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PHENOTYPICAL AND FUNCTIONAL ANALYSIS OF HIV SPECIFIC CD4$^+$ AND CD8$^+$ T-CELLS DURING HIV-1 INFECTION: CLINICAL IMPLICATIONS

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CHAPTER 1

1.0 THE DISCOVERY OF HUMAN IMMUNODEFICIENCY VIRUS

On July 3, 1981, the *New York Times* published an article detailing the diagnosis of a rare form of cancer called Kaposi's Sarcoma among 41 homosexual men primarily in New York City and the San Francisco Bay Area over a period of 30 months [1]. Until that time, the nationwide incidence of this disease was one case in every 1.5 million people as reported by the Centers for Disease Control (CDC). Two years later, with 1,350 reported cases, viral DNA from a human T-lymphotrophic retrovirus was isolated from the T cells of several patients exhibiting symptoms common to what is now called Acquired Immune Deficiency Syndrome (AIDS) [2]. The enveloped virus was named Human Immunodeficiency Virus (HIV), and its discovery earned Drs. Françoise Barré-Sinoussi and Luc Montagnier the Nobel Prize in Physiology or Medicine in 2008. It was classified as belonging to genus *Lentivirus* in the *Retroviridae* family. The lentiviral classification denotes the protracted period of latency which, in the case of HIV, can exceed seven-ten years before the emergence of opportunistic infections that are collectively referred to as AIDS [3].

Since 1985, the availability of serologic tests to detect HIV infection has allowed to estimate the real size of the epidemic.

1.1 AN OVERVIEW OF HIV/AIDS EPIDEMIC

Today, HIV is a global pandemic. It is estimated that HIV/AIDS has claimed the lives of 25 million people with an estimated 33.3 million people living with the disease in 2009 [4]. The majority of people with HIV/AIDS are living in developing countries. In particular, the sub-Saharan Africa region, which has adult prevalence of 5%, has continued to bear the overwhelming burden of the epidemic. Sub-Saharan Africa has about two-thirds of all people living with HIV/AIDS globally [4]. Table 1 below shows the summary of estimates of HIV and AIDS at the end of the year 2009.

Table 1: Estimates of HIV and AIDS at the end of year 2009

	Global number of people (in millions)	**Sub-Saharan Africa (in millions)**
Living with HIV/AIDS in year 2009	33.3	22.5
Newly infected with HIV in year 2009	2.6	1.8
Died due to AIDS in year 2009	1.8	1.3

Source: (UNAIDS/WHO, 2010). AIDS epidemic update, December 2010

In Italy the surveillance system of new HIV infections was established by a decree of the Ministry of Health in 2008 and receives data from the country's regions and provinces. Some regions had already collected data on new diagnoses before the system existed, as early as 1985. According to these data, the incidence of HIV infection in Italy peaked at the end of the 1980s and followed by a progressive decrease until the end of the 1990s.

Afterwards, the number of new infections stabilized. In 2009 it was estimated an incidence of new HIV diagnoses of 4.6 per 100,000 population among persons with Italian nationality and of 22.5 per 100,000 population among non-nationals (Fig. 1). Moreover, in the same year, it was evaluated that almost one third of the persons with HIV infection were of foreign nationality (Fig. 2). The median age of newly diagnosed cases has increased over time: in 2009, it was of 39 years for males and 36 years for females (Fig. 3). Cases attributable to heterosexual contact or contact among men who have sex with men (MSM) are steadily increasing, accounting for 79% of all reported cases in 2009 (Fig. 4). In 2009, one third of the persons with a new HIV diagnosis were diagnosed at an advanced stage of illness, with significant impairment to their immune systems (Fig. 5). Regarding to AIDS cases, the surveillance system, that was created in 1984, recorded that since the beginning of the epidemic in 1982, about 63,000 cases of AIDS have been reported and nearly 40,000 of them have died. The number of new AIDS cases reported each year has decreased since 1996, mainly because of antiretroviral therapies introduced in Italy in the same year [5].

Although the development of highly active antiretroviral therapy (HAART) in 1996 is credited for the precipitous drop in AIDS-related morbidity and mortality in developed countries such as the United States and Europe, the costs associated with HAART and the lack of robust health care infrastructures has hindered their widespread use in most developing countries. It was reported that by the end of 2007, the number of people receiving HAART in developing countries had reached the 3 million – roughly 10% of

those in need [4]. The combination of economic, logistical, and political barriers to treatment is particularly alarming given that nearly 95% of all HIV-infected people live in developing regions of the world such as sub-Saharan Africa and Asia. Education and sexually transmitted disease control programs have proven to be effective strategies for reducing the spread of HIV, but the need to develop an inexpensive vaccine capable of preventing or controlling an infection remains one of the most important challenges in combating the pandemic.

1.2 MOLECULAR AND BIOLOGIC FEATURES OF HIV

1.2.0 HIV Structure and Genes

HIV is a member of the lentivirus family of animal retroviruses. Two closely related types of HIV, designated HIV-1 and HIV-2, have been identified. HIV-1 is by far the most common cause of AIDS, but HIV-2, which differs in genomic structure and antigenicity, causes a similar clinical syndrome.

HIV consists of two identical strands of RNA packaged within a core of viral proteins and surrounded by a phospholipid bilayer envelope derived from the host cell membrane but including virally encoded membrane proteins (Fig. 6). Long terminal repeats (LTRs) at each end of the genome regulate viral gene expression, viral integration into the host genome, and viral replication. The *gag* sequences encode core structural proteins. The *env* sequences encode the envelope glycoproteins gp120 and gp41, which are required for infection of cells. The *pol* sequences encode reverse trascriptase, integrase, and viral

protease enzymes required for viral replication. In addition to these typical retrovirus genes, HIV-1 also includes six other regulatory genes, namely, the *tat*, *rev*, *vif*, *nef*, and *vpu* genes, whose products regulate viral reproduction in various ways [6].

1.2.1 Viral Life Cycle

HIV infection of cells begins when the envelope glycoprotein (Env) of a viral particle binds to both CD4 and coreceptor that is a member of the chemokine family. The viral particles that initiate infection are usually in the blood, semen, or other body fluid of one individual and are introduced into another individual by sexual contact, needle stick, or transplacental passage. Env is a complex composed of a transmembrane gp41 subunit and an external, nonconvalently associated gp120 subunit. The first step of viral life cycle is the binding of gp120 subunits to CD4 molecules [7], which induces a conformational change that promotes secondary gp120 binding to a chemokine coreceptor. Coreceptor binding induces a conformational change in gp41 that exposes a hydrophobic region, called the fusion peptide, that inserts into the cell membrane and enables the viral membrane to fuse with the target cell membrane. After the virus completes its life cycle in the infected cell, free viral particles are released from one infected cell and bind to an uninfected cell, thus propagating the infection. In addition, gp120 and gp41, which are expressed on the plasma membrane of infected cells before virus is released, can mediate cell-fusion with an uninfected cell that expresses CD4 and coreceptor, and HIV genomes can then be passed between the fused cells directly.

More than seven different chemokine receptor have been shown to serve as coreceptors for HIV entry into cells, but those most important are CCR5 and CXCR4 [8]. HIV-1 isolates can be divided in three broad groups based on coreceptor used. Variants with singular use of CCR5 and CXCR4 are termed R5 and X4 viruses, respectively, while those capable using both coreceptors are termed R5X4.

Because CCR5 is expressed on the surface of the macrophages (and some memory T cells), HIV strains that use this coreceptor are macrophages-tropic (M-tropic) whereas those that use CXCR4, expressed on the surface of the T cells, are T-tropic. HIV strains the use both coreceptor are dual-tropic viruses. In many HIV-infected individuals, there is a change from the production of virus that uses CCR5, in the early stages of the disease, to virus that binds to CXCR4, in the later stages of the disease. The T-tropic strains tend to be more virulent, presumably because they infect and deplete T cells more than do M-tropic strains.

Once an HIV virion enters a cell, the enzymes within the nucleoprotein complex become active and begin the viral reproductive cycle. The nucleoprotein core of the virus becomes disrupted, the RNA genome of HIV is transcribed into a double-stranded DNA form by viral reverse transcriptase, and the viral DNA enters the nucleus. The viral integrase also enters the nucleus and catalyzes the integration of viral DNA into the host cell genome. The integrated HIV DNA is called the provirus. The provirus may remains transcriptionally inactive for months or years, with little or no production of new viral proteins or virions, and in this ways HIV infection of an individual cell can be latent.

Initiation of HIV gene transcription in T cells is linked to activation of the T cells by antigen or cytokines [9]. For example, cytokines such us IL2, TNF, and lymphotoxin stimulate HIV gene expression in infected T cells, and IL1, IL3, IL6, TNF, lymphotoxin, and IFN-γ stimulate HIV gene expression and viral replication in infected monocytes and macrophages. This phenomenon is significant to the pathogenesis of AIDS because the normal response of a latently infected T cell to a microbe involves the end of the latency. The multiple infections that AIDS patients acquire thus stimulate production and infection of additional cells.

