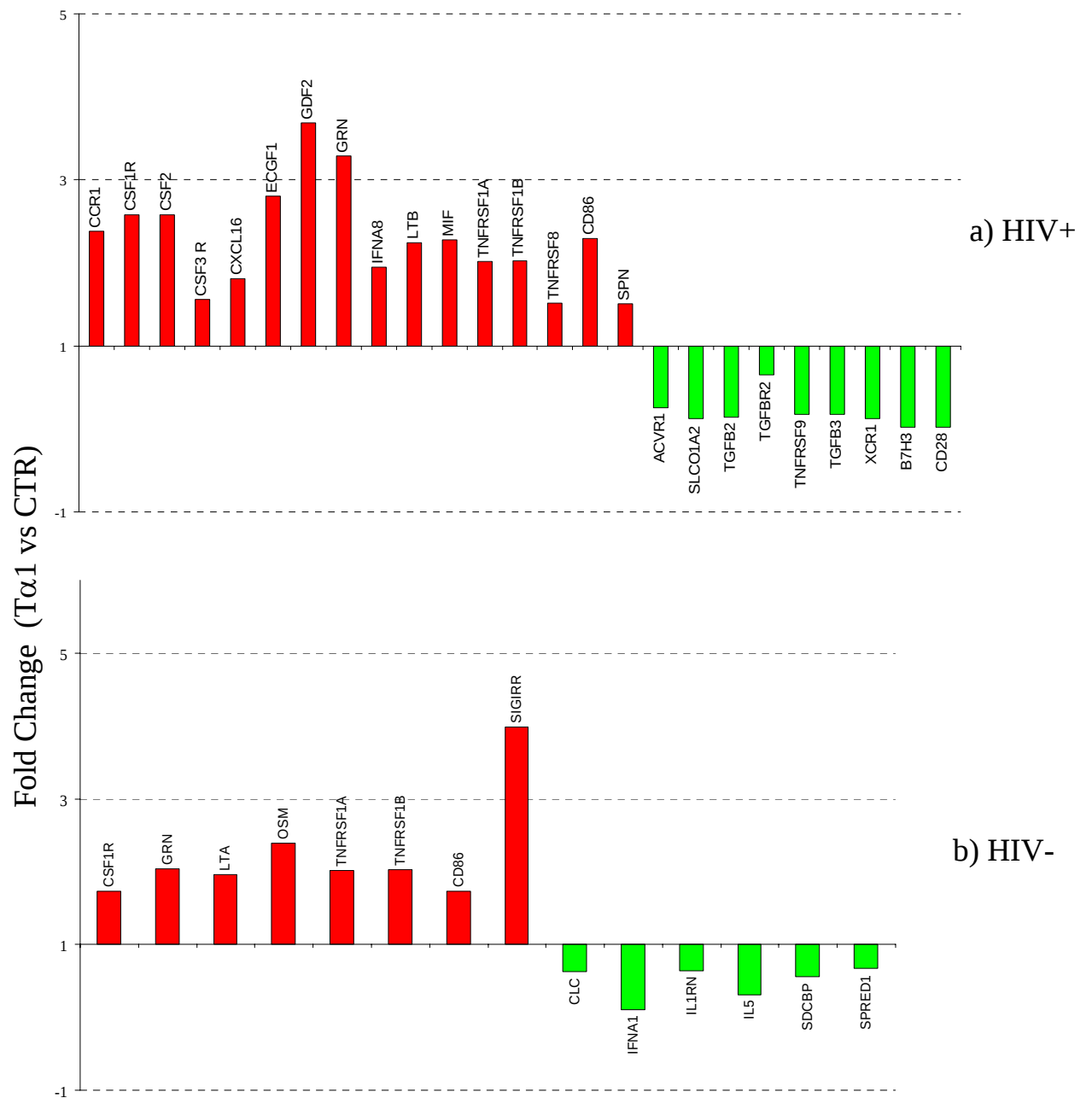


Figure 6

Cytokine-cytokine receptors interaction.

Expression of positively modulated genes (red) and negatively modulated genes (green) after treatment with Thymosin alpha 1 in PBMCs from HIV+ patients (a) and HIV- donors (b).