1.3 PATHOGENESIS OF HIV INFECTION AND AIDS

HIV disease begin with acute infection and advances to chronic progressive infection of peripheral lymphoid tissues. Acute infection is characterized by infection of memory CD4+ T cells (which express CCR5) in mucosal lymphoid tissues, and death of many infected cells [10].

The transition from acute phase to a chronic phase of infection is characterized by dissemination of the virus (viremia) and the development of host immune response. This immune response partially control the infection and viral production, and such control is reflected by a drop in viremia to low but detectable levels by approximately 12 weeks after the primary exposure.

In the next chronic phase of the disease, lymph nodes and the spleen are sites of continuous HIV replication and cell destruction. During this period of the disease, the

immune system remains competent and few or no clinical manifestations of the HIV infection are present. Therefore, this phase of HIV disease is called the clinical latency period. Although the majority of peripheral blood T cells do not harbor the virus, destruction of CD4+ T cells within lymphoid tissues progresses during the latent period, and the number of circulating blood CD4+ T cells steadily declines [11].

1.3.0 Mechanisms of Immunodeficiency caused by HIV

HIV infection ultimately results in impaired function of both the adaptive and innate immune systems. The most prominent defects are in cell-mediated immunity, and they can be attributed to several mechanisms, including direct cytopathic effects of the virus and indirect effects.

Death of CD4+ T cells is a major cause of the decline in the number of these cells, especially in the acute phase of the infection. Several direct toxic effects of HIV on infected CD4+ cells have been described [11]:

- The process of virus production, with expression of gp41 in the plasma membrane and budding of viral particles, may lead to increased plasma membrane permeability and the influx of lethal amounts of calcium, which induces apoptosis, or osmotic lysis of the cell caused by the influx of water.
- Viral production can interfere with cellular protein synthesis and thereby lead to cell death.

- The plasma membrane of HIV-infected T cells fuse with uninfected CD4+ T cells by virtue of gp120-CD4 interaction, and multinucleated giant cells or syncytia are formed. The process of HIV-induced syncytia formation can be lethal to HIV-infected T cells as well as to uninfected CD4+ T cells that fuse to the infected cells. However, this phenomenon has largely been observed in vitro, and syncytia are rarely seen in the tissues of patients with AIDS.

Other mechanisms in addition to direct lysis of infected CD4+ T cells by virus have been proposed for the depletion and loss of function of these cells in HIV-infected individuals:

- Chronic activation of the T cells may predispose the cells to apoptosis.
- HIV-specific CTLs are present in many patients with AIDS, and these cells can kill infected CD4+ T cells.
- Antibodies against HIV envelope proteins may bind to HIV-infected CD4+ T cells and target the cells for antibody-dependent cell-mediated cytotoxicity (ADCC).
- Binding of gp120 to newly synthesized intracellular CD4 may interfere with normal protein processing in the endoplasmic reticulum and block cell surface expression of CD4, making the cells incapable of responding to antigenic stimulation.
- Defective maturation of CD4+ T cells in the thymus.

1.4 CLINICAL FEATURES OF HIV DISEASE

As antiretroviral therapy is improving, many of the clinical manifestations are changing.

1.4.0 Transmission of HIV and Epidemiology of AIDS

HIV is transmitted from one individual to another by three major routes:

- Sexual contact is the most frequent mode of transmission, either between heterosexual couples (the most frequent mode of transmission in Africa and Asia) or between homosexual partners.
- Mother-to-child transmission of HIV accounts for the majority of pediatric cases of AIDS. This type of transmission occurs most frequently *in utero* or during childbirth, although transmission through breast milk is also possible.
- Inoculation of a recipient with infected blood or blood products is also a frequent mode of HIV transmission. Needles shared by intravenous drug abuser account for most cases of this form of transmission. With the advent of routine laboratory screening, transfusion of blood or blood products in a clinical setting accounts for a small portion of HIV infections.
- Major groups at risk for the development of AIDS in the United States include homosexual or bisexual males, intravenous drug abusers, heterosexual partners of members of the other risk groups, and babies born of infected mothers. Health care workers have a small increased risk for infection.

1.4.1 Clinical Course of HIV Infection

The acute phase of the illness, also called the acute HIV syndrome, is the period of viremia characterized by non-specific symptoms of infection. It develops in 50% to 70% of infected adults tipically 3 to 6 weeks after infection. There is a spike of plasma virus and a modest reduction in CD4+ T cell counts, but the number of blood CD4+ T cells often returns to normal. In many patients, however, the infection is occult and there are no symptoms [12].

The chronic phase of clinical latency may last for many years. During this time, the virus is contained within lymphoid tissues, and the loss of CD4+ T cells is corrected by replenishment from progenitors. Patients are asymptomatic or suffer from minor infections [12]. Within 2 to 6 months after infection, the concentration of plasma virus stabilizes at a particular set-point, which differs among patients. The level of the viral set-point and the number of blood CD4+ T cells are clinically useful indicators of the progression of disease. As the disease progresses, patients become susceptible to other infections, and immune responses to these infections may stimulate HIV production and accelerate the destruction of lymphoid tissues. Cytokines, such as TNF, that are produced by the innate immune system in response to microbial infections are particularly effective in boosting HIV production. Thus, as the immune system attempts to eradicate other microbes, it brings about its own destruction by HIV.

HIV disease progresses to the final and almost invariable lethal phase, called AIDS, when the blood CD4+ T cell count drops below 200 cells/mm^3. HIV viremia may climb

dramatically as viral replication in other reservoirs accelerates unchecked. Patients with AIDS suffer from combinations of opportunistic infections, neoplasm, cachexia (HIV wasting syndrome), kidney failure, (HIV nephropathy), and CNS degeneration (AIDS encephalopathy) [12]. Because CD4+ helper T cells are essential for both cell-mediated and humoral immune response to various microbes, the loss of these lymphocytes is the main reason that patients with AIDS become susceptible to many different type of infections. Furthermore, many of the tumors that arise in patients with AIDS have a viral etiology, and their prevalence in the setting of AIDS reflects an inability of the HIV infected patient to mount an effective immune response against oncogenic viruses. Most of the opportunistic infections and neoplasms associated with HIV infection occur after the blood CD4+ T cell count drops below 200 cells/mm^3. Cachexia is often seen in patients with chronic inflammatory diseases and may result from effects of inflammatory cytokines (such as TNF) on appetite and metabolism. The CNS disease in AIDS may be due to neuronal damage by the virus or by shed viral proteins such as gp120 and Tat as well as to the effects of cytokines elaborated by infected microglial cells. Many of these devastating consequences of HIV infection, including opportunistic infections and tumors, have been significantly reduced by highly active anti-retroviral therapy (HAART).

Although this summary of the clinical course is true for the most severe cases, the rate of progression of the disease is highly variable, and some individuals are long-term non progressors (LTNPs) [13]. The immunologic correlates of variable progression remain

unknown. Also, recent anti-retroviral therapy as changed the course, and greatly reduced the incidence, of severe opportunistic infections (such as *Pneumocystis*) and tumors (such as Kaposi's sarcoma).

1.5 LABORATORY TESTING FOR INITIAL ASSESSMENT AND MONITORING OF ANTIRETROVIRAL THERAPY

A number of laboratory tests are important for initial evaluation of HIV-infected patients upon entry into care, during follow-up if antiretroviral therapy (ART) has not been initiated, and prior to and after initiation or modification of therapy to assess virologic and immunologic efficacy of ART and to monitor for laboratory abnormalities that may be associated with antiretroviral (ARV) drugs.

Two surrogate markers are used routinely to assess the immune function and level of HIV viremia: CD4 T-cell count (CD4 count) and plasma HIV RNA (viral load).

1.5.0 CD4 T-cell count

The CD4 T-count is the major laboratory indicator of immune function in patients with HIV infection. It is one of the key factors in deciding whether to initiate ARV therapy and prophylaxis for opportunistic infections, and currently it is the strongest predictor of disease progression and survival according to clinical trials and cohort studies [14, 15].

In general, CD4 T-cell count should be monitored every 3–4 months to:

1. determine when to start ARV therapy in untreated patients (ARV therapy should be initiated in all patients with a CD4 count <350 cells/mm^3 or with history of an AIDS-defining illness),
2. assess immunologic response to ART, and
3. assess the need for initiation or discontinuation of prophylaxis for opportunistic infections.

The absolute CD4 count is a calculated value based on the total white blood cell (WBC) count and the percentages of total and CD4+ T lymphocytes. This absolute number may fluctuate among individuals or may be influenced by factors that may affect the total WBC and lymphocyte percentages, such as use of bone marrow–suppressive medications or the presence of acute infections. In all these cases, CD4 percentage remains stable and may be a more appropriate parameter to assess the patient's immune function.