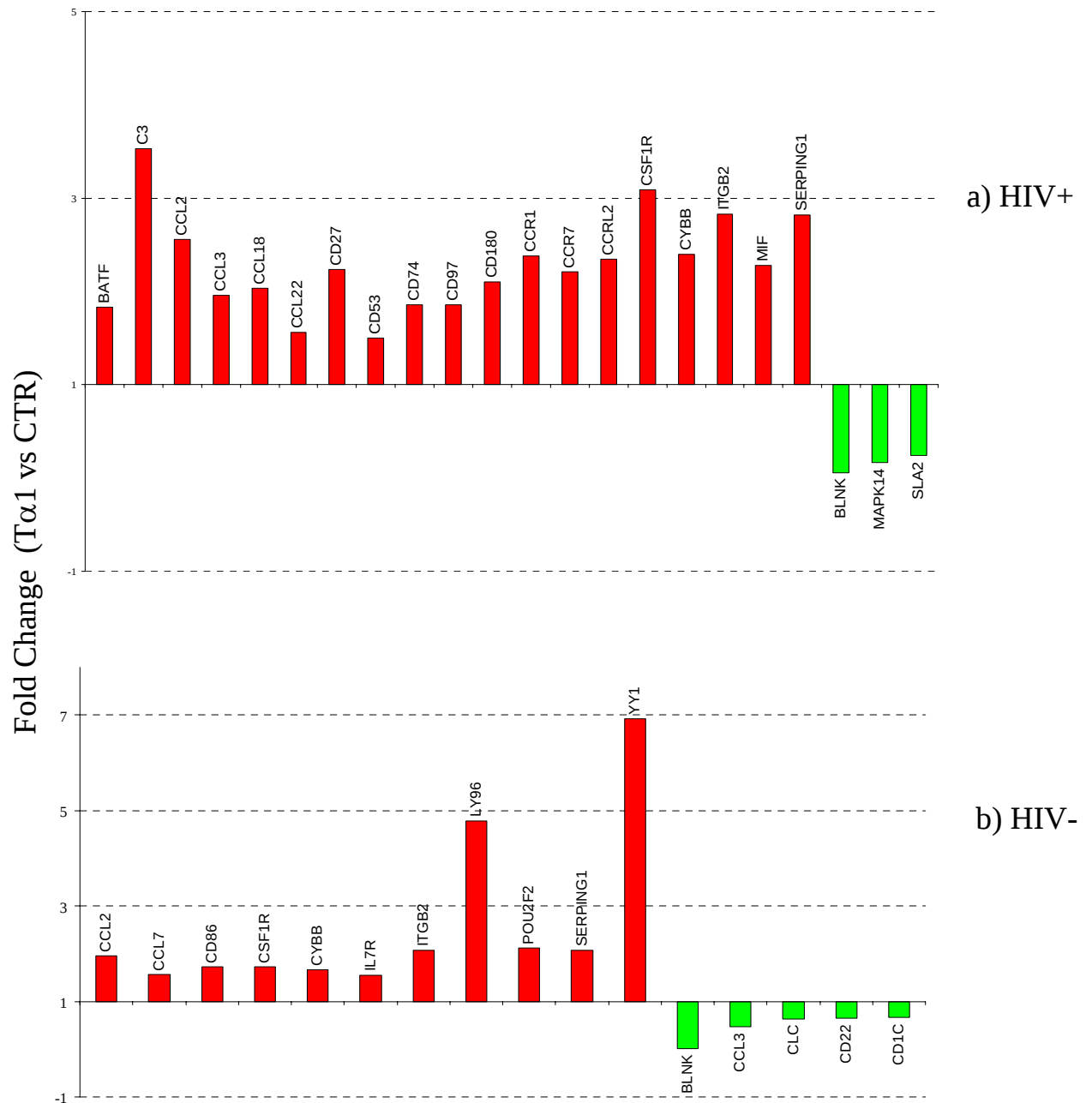


Figure 7

Humoral immune response.

Expression of positively modulated genes (red) and negatively modulated genes (green) after treatment with Thymosin alpha 1 in PBMCs from HIV+ patients (a) and HIV- donors (b).

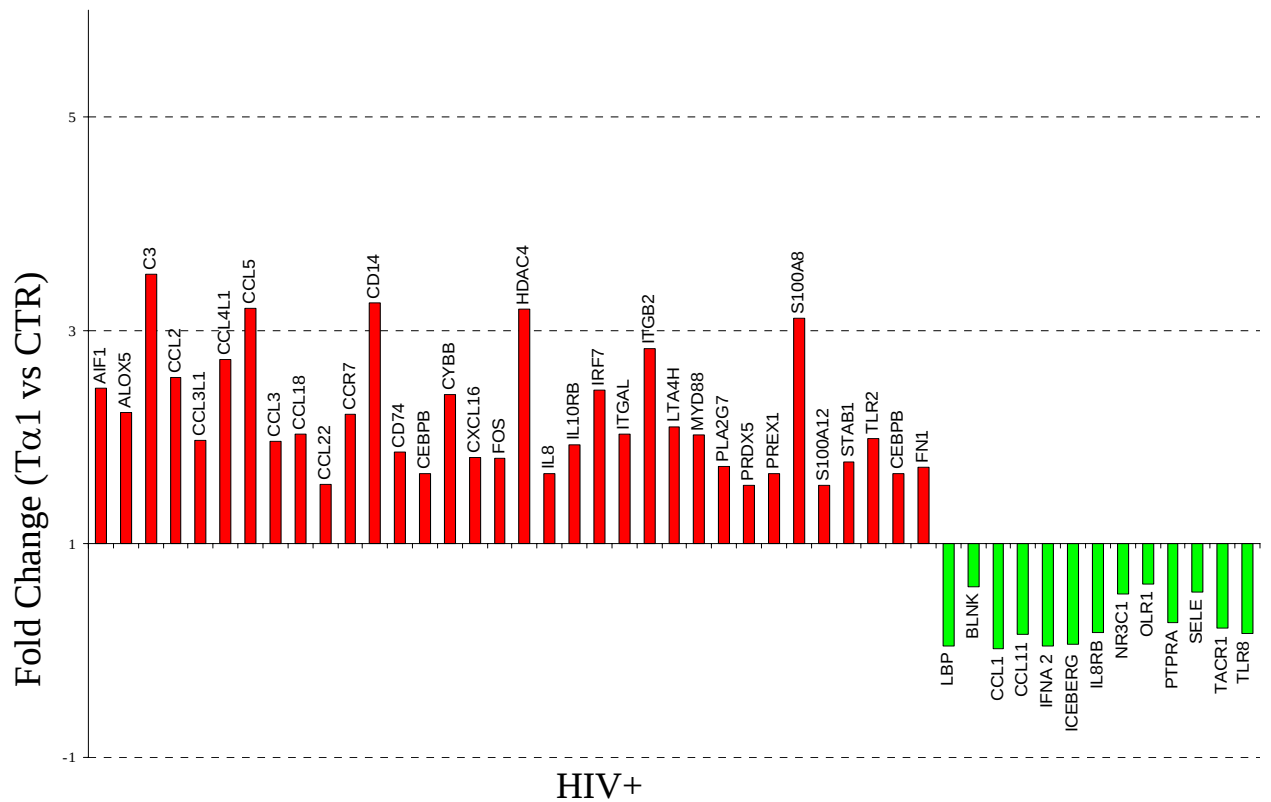


Figure 8

Inflammatory response.

Expression of positively modulated genes (red) and negatively modulated genes (green) after treatment with Thymosin alpha 1 in PBMCs from HIV+ patients.