1.5.1 Plasma HIV RNA Testing

Plasma HIV RNA (viral load or HIV viremia) should be measured in all patients at baseline and on a regular basis thereafter, especially in patients who are on treatment, because viral load is the most important indicator of response to antiretroviral therapy. Thus, viral load testing serves as a surrogate marker for treatment response [16] and can be useful in predicting clinical progression [17, 18]. Optimal viral suppression is generally defined as a viral load persistently below the level of detection (<20–

75copies/mL, depending on the assay used). However, isolated “blips” (viral loads transiently detectable at low levels, typically <400 copies/mL) are not uncommon in successfully treated patients and are not thought to represent viral replication or to predict virologic failure [19]. For most individuals who are adherent to their antiretroviral regimens and who do not harbor resistance mutations to the prescribed drugs, viral suppression is generally achieved in 12–24 weeks, even though it may take longer in some patients.

Plasma viral load should be measured before initiation of therapy and preferably within 2–4 weeks, and not more than 8 weeks, after treatment initiation or after treatment modification. Repeat viral load measurement should be performed at 4–8-week intervals until the level falls below the assay’s limit of detection.

Viral load should be repeated every 3–4 months or as clinically indicated. Some clinicians may extend the interval to every 6 months for adherent patients who have suppressed viral loads for more than 2–3 years and whose clinical and immunologic status is stable.

CHAPTER 2

2.0 T-CELL IMMUNE RESPONSE AGAINST HIV INFECTION

HIV infection may develop to AIDS at different rates in different individuals, with a spectrum varying from rapid progression to long-term non-progression. Therefore, owing to this variable evolution, it is essential to have tests which can accurately assess the stage of infection in an individual, as well as predict its course and its progression. Laboratory tests, used routinely in the management of HIV infected patients to assess the immune function and level of HIV viremia (CD4 T-cell count and plasma HIV RNA), are only indicators and not predictors of disease progression.

In the last few years there has been great interest for the study of T-cell immune response against different viruses that are able to establish chronic infection (such as CMV, EBV, HSV, epatitis viruses, HIV) in order to identify immunologic markers of control/progression.

These studies have shown great phenotypic and functional heterogeneity of the T-cell responses against these viruses in both animal and human models and some authors have suggested that this heterogeneity is regulated by the levels of viral/antigen load [20].

With regard phenotypic heterogeneity, a large number of surface markers have been used over time to define sub-populations of antigen-specific CD4+ and CD8+ T cells at different stages of differentiation. These markers include CD45RO, CD45RA, CD62L, CD28, CD27, CD7, CD57, CD127, and CCR7 [21, 22].

Combined use of CD45RA (cell surface glycoprotein associated with the naïve T-cell phenotype) and CD27 (member of the tumor necrosis factor receptor family), has been used in previous studies in order to separate four gamma delta T-cell subsets with distinct homing capacity and effector function, by flow cytometry [23, 24]:

1) CD27+ CD45RA+, naïve cells;

2) CD27+ CD45RA-, central memory cells;

3) CD27- CD45RA-, effector memory cells;

4) CD27- CD45RA+, terminally differentiated cells.

In particular, naive and memory cells express lymph node homing receptors, abound in lymph nodes, and lack immediate effector functions. Conversely, memory and terminally differentiated cells, which express receptors for homing to inflamed tissues, are poorly represented in the lymph nodes while abounding at sites of inflammation, and display immediate effector functions [23].

With regard functional heterogeneity, distinct populations of specific-CD4+ and -CD8+ T-cells have been identified on the basis of the ability to secrete interleukin-2 (IL-2) and interferon-γ (IFN-γ): three populations of $CD4^+$ T-cells (single IL-2, dual IL-2/IFN-γ, and single IFN-γ), and two of $CD8^+$ T-cells (dual IL-2/IFN-γ and single IFN-γ).

There is a correlation between the patterns of cytokine secretion and the proliferation capacity. Antigen-specific CD4+ T-cell populations secreting only IL-2 (single IL-2) and both IL-2 and IFN-γ (dual IL-2/IFN-γ) and antigen-specific CD8+ T-cell population secreting both IL-2 and IFN-γ retain proliferation capacity, while CD4+ and CD8+ T-

cells secreting only IFN-γ (single IFN-γ) have poor proliferation capacity [20, 25] (Fig. 7A). However, proliferation of single IFN-γ secreting CD4+ and CD8+ T-cells can be promoted in the presence of an exogenous source of IL-2.

Regarding to HIV-1 infection, recent studies have shown that virus-specific CD4+ T-cell response, during primary infection, was typically an effector response, consisting of single IFN-γ secreting CD4+ T-cells (monofunctional response) [26] and that, in the chronic phase of infection, it was strictly dependent upon a series of factors [27], including the lack of control of virus replication, the initiation of ARV therapy and the spontaneous control of virus replication as it may occur in a small (1%) percentage of subjects known as long-term non-progressors (LTNPs). Specifically, during chronic infection, in the absence of controlled virus replication, the HIV-1-specific CD4+ T-cell response, remained predominantly an effector response [26, 28] whereas, in subjects successfully treated with ARV therapy or in LTNPs, who spontaneously control virus replication with viremia levels below 50 HIV-1 RNA copies per ml of plasma (the limit of detection of the standard PCR assay), it switched to a memory response consisting of single IL-2-, dual IL-2/IFN-γ, and single IFN-γ secreting CD4+ T-cells (polyfunctional response) [26, 28]. Similarly, the HIV-1-specific CD8+ T-cell response during primary infection was typically an effector response consisting of single IFN-γ secreting CD8+ T-cells (monofunctional response) and remained so during the chronic phase of infection, in the absence of controlled virus replication. In LTNP patients and only in 30-40% of subjects successfully treated with antiviral therapy, the HIV-1-specific CD8+

T-cell response was a memory response consisting of dual IL-2/IFN-γ and single IFN-γ secreting CD8+ T-cells (polyfunctional response) [26, 28, 29].

Therefore, the above results show that antiviral therapy-mediated suppression of virus replication is not associated with consistent detection of the IL-2 CD8+ T-cell response (dual-IL-2/IFN-γ cells), differently from HIV-1-specific CD4+ T-cell response that is constantly associated with the detection of single-IL-2 and dual-IL-2/IFN-γ secreting cells.

Although there is not formal demonstration, it seems that polyfunctional T-cell responses, mediated by multiple-cytokine producing T-cells, are directly responsible for control of virus replication and prevention of virus-associated disease [30].

2.1 AIMS OF THE STUDY

The aims of my study were to carry-out a phenotypic and functional analysis of CD4+ and CD8+ T-cells in HIV-1 infected patients, enrolled in Policlinico Hospital, Palermo, treated and untreated with antiretroviral therapy in order to evaluate their immunological status and to analyse the serum cytokine profile in these patients in order to establish their immune-activation state.

Our proposal was to correlate the immunological status with the viral activity (antigen load routinely used during the follow up of these patients) and the efficacy of ARV therapy in order to identify immunologic markers that help the clinician to predict progression of HIV disease and to define the most opportune time to initiate, modulate or interrupt therapeutic measures.

2.2 MATERIALS AND METHODS

2.2.0 Study population

HIV-1 mono-infected adults, on regular follow-up at the AIDS Center, Infectious Disease Department of the University of Palermo, were enrolled in this study after written informed consent was obtained.

All patients were admitted to the hospital every 3 months for physical examination and routine laboratory tests, including HIV viremia and CD4 T-cell count.

From this cohort, we selected participants meeting criteria for one of five groups:

I. **“naïve” patients**, defined as individuals never treated with ARV drugs;

II. **virologic "controllers"**, defined as ARV drug-untreated individuals with plasma HIV viremia below 10,000 copies RNA/ml;

III. **virologic "non controllers"**, defined as ARV drug-untreated individuals with plasma HIV viremia above 10,000 copies/ml;

IV. **virologic "responders"**, defined as ARV drug-treated individuals with undetectable plasma HIV viremia (<47 copies/ml);

V. **virologic "non responders"**, defined as ARV drug-treated individuals with detectable plasma HIV viremia.

In general, the "controllers" are clinically stable patients not receiving ARV therapy more than 3 years; the "non controllers" are immunocompromised patients, which experienced opportunistic infections (most frequently *Mycobacterium avium complex* infection, *Cytomegalovirus* retinitis, *Pneumocystis jiroveci* pneumonia), not receiving antiretroviral therapy because not compliant; the "responders" are patients on antiretroviral therapy, compliant, in excellent clinical conditions; the "non responders" are immunocompromised patients with high-level of phenotypic resistance to antiretroviral drugs, which experienced in the past opportunistic infections; finally, the "naïve" patients are all newly diagnosed asymptomatic cases of HIV-1 infection.

Based on the foregoing it is clear that "virologic controllers" represent a unique natural human model in which to investigate correlates of immune protection.

Informations about demographic characteristics (age, gender, risk factors for HIV infection, ARV therapy) were all recorded in a database designed for this study and are summarized in Table 2.

Table 2: Demographic characteristics of the study groups, *n* (%)

	Naïve ***n* = 5**	**Controllers** ***n* = 5**	**Non controllers** ***n* = 10**	**Responders** ***n* = 20**	**Non responders** ***n* = 10**
Male gender	3 (60)	3 (60)	7 (70)	12 (60)	6 (60)
Risk group					
Blood Transfusion	-	-	-	-	-
Homosexual men	3 (60)	1 (20)	3 (30)	4 (20)	2 (20)
Intravenous drug use	-	2 (40)	4 (40)	9 (45)	4 (40)
Others	2 (40)	2 (40)	2 (20)	6 (30)	4 (40)
Unknown	-	-	1 (10)	1 (5)	-
ARV therapy	No	No	No	Yes	Yes

Most patients were males and the most frequent risk factors for HIV infection were homosexual contact and intravenous drug use. Furthermore, in our cohort the 60% of patients were under antiretroviral therapy and the 40% of these had an undetectable HIV-RNA load (<47 copies/mL).

Baseline mean value of CD4 T-cell counts and HIV-RNA loads for each group of patients is shown in Table 3.

Table 3: Baseline media of CD4 T-cell counts and HIV-RNA loads in the study groups

	Naïve *n* = 5	Controllers *n* = 5	Non controllers *n* = 10	Responders *n* = 20	Non responders *n* = 10
CD4 T-cell counts	540	978	105	1288	62
HIV RNA load	23100	5250	20000	<47	2250

CD4 T-cell count normal values 500-1500 cells/mm^3

2.2.1 Flow cytometry analysis

Functional pattern. To study intracellular production of IFN-γ and IL-2, peripheral blood mononuclear cells (PBMC, 10^6/ml) were stimulated with HIV-1IIIB p55 Gag (5 μg/ml, final concentration, NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), in the presence of monensin for 6 hrs at 37°C in 5% CO_2. Subsequently, the cells were harvested, washed and stained with APC-conjugated anti-CD8 or anti-CD4 mAb (Becton Dickinson) in incubation buffer (PBS-1% FCS-0.1% Na azide) for 30 min at 4°C. Later on, the cells were washed twice in PBS-1% FCS and fixed with PBS-4% paraformaldehyde overnight at 4°C. Fixation was followed by permeabilization with PBS-1% FCS-0.3% saponin–0.1% Na azide for 15 min at 4°C. Staining of intracellular cytokines was performed by incubation of fixed permeabilized cells with PE-labelled anti-IFN-γ (clone B27) and FITC-labelled IL-2 antibody (clone MQ1-17H12) or an isotype-matched control mAb. All mAbs were from BD Biosciences. After two more washes in PBS containing 1% FCS, the cells were analyzed by FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA)

and analyzed using FlowJo 8.3.3 software (Treestar). Initial gating was on the lymphocyte population by forward and side scatter, and analysis was performed on 100,000 acquired events for each sample. For every sample 100,000 viable nucleated cells were acquired within each population. Data are reported after background correction and the percent of cytokine secreted CD8 and CD4 T cells had to be at least > 2-fold higher than background for individual cytokine**.**

2.2.2 Phenotypic analysis. To assess the phenotype of T cells, before and after HIV antigen stimulation as above reported, PBMC were harvested, washed and stained with anti-CD8 or anti-CD4 mAb and anti-CD45RA and anti-CD27 mAbs, conjugated with different fluorochromes in incubation buffer (PBS-1% FCS-0.1% Na azide) for 30 min at 4°C. Cells were then washed twice in PBS with 1% FCS and analyzed by flow cytometry.

2.2.3 Cytokine assay. Different cytokines **(**IL-1β, IL-2, IL-6, TNF-β, IL-4, IL-10, IL-13, IP-10, IL-8, MIP-1 α, MIP-1β, MCP-1 and more) levels in sera from HIV patients was evaluated by xMAP multiplex technology on the Luminex platform (Luminex, Austin TX, USA), using Biorad reagents (Bio-Rad Life Sciences, CA, USA). Data were acquired and analyzed with the Bioplex Manager Software (Bio-Rad). Responses were scored positive if the value was 2 fold over the negative control.

Preparation of the samples before analysis on the Luminex machinery was performed according to the '*Sample protocol for washed capture sandwich immunoassay*' (January

2006) downloaded from http://www.luminexcorp.com, with a few modifications. Briefly, 50 µl of bead-solution (containing Assay buffer and 5000 beads) was added to the appropriate wells in a 96-wells Millipore filter plate. Fifty microlitre Assay buffer was added to each background well, 50 µl of a diluted standard serum pool, diluted twofold from 1:25 to 1:3200 to each standard well and 50 µl of diluted positive serum control, diluted 1:25 to each positive control well. Fifty microlitre sample, diluted to 1:25 and 1:400, respectively was added to each sample well. Standards and positive controls were diluted in Assay buffer and samples were diluted in Assay buffer with 10%Sample blocking buffer. After 30 min incubation at room temperature on a plate shaker and two washes, 50 µl of biotinylated detection antibody, diluted 1:1000 in Assay buffer with a final antibody concentration of 1.0 µg/ml was added to each well. After further 30 min incubation at room temperature on a plate shaker and two washes, 50 µl of diluted streptavidin-R-phycoerythrin, diluted 1:250 in Assay buffer was added to each well. After further 30 min incubation at room temperature on a plate shaker and two washes, the samples were analysed on the Luminex machinery.

2.2.4 Statistical analysis

Values of p were derived from two-tailed ANOVA tests. Values of $p < 0.05$ were considered significant.

2.3 RESULTS

A total of 50 subjects were studied, including 5 “naïve” patients, 5 “controllers”, 10 “non controllers”, 20 “responders” and 10 “non responders”.

All patients were followed for a period of 12 months, except for the naïve patients which were followed only 3 months because, on the basis of their clinical and immune-virologic characteristics, it was necessary to start ARV therapy early.

2.3.1 Functional analysis of CD4+ and CD8+ T-cells. For each group of patients, we measured the average percentage of CD4+ and CD8+ T-cells producing IFN-γ and/or IL-2, at different observation time (T0, T3, T6, T9 and T12), to evaluate the different state of T-cells and to find a correlation between immunological and clinical status of the patients over time.

We compared virologic “controllers” with virologic “non controllers” (Fig. 8). In the first group of patients we observed during the period of study that all T-cell subsets were represented, the average percentage of single IFN-γ producing T-cells did not change with regard to CD4+ T-cells whereas it was reduced with regard CD8+ T-cells and finally double cytokine producing T-cells (both CD4+ and CD8+) always were well represented, reaching values around 40%.

On contrary, in the second group of patients we observed that single IFN-γ producing T-cells (both CD4+ and CD8+) were much more represented than other T-cell populations,

reaching values around 80%, and double cytokine producing T-cells (both CD4+ and CD8+) were virtually absent.

We also compared virologic "responders" with virologic "non responders" (Fig. 9). In the first group of patients we observed during the period of study that all T-cell populations were represented, the average percentage of single IFN-γ producing T-cells (both CD4+ and CD8+) reduced reaching lower levels that baseline after 6 months of therapy and finally double cytokine producing T-cells (both CD4+ and CD8+) were well represented reaching values 20-30%.

In the second group of patients we observed that single IFN-γ producing T-cells were more represented than other T-cell populations (expecially with regard to CD8+), sometimes reaching values of 100%, and double cytokine producing T-cells (both CD4+ and CD8+) were virtually absent.

In order to establish if the double cytokine producing cells are simply a consequence or a cause of the virologic control (as suggested by Pantaleo et al.), we evaluated the frequency of these cells both in patients with low viremia in the absence of therapy ("controllers") and in those who were aviremic as a consequence of ARV therapy ("responders"). Through this comparison (Fig. 10), we found that the "controllers" had slightly higher total levels of double cytokine producing T-cells (mean total values of 36.4 vs 35.9%). These data suggest but do not prove that these cells contribute to virologic control, at least in this group of patients. However, we can safely assume that

the absence of viremia does not in and of itself result in higher levels of double-cytokine producing cells.

Finally, functional analysis of T-cell immune response in naïve patients was possible only for 3 months (T0 and T1) because these patients, on the basis of their clinical and immune-virologic characteristics, started early ARV therapy. However, despite the short period of study, we observed that double cytokine producing T-cells were very poorly represented compared to those producing a single cytokine (Fig. 11).

2.3.2 Phenotype analysis of CD4+ and CD8+ T-cells.

We also asked whether there were differences in the differentiation status of T-cells in "controller" and "responder" patients before and after HIV antigen stimulation. To perform this analysis, CD4+ and CD8+ T-cells were characterized by CD45RA and CD27, allowing discrimination of 4 phenotypically distinct subpopulations of T-cells:

1) CD27+ CD45RA+, naïve cells (TNAIVE);

2) CD27+ CD45RA-, central memory cells (TCM);

3) CD27- CD45RA-, effector memory cells (TEM);

4) CD27- CD45RA+, terminally differentiated cells (TEMRA).

With regard to the phenotypic pattern of T-cells in "controllers", we observed no significant difference after stimulation of both CD4+ and CD8+ T-cells with HIV-1IIIB p55. In particular, we found that in these patients among CD4+ T-cells there was a higher percentage of cells within the less differentiated phenotype (CD27+ CD45RA+/-)

and a lower percentage of cells within the more differentiated phenotype (CD27- CD45RA+/-) (Fig. 12A), whereas among CD8+ T-cells there was a higher proportion of effector memory cells (CD27- CD45-) (Fig. 12B).