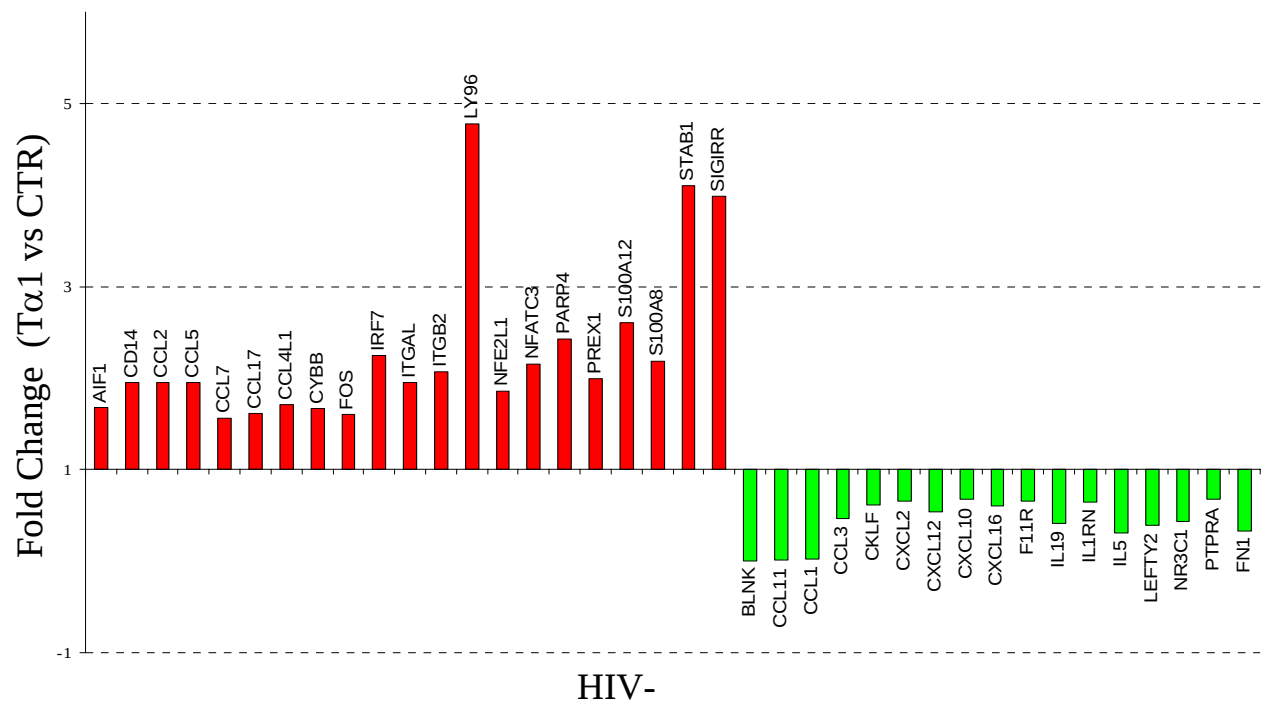


Figure 9

Inflammatory response.

Expression of positively modulated genes (red) and negatively modulated genes (green) after treatment with Thymosin alpha 1 in PBMCs from healthy donors.

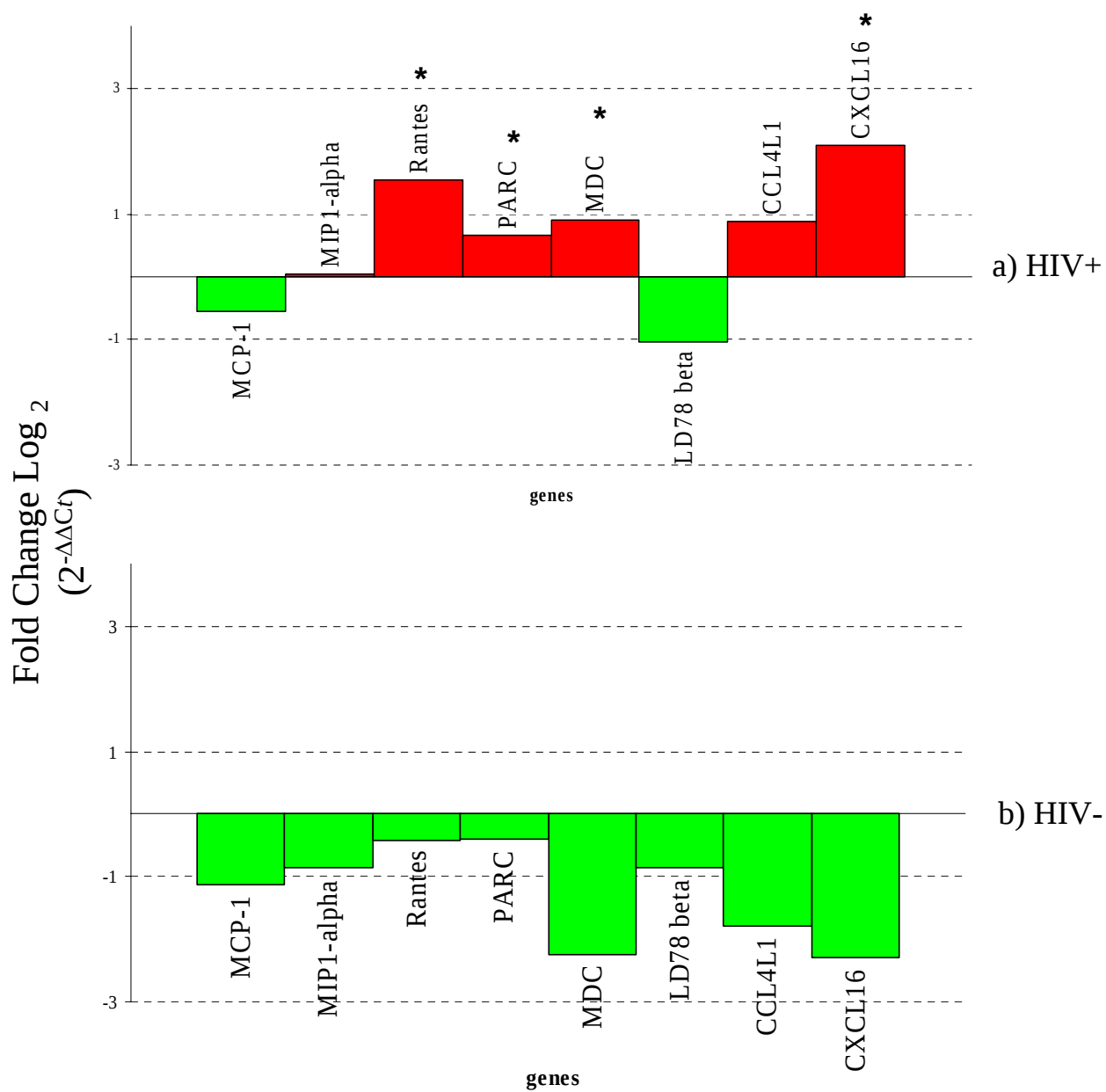


Figure 10

Real time analysis of the gene expression of chemokines after treatment with Thymosin alpha 1 in PBMCs from HIV+ patients (a) and healthy donors (b).