Instead, with regard to the phenotypic pattern of T-cells in "responders", we observed any difference after stimulation of both CD4+ and CD8+ T-cells with HIV-p55. Compared to baseline CD4+, stimulated CD4+ and CD8+ T-cells shown a slight decrease of naïve T-cells and a slight increase of central memory and effector memory T-cells (Fig. 13).

2.3.3 Cytokine production. By X-MAP technology, we analyzed 11 cytokines levels (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IFN-γ, IP-10, TNF-α) in the sera of "responder" and "non responder" patients, in order to evaluate if there was some correlation between their clinical and immune-virologic status and cytokine profile.

Fig. 14 shows the average cytokines levels (in pg/ml) in "responder" (in blue) and "non responder" (in red) patients, during the 12 months of study.

All patients showed a state of immune-activation, the degree of which correlated with viral load, being higher in the "non responders" group.

In particular, we found that "non responder" patients had higher levels of inflammatory cytokines (IL-1, IL-2, IL-6, IFN-γ) and slight higher levels of immunoregulatory cytokines (IL-10) compared with "responders".

IL-10 is a major regulator of innate and adaptive immunity because on the one hand it interferes with the production of inflammatory mediators by polymorphonuclear neutrophils, monocytes and macrophages and on the other hand it down-regulates antigen presentation and affects the function of CD4+ T-cells [31]. Although several reports suggest an increase of IL-10 levels during disease progression in HIV-infected patients, others have not confirmed this [32]. Nevertheless, several studies have shown that IL-10 production is induced *in vivo* and *in vitro* during HIV infection and that neutralization of endogenous IL-10 may improve defective antigen-specific T cell function in HIV-infected patients. On the other hand, IL-10 may inhibit T-cell apoptosis [33], a potential beneficial effect in these patients. Moreover, while IL-10 has been found to inhibit HIV replication in acutely infected macrophages at concentration that block endogenous cytokine secretion, lower IL-10 concentration appear to enhance HIV replication. Thus, the present data are conflicting and the contribution of IL-10 to the immunopathogenesis of HIV infection remains unclear, even though it is important to underline that we evaluated the serum profile of IL-10 and this analysis requires further study, e.g. the intracellular analysis of HIV-specific CD4+ and CD8+ T-cells.

2.4 DISCUSSION

In this study, we have analysed the phenotypical and functional patterns of circulating T-cells and the serum cytokine profile in HIV-1 mono-infected patients with varying levels of viremia, on and off ARV therapy.

Although we have a modest study population, our results are consistent with recent observations from other authors which suggest an important role of polyfunctional immune responses in the viral replication control. Indeed, we have found that double cytokine producing T-cells are only represented in immunocompetent patients, both in the absence ("controllers") and in the presence of ARV therapy ("responders"). Moreover, our data show that low levels of T-cell activation are evident in immunocompetent patients, both in the absence ("controllers") and in the presence of ARV therapy ("responders") and lower levels of inflammatory cytokines are evident in "responders" compared with "non responders".

This immunologic state suggests that in patients which control virus replication, the host's immune system responds to HIV by expanding but not exhausting T-cells while maintaining a relatively quiescent immune system. One model to explain the relevance of double cytokine producing cells is that these, through IL-2 production, are associated with an improved proliferative capacity [25]. This model suggests that IL-2 production facilitates constant replenishment of T-cells providing ultimately an adequate immune-competence.

Polyfunctional immune response could represent either the cause of viral replication control or the consequence of low viremia. Recent studies suggest that the functional profile of T-cell immune response is largely determined by the duration and intensity of antigenic exposure, and is therefore mainly a consequence of viremia [20]. In our study, to establish if the double-cytokine producing cells are or not an effect of the virologic

control, we compared “controllers” with “responders”. Through this comparison (Fig. 10), we observed that the former have slightly higher total levels of double cytokine producing T-cells. These data suggest but don’t prove that these cells contribute to virologic control because otherwise the absence viremia should result in higher levels of double cytokine producing T-cells. However, we can safely assume that the absence of viremia does not in and of itself result in higher levels of double cytokine producing cells.

Viral replication is regulated by a complex network of both inflammatory and regulatory cytokines. Although there are conflicting reports regarding the role of certain cytokines, their contribution to the state of generalized immune activation is well acknowledged. Through serum cytokine analysis, we noted that all HIV-1 infected patients show a certain state of immune-activation, the degree of which correlates with viral load. In particular we found that “responder” have lower levels of inflammatory cytokines (IL-1, IL-2, IL-6, IFN-γ) and slight lower levels of immunoregulatory cytokines (such as IL-10) compared with “non responders”, as consequence of virus replication control and therefore of less stimulation of immune system. This lends further credence to the hypothesis that IL-10 may play a role in the pathogenesis of HIV disease and it supports the notion that full immunological normalization may not be achieved during antiretroviral therapy.

2.5 CONCLUSION

Our analysis on HIV-infected patients, according to the results shown by recent experimental studies, suggests that the presence of polyfunctional immune responses is a strong correlate of HIV control, both in the absence and in the presence of therapy.

Therefore, the study of T-cell immune response together to analysis of serum cytokine profile, associated with two surrogate markers used routinely (CD4 T-cell count and HIV viremia) in the management of HIV-1 infected patients, could help the clinicians to predict progression of HIV disease and to define the most opportune time to initiate or interrupt therapeutic measures. In the first case, the absence of polyfunctional immune responses in naïve HIV-1 infected patients, on regular follow-up, may be an indicator for an earlier initiation of antiretroviral therapy and thus preserve immunologic function.

In this regard, we report the case of M.P., an asymptomatic 25 year-old man who presented to our AIDS Center for HIV high risk-behaviours. After diagnosis of HIV-1 infection, he initiated a regular follow-up at our Center. At baseline, he presented a CD4 T-cell count of 550 cells/mm3 and an HIV viremia of 25,000 copies/ml. We enrolled this patients in our study and we carried-out a functional analysis of his T-cell immune response. Fig. 15 shows that at T0, double-cytokine producing T cells were absent whereas those producing a single cytokine (IFN-γ or IL-2) were predominant. Three months after, because of a rapid fall of CD4 T-cell count (<200 cells/mm3), M.P. initiated antiretroviral therapy.

In the second case, the presence of polyfunctional immune responses in HIV-1 infected patients on antiretroviral therapy, may be an important indicator to interruption or simplification of ARV therapy in order to avoid his long-term toxicity. In this regard, we report the case of A.G, a 43-year old HIV-1 infected woman in A3 CDC class (this class includes patients with asymptomatic infection and pre-treatment nadir CD4 T-cell count <200 cells/mm3), in excellent clinical conditions, receiving antiretroviral therapy from 2005 with good immune-virologic results (CD4 T-cell count >1000 cells/mm3 and viral load <47 copies/ml). We enrolled this patients among "responder" patients and we carried-out a functional analysis of his T-cell immune response. Fig. 16 shows that at T0, double cytokine producing T-cells were much more represented that single cytokine producing T-cells. Five months later, our patient reported concerns about a loss of muscle mass and she was clinically diagnosed as having lipoatrophy of the upper and lower limbs.

Finally, these results are very important for other two reasons. First, for the formulation of effective vaccines whose design requires knowledge of the types of immune responses that are most likely to be protective, and second for the development and assessment of new therapeutic approaches, particularly immunomodulating.

Fig. 1 Italian incidence of new HIV diagnoses (per 100,000 population), 2009

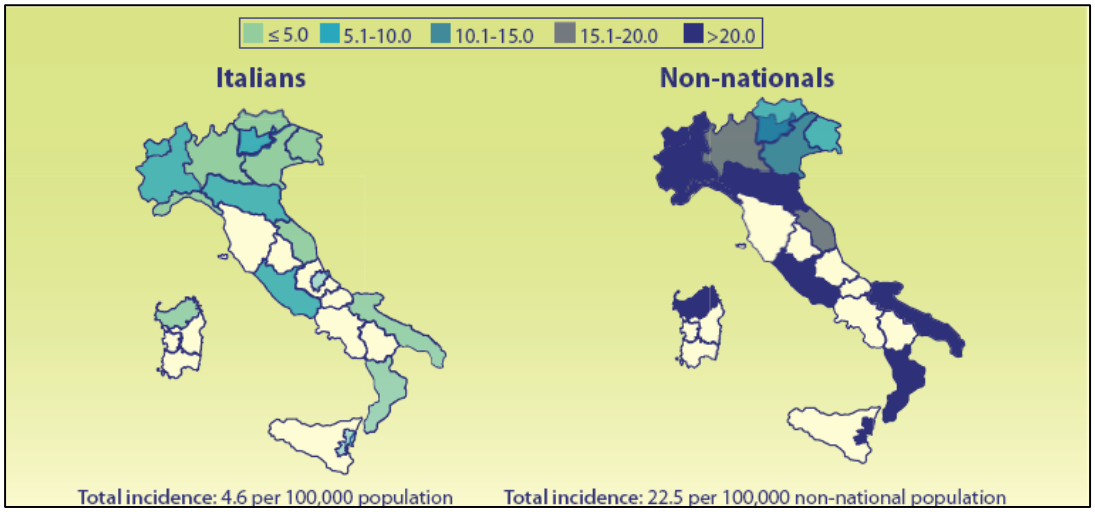


Fig. 2 Percent distribution of new HIV diagnoses among Italians and non nationals, by year of diagnosis