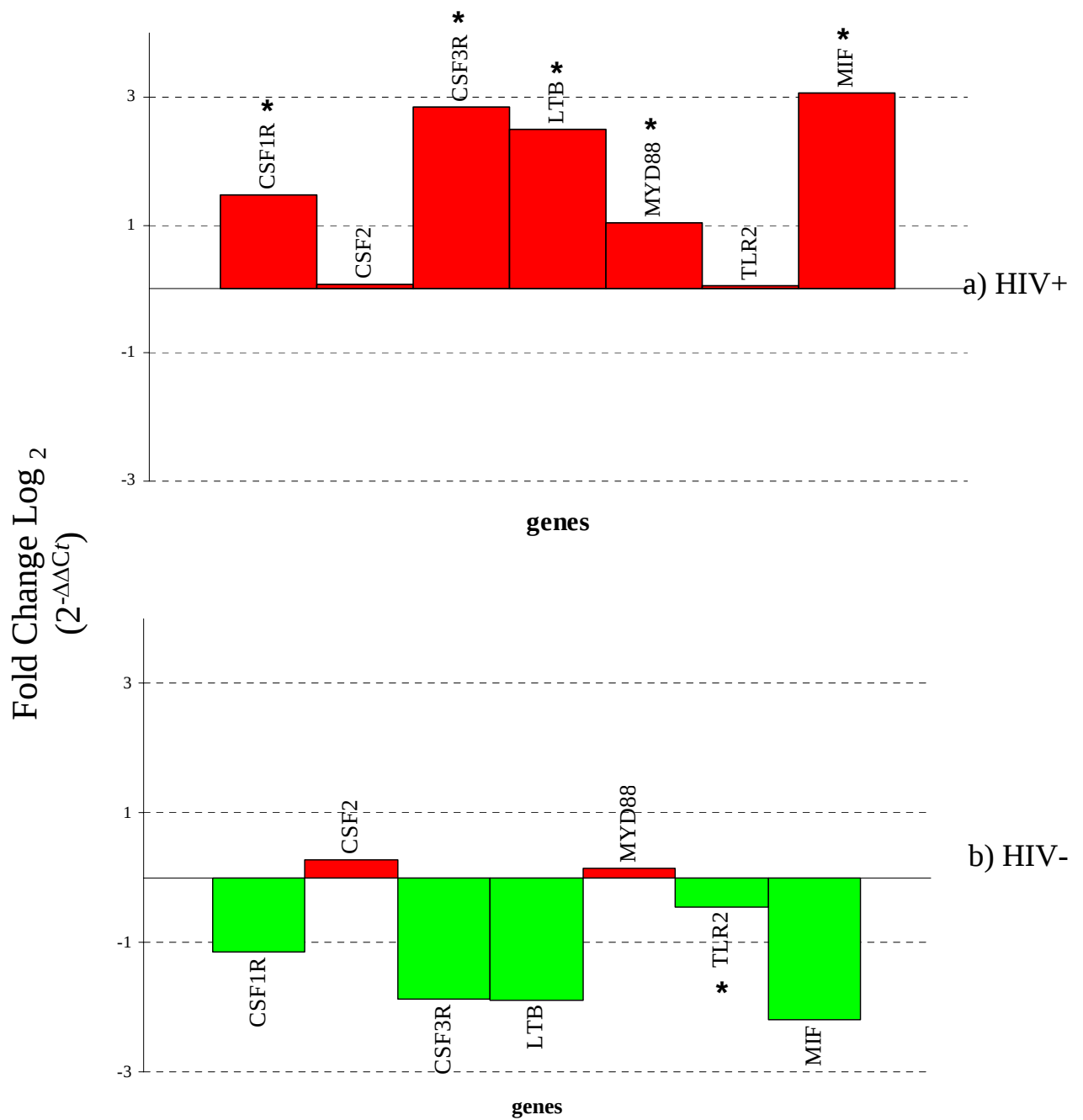


Figure 11

Real time analysis of gene involved in Inflammatory response and in cytokine-cytokine receptors interaction after treatment with Thymosin alpha 1 in PBMCs from HIV+ patients (a) and healthy donors (b).