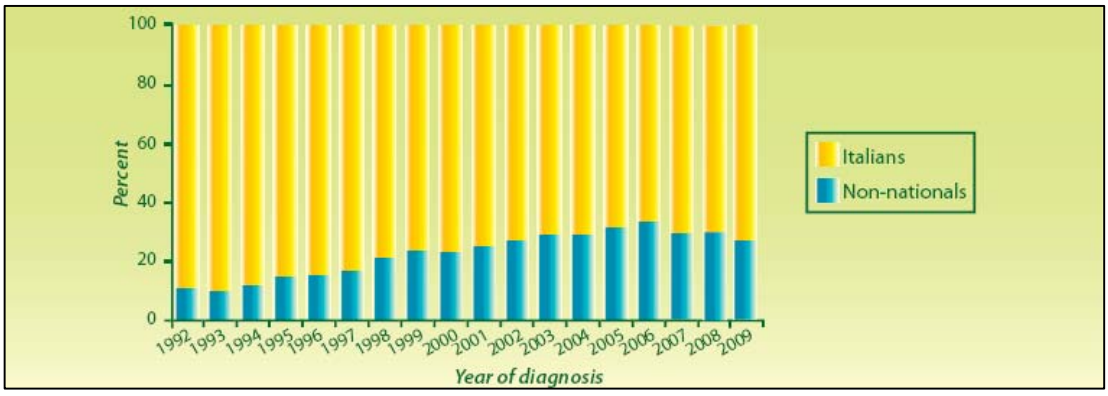


Fig. 3 Median age of new HIV diagnoses, by year of diagnosis

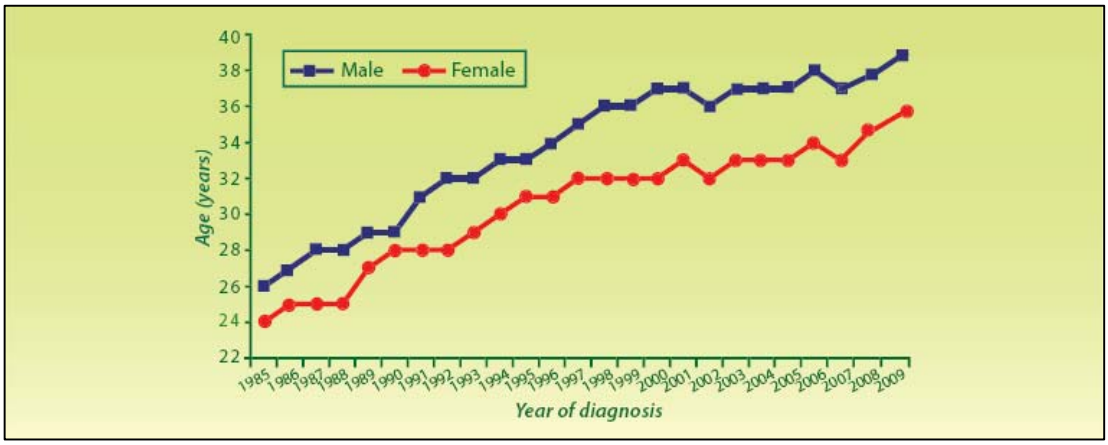


Fig. 4 Percent distribution of new HIV diagnoses, by exposure category and year of diagnosis

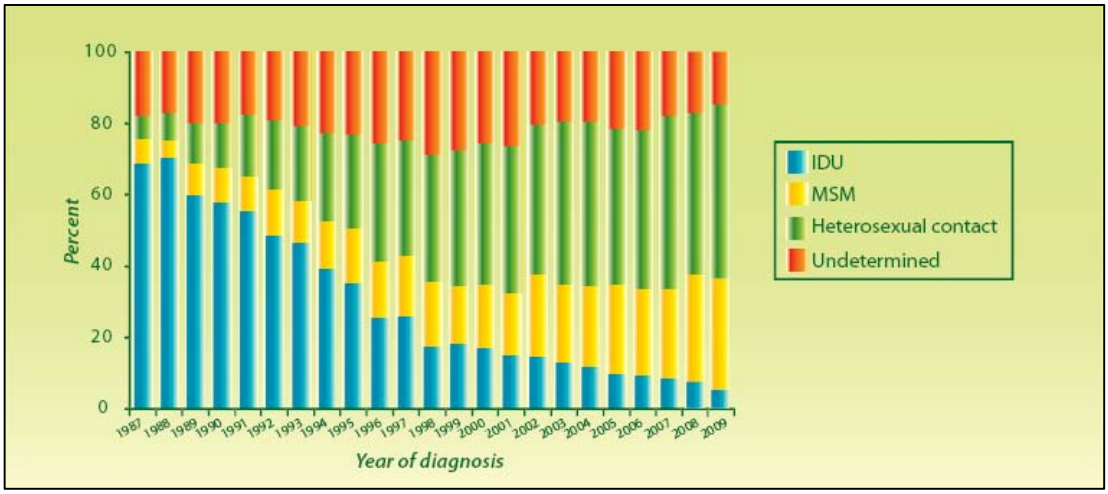


Fig. 5 New HIV diagnoses: CD4 count at diagnosis, 2009

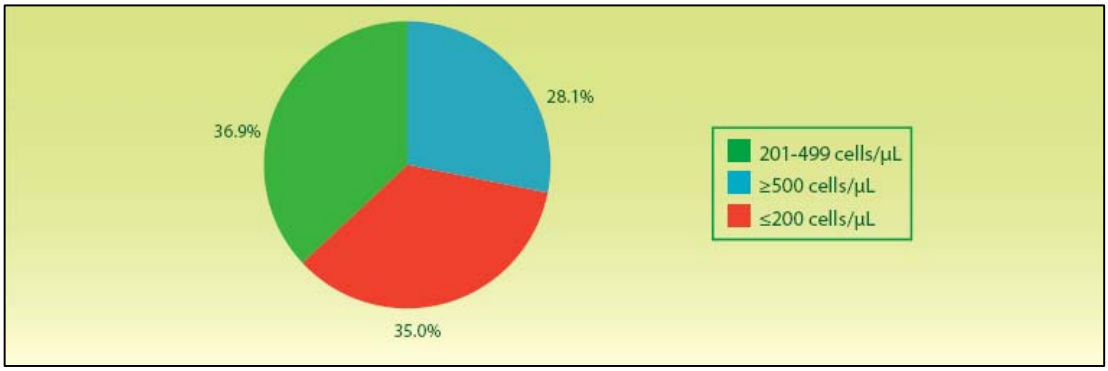


Fig. 6 HIV structure

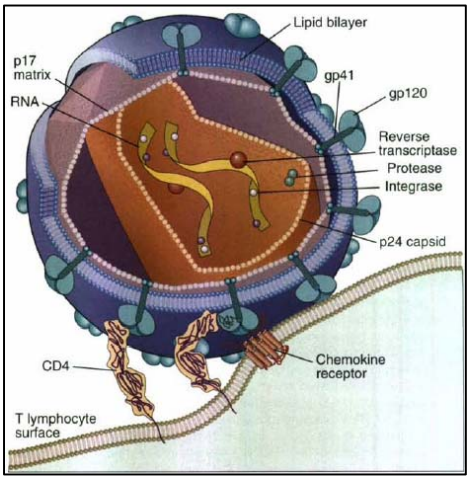


Fig. 7 Functionally distinct populations of Ag-specific CD4+ and CD8+ T cells identified by the assessment of IL-2 and IFN-γ secretion