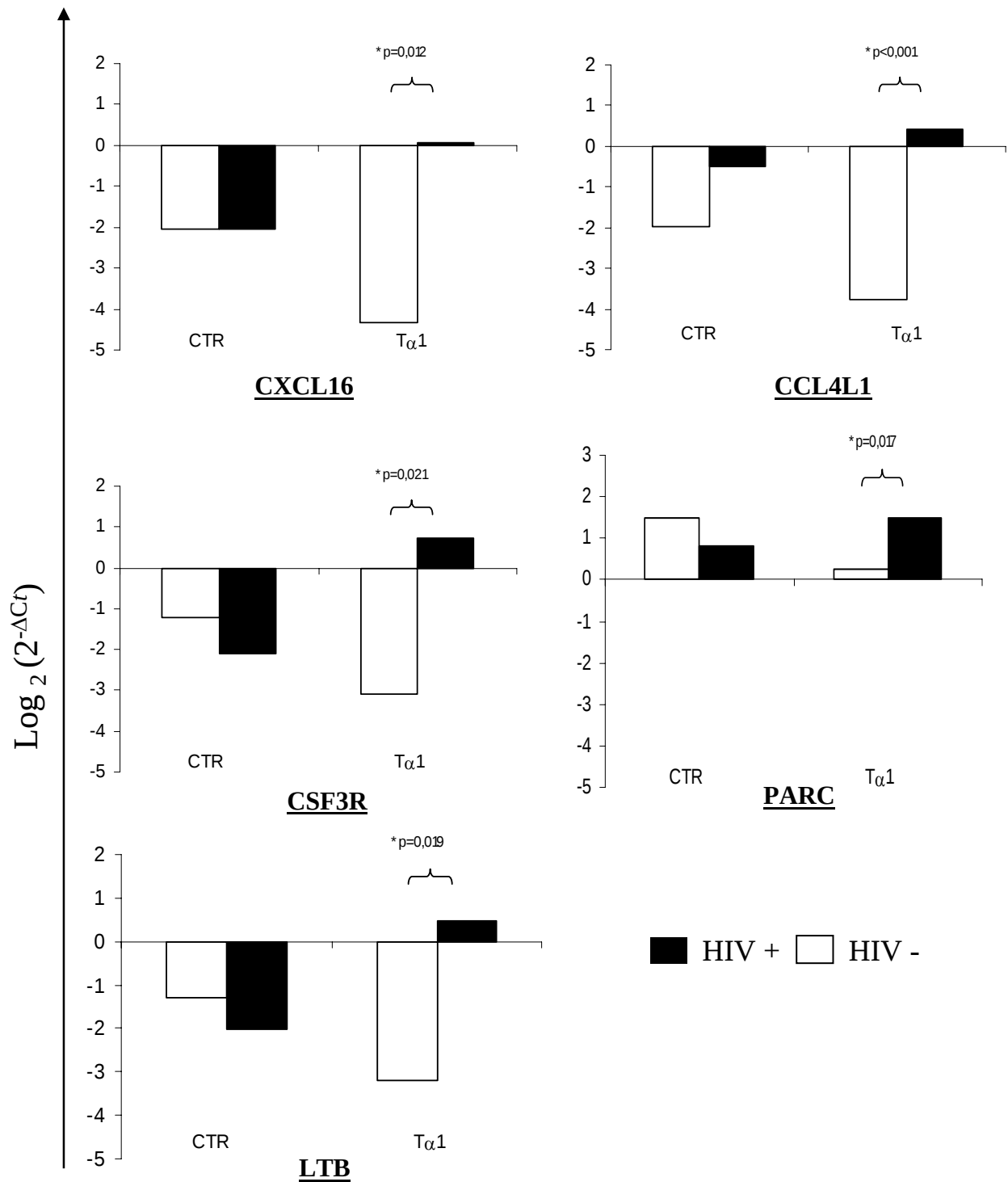


Figure 12a

Real time analysis of gene expression after treatment with Thymosin alpha 1 in PBMCs from HIV+ patients and healthy donors; values are normalized for the housekeeping gene and expressed as Log₂(2^{-ΔCt})

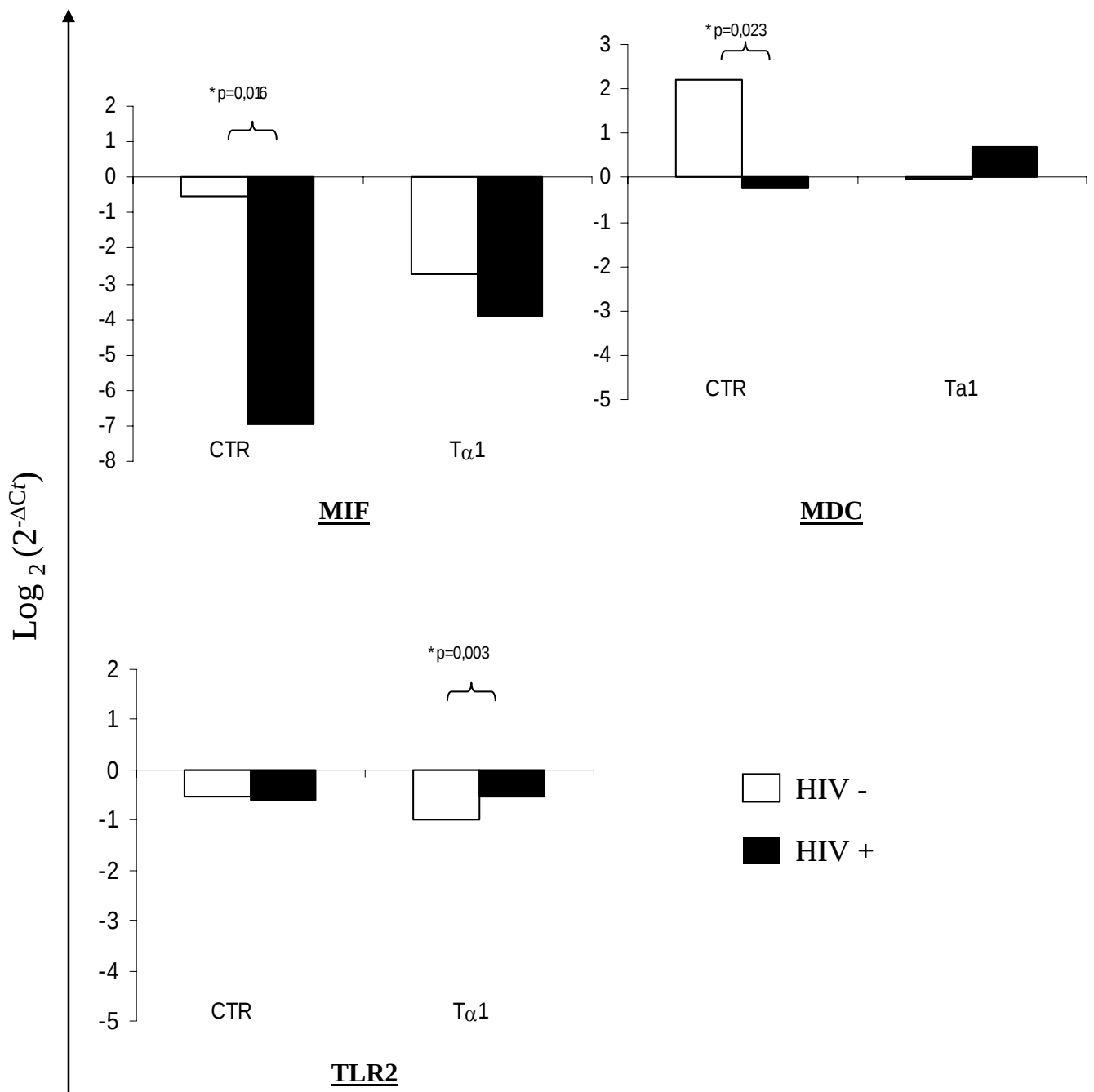


Figure 12b

Real time analysis of gene expression after treatment with Thymosin alpha 1 in PBMCs from HIV+ patients and healthy donors; values are normalized for the housekeeping gene and expressed as Log₂(2^{-ΔCt})