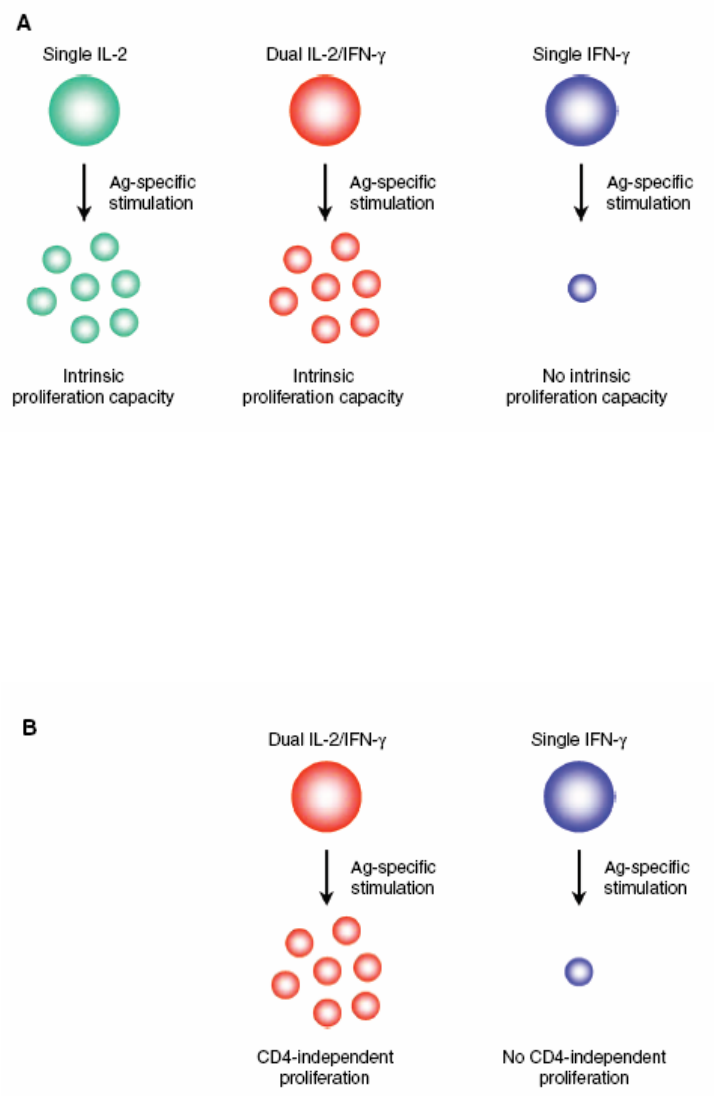


Fig. 8 Functional analysis of T-cells. Comparison between Controllers and non Controllers

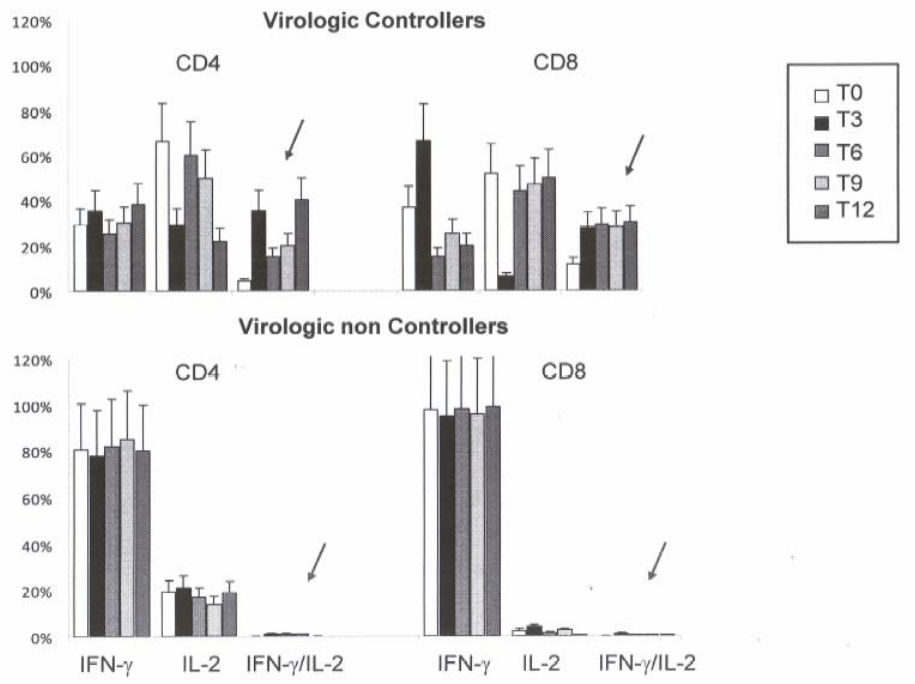


Fig. 9 Functional analysis of T-cells. Comparison between Responders and non Responders

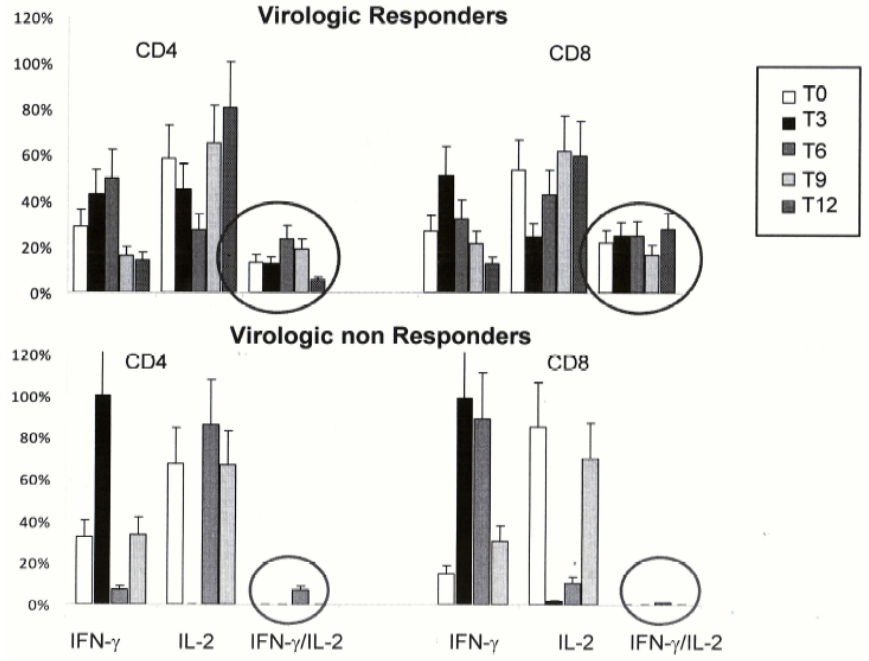


Fig. 10 Functional analysis of T-cells. Comparison between Controllers and Responders

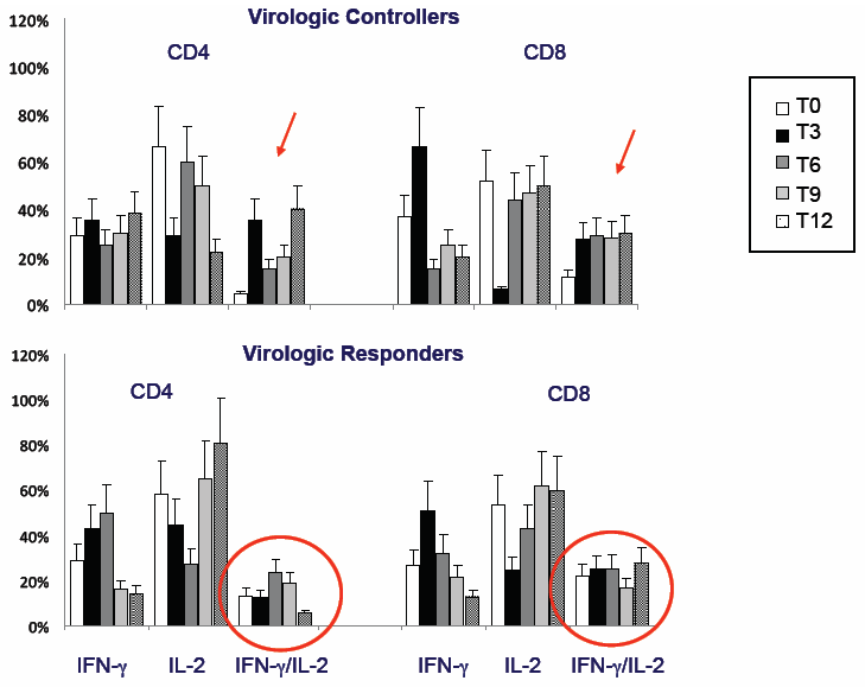


Fig. 11 Functional analysis of T-cells in Naïve patients

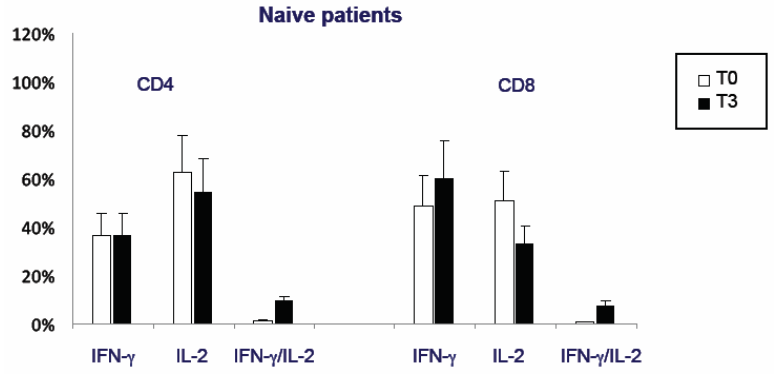


Fig. 12 Phenotypic analysis of CD4+ and CD8+ T-cells in Controllers before and after HIV-p55 stimulation