Table 5: Genes found to be regulated by Thymosin alpha 1 in PBMCs from HIV infected patients (1)

	Gene		Gene Bank Access number	SuperArray fold change
Cytokine , Chemokines and receptors				
UP	CCL2	Chemokine (C-C motif) ligand 2 (MCP-1/ MCAF)	NM_002982	2,56
	CCL3	Chemokine (C-C motif) ligand 3 (MIP-1 alpha)	NM_002983	1,96
	CCL3L1	Chemokine (C-C motif) ligand 3-like 1 (LD78 beta)	NM_021006	1,97
	CCL4L1	Chemokine (C-C motif) ligand 4-like 1	NM_001001435	2,73
	CCL5	Chemokine (C-C motif) ligand 5 (Rantes)	NM_002985	3,21
	CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated) (MIP-4/PARC)	NM_002988	2,03
	CCL22	Chemokine (C-C motif) ligand 22 (MDC)	NM_002990	1,56
	IL8	Interleukin 8	NM_000584	1,66
	TRAP1	TNF receptor-associated protein 1	NM_016292	1,51
	VEGFB	Vascular endothelial growth factor B	NM_003377	3,13
	CCR1	Chemokine (C-C motif) receptor 1	NM_001295	2,38
	CCR7	Chemokine (C-C motif) receptor 7	NM_001838	2,21
	CCR2	Chemokine (C-C motif) receptor-like 2	NM_003965	2,35
	IL4R	Interleukin 4 receptor	NM_000418	1,66
	IL6R	Interleukin 6 receptor	NM_000565	1,9
	IL10RA	Interleukin 10 receptor, alpha	NM_001558	1,56
	IL10RB	Interleukin 10 receptor, beta	NM_000628	1,93
DOWN	CCL1 (I-309)	Chemokine (C-C motif) ligand 1	NM_002981	0,02
	CCL11	Chemokine (C-C motif) ligand 11 (Eotaxin)	NM_002986	0,15
	CMTM1 (CKLF)	CKLF-like MARVEL transmembrane domain containing 1	NM_181269	0,11
	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	NM_001511	0,61
	IFNA1	Interferon, alpha 1	NM_024013	0,45
	IFNA 2	Interferon, alpha 2	NM_000605	0,04
	IL5RA	Interleukin 5 receptor, alpha	NM_000564	0,16
	LEFTY1	Left-right determination factor 1	NM_020997	0,02
	LEFTY2	Left-right determination factor 2	NM_003240	0,07
	OSMR	Oncostatin M receptor	NM_003999	0,24
	CCR2	Chemokine (C-C motif) receptor 2	NM_000648	0,17
	CXCR6	Chemokine (C-X-C motif) receptor 6	NM_006564	0,54
	IL2RG	Interleukin 2 receptor, gamma (severe combined immunodeficiency)	NM_000206	0,56

Table 6: Genes found to be regulated by Thymosin alpha 1 in PBMCs from HIV infected patients (2

	Gene		Gene Bank Access number	SuperArray fold change
	Other genes involved in Cytokine-cytokine receptor interaction			
UP	CCR1	Chemokine (C-C motif) receptor 1	NM_001295	2,38
	CSF1R	Colony stimulating factor 1 receptor	NM_005211	2,58
	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	NM_000758	2,58
	CSF3 R	Colony stimulating factor 3 receptor (granulocyte)	NM_000760	1,56
	CXCL16	Chemokine (C-X-C motif) ligand 16 (small inducible cytokine B6)	NM_022059	1,81
	ECGF1	Endothelial cell growth factor 1 (platelet-derived)	NM_001953	2,8
	GDF2	Growth differentiation factor 2	NM_016204	3,69
	GRN	Granulin	NM_002087	3,29
	IFNA8	Interferon, alpha 8	NM_002170	1,95
	LTB	Lymphotoxin beta (TNF superfamily, member 3)	NM_002341	2,24
	MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	NM_002415	2,28
	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	NM_001065	2,02
	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	NM_001066	2,03
	TNFRSF8	Tumor necrosis factor receptor superfamily, member 8	NM_001243	1,52
	CD86	CD86 molecule	NM_006889	2,29
	SPN	Sialophorin (leukosialin, CD43)	NM_003123	1,51
DOWN	ACVR1	Activin A receptor, type I	NM_001105	0,25
	SLCO1A2	Solute carrier organic anion transporter family, member 1A2	NM_005075	0,12
	TGFB2	Transforming growth factor, beta 2	NM_003238	0,14
	TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	NM_003242	0,65
	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	NM_001561	0,18
	TGFB3	Transforming growth factor, beta 3	NM_003239	0,18
	XCR1	Chemokine (C motif) receptor 1	NM_005283	0,12
	B7H3	Costimulatory B7 molecule	NM_025240	0,018
	CD28	CD28 molecule	NM_006139	0,016

Table 7: Genes found to be regulated by Thymosin alpha 1 in PBMCs from HIV infected patients (3

	Gene		Gene Bank Access number	SuperArray fold change
	Humoral Immune response			
UP	BATF	Basic leucine zipper transcription factor, ATF-like	NM_006399	1,83
	C3	Complement component 3	NM_000064	3,53
	CCL2	Chemokine (C-C motif) ligand 2 (MCP-1/ MCAF)	NM_002982	2,56
	CCL3	Chemokine (C-C motif) ligand 3 (MIP-1 alpha)	NM_002983	1,96
	CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated) (MIP-4)	NM_002988	2,03
	CCL22	Chemokine (C-C motif) ligand 22 (MDC)	NM_002990	1,56
	CD27	CD27 molecule	NM_001242	2,24
	CD53	CD53 molecule	NM_000560	1,5
	CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	NM_004355	1,86
	CD97	CD97 molecule	NM_001784	1,86
	CD180	Ly 78	NM_005582	2,1
	CCR1	Chemokine (C-C motif) receptor 1	NM_001295	2,38
	CCR7	Chemokine (C-C motif) receptor 7	NM_001838	2,21
	CCRL2	Chemokine (C-C motif) receptor-like 2	NM_003965	2,35
	CSF1R	Colony stimulating factor 1 receptor	NM_005211	3,09
	CYBB	Cytochrome b-245, beta polypeptide (chronic granulomatous disease)	NM_000397	2,4
	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	NM_000211	2,83
	MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)		2,28
	SERPING1	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)	NM_000062	2,82
DOWN	BLNK	B-cell linker	NM_013314	0,06
	MAPK14	Mitogen-activated protein kinase 14	NM_001315	0,17
	SLA2	Src-like-adaptor 2	NM_175077	0,24

Table 8: Genes found to be regulated by Thymosin alpha 1 in PBMCs from HIV infected patients (4

	Gene	Gene Bank Access number	SuperArray fold change
UP	Inflammatory response		
	AIF1	Allograft inflammatory factor 1	NM_001623 2,46
	ALOX5	Arachidonate 5-lipoxygenase	NM_000698 2,23
	C3	Complement component 3	NM_000064 3,53
	CCL2	Chemokine (C-C motif) ligand 2 (MCP-1/ MCAF)	NM_002982 2,56
	CCL3L1	Chemokine (C-C motif) ligand 3-like 1 (LD78 beta)	NM_021006 1,97
	CCL4L1	Chemokine (C-C motif) ligand 4-like 1	NM_001001435 2,73
	CCL5	Rantes	NM_002985 3,21
	CCL3	Chemokine (C-C motif) ligand 3 (MIP-1 alpha)	NM_002983 1,96
	CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated) (MIP-4)	NM_002988 2,03
	CCL22	Chemokine (C-C motif) ligand 22 (MDC)	NM_002990 1,56
	CCR7	Chemokine (C-C motif) receptor 7	NM_001838 2,21
	CD14	CD14 molecule	NM_000591 3,26
	CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	NM_004355 1,86
	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	NM_005194 1,66
	CYBB	Cytochrome b-245, beta polypeptide (chronic granulomatous disease)	NM_000397 2,4
	CXCL16	Chemokine (C-X-C motif) ligand 16 (small inducible cytokine B6)	NM_022059 1,81
	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	NM_005252 1,8
	HDAC4	Histone deacetylase 4	NM_006037 3,2
	IL8	Interleukin 8	NM_000584 1,66
	IL10RB	Interleukin 10 receptor, beta	NM_000628 1,93
	IRF7	Interferon regulatory factor 7	NM_001572 2,44
	ITGAL	Integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1	NM_002209 2,03
	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	NM_000211 2,83

Table 9: Genes found to be regulated by Thymosin alpha 1 in PBMCs from HIV infected patients (5

		Gene		Gene Bank Access number	SuperArray fold change
UP	Inflammatory	LTA4H	Leukotriene A4 hydrolase	NM_000895	2,1
		MYD88	Myeloid differentiation primary response gene (88)	NM_002468	2,02
		PLA2G7	Phospholipase A2, group VII (plat-acti factor acetylhydrolase, plasma)	NM_005084	1,73
		PRDX5	Peroxiredoxin 5	NM_181652	1,55
		PREX1	Phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1	NM_020820	1,66
		S100A8	S100 calcium binding protein A12	NM_002964	3,11
		S100A12	S100 calcium binding protein A8	NM_005621	1,55
		STAB1	Stabilin 1	NM_015136	1,77
		TLR2	Toll-like receptor 2	NM_003264	1,99
		CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	NM_005194	1,66
DOWN		FN1	Fibronectin 1	NM_002026	1,72
		BLNK	B-cell linker	NM_013314	0,6
		CCL1	Chemokine (C-C motif) ligand 1	NM_002981	0,02
		CCL11	Chemokine (C-C motif) ligand 11 (Eotaxin)	NM_002986	0,15
		IFNA 2	Interferon, alpha 2	NM_000605	0,04
		ICEBERG	ICEBERG caspase-1 inhibitor	NM_021571	0,06
		IL8RB	Interleukin 8 receptor, beta	NM_001557	0,17
		NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	NM_000176	0,53
		OLR1	Oxidized low density lipoprotein (lectin-like) receptor 1	NM_002543	0,62
		PTPRA	Protein tyrosine phosphatase, receptor type, A	NM_002836	0,26
		SELE	Selectin E (endothelial adhesion molecule 1)	NM_000450	0,55
		TACR1	Tachykinin receptor 1	NM_001058	0,21
		TLR8	Toll-like receptor 8	NM_016610	0,16
		LBP		NM_004139	0,04

Table 10: Genes found to be regulated by Thymosin alpha 1 in PBMCs from healthy donors (1)

	Gene	Gene Bank Access number	fold change	
	Cytokine, Chemokines and receptors			
UP	CCL2	Chemokine (C-C motif) ligand 2	NM_002982	1,95
	CCL5	Chemokine (C-C motif) ligand 5	NM_002985	1,95
	CCL7	Chemokine (C-C motif) ligand 7	NM_006273	1,57
	CCL17	Chemokine (C-C motif) ligand 17	NM_002987	1,61
	CCL19	Chemokine (C-C motif) ligand 19	NM_006274	1,62
	CCL4L1	Chemokine (C-C motif) ligand 4-like 1	NM_001001435	1,70
	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11	NM_003701	6,07
	TNFSF13B	Tumor necrosis factor (ligand) superfamily, member 13b	NM_006573	3,57
	TRAP1	TNF receptor-associated protein 1	NM_016292	3,28
	VEGFB	Vascular endothelial growth factor B	NM_003377	3,16
	BLR1	Burkitt lymphoma rec 1, GTP bind prot (chemokine (C-X-C motif) rec 5)	NM_001716	1,51
	IL11RA	Interleukin 11 receptor, alpha	NM_004512	1,81
	IL4R	Interleukin 4 receptor	NM_000418	2,07
	IL7R	Interleukin 7 receptor	NM_002185	1,54
	TTN	Titin	NM_003319	1,51
DOWN	CCL11	Chemokine (C-C motif) ligand 11	NM_002986	0,01
	CCL1	Chemokine (C-C motif) ligand 1	NM_002981	0,02
	CCL3	Chemokine (C-C motif) ligand 3	NM_002983	0,46
	CXCL2	Chemokine (C-X-C motif) ligand 2	NM_002089	0,65
	CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	NM_000609	0,53
	CXCL10	Chemokine (C-X-C motif) ligand 10	NM_001565	0,68
	CXCL16	Chemokine (C-X-C motif) ligand 16	NM_022059	0,60
	IL8	Interleukin 8	NM_000584	0,55
	CKLF	Chemokine-like factor	NM_181641	0,62
	CSF3R	Colony stimulating factor 3 receptor (granulocyte)	NM_000760	0,63
	GHR	Growth hormone receptor	NM_000163	0,05
	IL3RA	Interleukin 3 receptor, alpha (low affinity)	NM_002183	0,28
	IL2RA	Interleukin 2 receptor, alpha	NM_000417	0,29