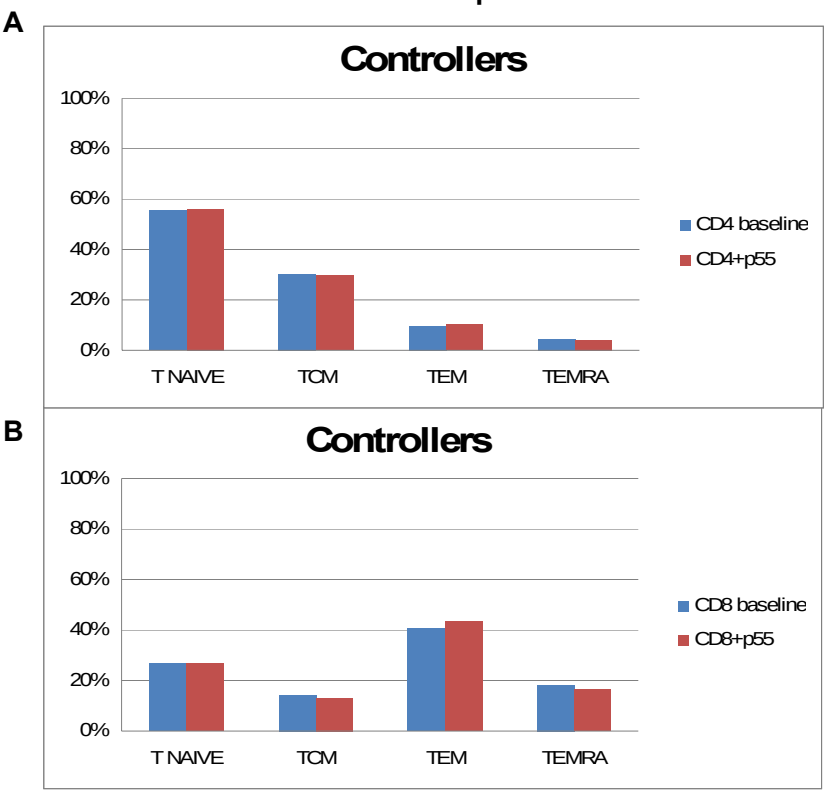


Fig. 13 Phenotypic analysis of CD4+ and CD8+ T-cells in Responders before and after HIV-p55 stimulation

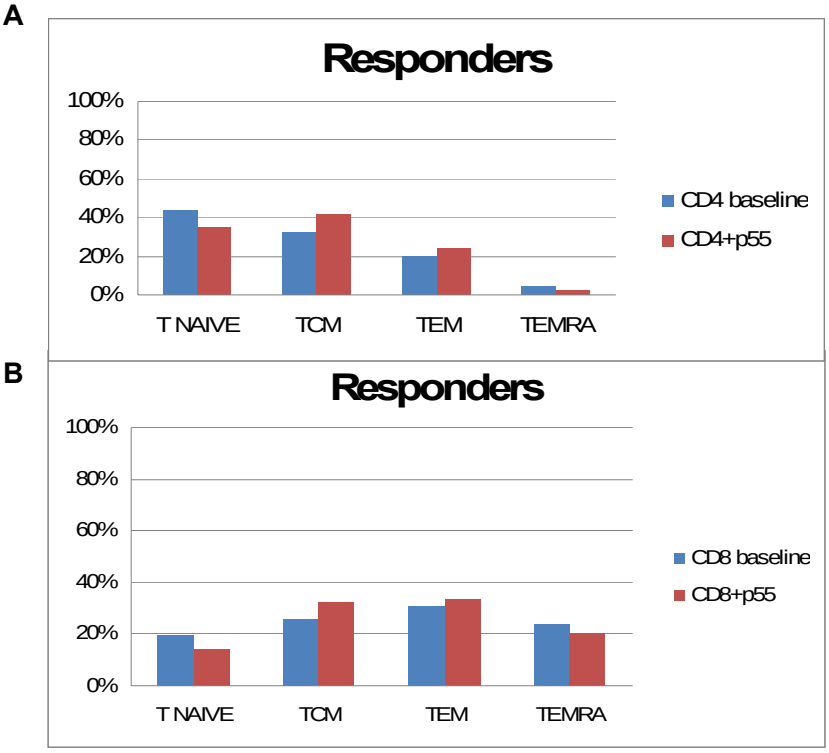


Fig. 14 Average cytokines levels in “responders” (in blue) and “non responders” (in red), during the period of study

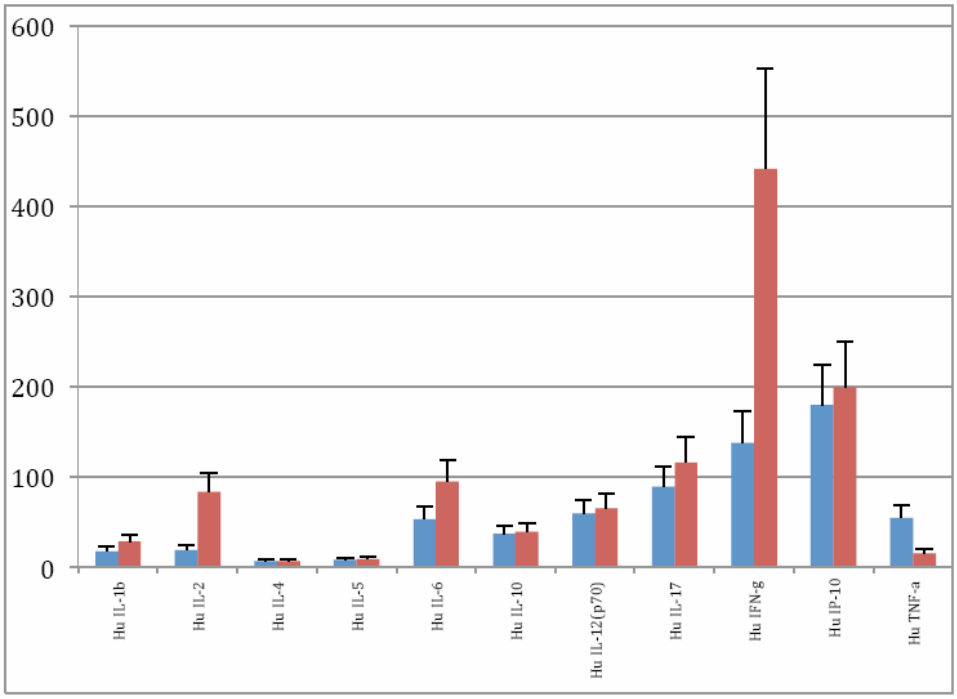


Fig. 15 Functional analysis of CD4+ and CD8+ T-cells in a naive patient at T0

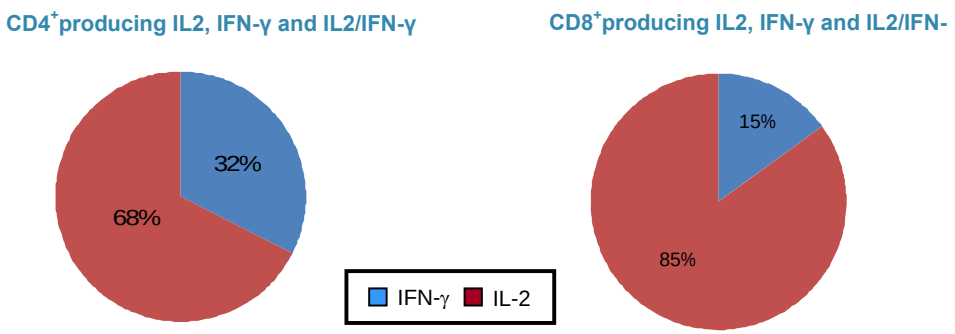
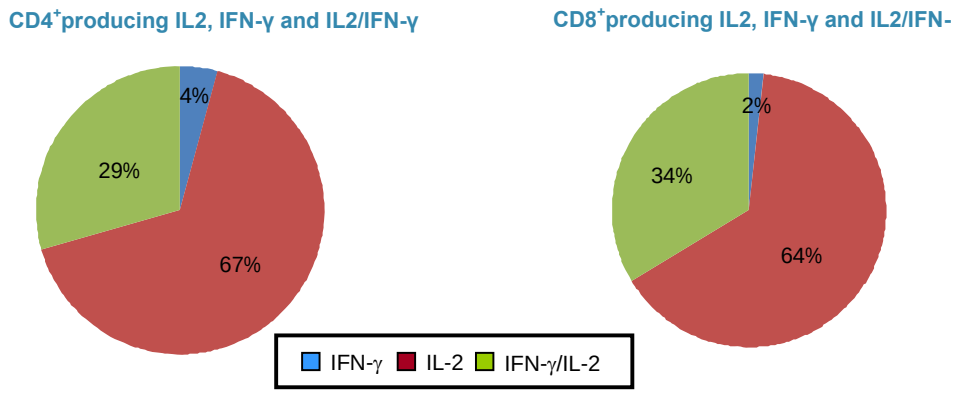


Fig. 16 Functional analysis of CD4+ and CD8+ T-cells in a responder patient at T0



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