Table 11: Genes found to be regulated by Thymosin alpha 1 in PBMCs from healthy donors (2)

	Gene		Gene Bank Access number	SuperArray fold change
Other genes involved in Cytokine-cytokine receptor interaction				
UP	CSF1R	Colony stimulating factor 1 receptor	NM_005211	1,74
	GRN	Granulin	NM_002087	2,04
	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	NM_000595	1,96
	OSM	Oncostatin M	NM_020530	2,39
	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	NM_001065	2,02
	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	NM_001066	2,03
	CD86	CD86 molecule	NM_006889	1,73
	SIGIRR	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	NM_021805	3,99
DOWN	CLC	Charcot-Leyden crystal protein	NM_001828	0,63
	IFNA1	Interferon, alpha 1	NM_024013	0,10
	IL1RN	Interleukin 1 receptor antagonist	NM_000577	0,64
	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	NM_000879	0,31
	SDCBP	Syndecan binding protein (syntenin)	NM_005625	0,56
	SPRED1	Sprouty-related, EVH1 domain containing 1	NM_152594	0,67
Humoral Immune response				
UP	CCL2	Chemokine (C-C motif) ligand 2	NM_002982	1,95
	CCL7	Chemokine (C-C motif) ligand 7	NM_006273	1,57
	CD86	CD86 molecule	NM_006889	1,73
	CSF1R	Colony stimulating factor 1 receptor	NM_005211	1,74
	CYBB	Cytochrome b-245, beta polypeptide (chronic granulomatous disease)	NM_000397	1,67
	IL7R	Interleukin 7 receptor	NM_002185	1,54
	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	NM_000211	2,07
	LY96	Lymphocyte antigen 96	NM_015364	4,78
	POU2F2	POU domain, class 2, transcription factor 2	NM_002698	2,11
	SERPING1	Serpin peptidase inhibitor, clade G (C1 inhibitor)	NM_000062	2,07
DOWN	YY1	YY1 transcription factor	NM_003403	6,92
	BLNK	B-cell linker	NM_013314	0,00
	CCL3	Chemokine (C-C motif) ligand 3	NM_002983	0,46
	CLC	Charcot-Leyden crystal protein	NM_001828	0,63
	CD22	CD22 molecule	NM_001771	0,64
	CD1C	CD1c molecule	NM_001765?	0,67

Table 12: Genes found to be regulated by Thymosin alpha 1 in PBMCs from healthy donors (3)

	Gene	Gene Bank Access number	SuperArray fold change
UP	Inflammatory response		
	AIF1	Allograft inflammatory factor 1	NM_001623 1,67
	CD14	CD14 molecule	NM_000591 1,95
	CCL2	Chemokine (C-C motif) ligand 2	NM_002982 1,95
	CCL5	Chemokine (C-C motif) ligand 5	NM_002985 1,95
	CCL7	Chemokine (C-C motif) ligand 7	NM_006273 1,57
	CCL17	Chemokine (C-C motif) ligand 17	NM_002987 1,61
	CCL4L1	Chemokine (C-C motif) ligand 4-like 1	NM_001001435 1,70
	CYBB	Cytochrome b-245, beta polypeptide (chronic granulomatous disease	NM_000397 1,67
	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	NM_005252 1,61
	IRF7	Interferon regulatory factor 7	NM_001572 2,25
	ITGAL	Integrin, alpha L antigen CD11A (p180), lymphocyte function-associated antigen 1	NM_002209 1,95
	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit	NM_000211 2,07
	LY96	Lymphocyte antigen 96	NM_015364 4,78
	NFE2L1	Nuclear factor (erythroid-derived 2)-like 1	NM_003204 1,86
	NFATC3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent	NM_004555 2,15
	PARP4	Poly (ADP-ribose) polymerase family, member 4	NM_006437 2,43
	PREX1	Phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1	NM_020820 2,00
	S100A12	S100 calcium binding protein A12	NM_005621 2,61
	S100A8	S100 calcium binding protein A8	NM_002964 2,18
	STAB1	Stabilin 1	NM_015136 4,11
	SIGIRR	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	NM_021805 3,99
DOWN	BLNK	B-cell linker	NM_013314 0,00
	CCL11	Chemokine (C-C motif) ligand 11	NM_002986 0,01
	CCL1	Chemokine (C-C motif) ligand 1	NM_002981 0,02
	CCL3	Chemokine (C-C motif) ligand 3	NM_002983 0,46
	CKLF	Chemokine-like factor	NM_181641 0,62
	CXCL2	Chemokine (C-X-C motif) ligand 2	NM_002089 0,65
	CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1	NM_000609 0,53
	CXCL10	Chemokine (C-X-C motif) ligand 10	NM_001565 0,68
	CXCL16	Chemokine (C-X-C motif) ligand 16	NM_022059 0,60
	F11R	F11 receptor	NM_144503 0,66
	IL19	Interleukin 19	NM_013371 0,41
	IL1RN	Interleukin 1 receptor antagonist	NM_000577 0,64
	IL5	Interleukin 5 (colony-stimulating factor, eosinophil	NM_000879 0,31
	LEFTY2	Left-right determination factor 2	NM_003240 0,39
	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor	NM_000176 0,43
	PTPRA	Protein tyrosine phosphatase, receptor type, A	NM_002836 0,67
	FN1	Fibronectin 1	NM_002026 0,33