Anti-apoptotic effects of Interleukin 7 in Human Immunodeficiency Virus Type 1 infection: Mechanism and implications for immune reconstitution strategies

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ANTI-APOPTOTIC EFFECTS OF INTERLEUKIN 7 IN HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION: MECHANISM AND IMPLICATIONS FOR IMMUNE RECONSTITUTION STRATEGIES

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This thesis is presented in partial fulfillment of the requirements for a Doctor in Philosophy in Cellular and Molecular Biology from the Open University.

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ABSTRACT

Although HAART has introduced great improvements in the clinical outcome of HIV-1-infected individuals, the current protocols often fail in completely restoring the CD4+ T-cell lymphopenia. Therefore, innovative approaches based on the use of immune adjuvants to HAART, including cytokines like IL-2, IL-15 and IL-7, are being explored. The present thesis aims at investigating the immunomodulatory effects of IL-7 in HIV/SIV infection, focusing on its pro-survival properties.

The first part of this work describes the results of a study in vitro to evaluate the effects of IL-7 on T cells from HIV-1-infected individuals, and shows that this cytokine strongly protects both CD4+ and CD8+ T cells from spontaneous apoptosis. IL-7-mediated apoptosis reduction ex vivo inversely correlated with the CD4+ T-cell count of the patient in vivo, suggesting that IL-7 treatment could be useful also for patients at with advanced disease. Moreover, the protective effect of IL-7 was not associated with the induction of cellular proliferation or viral replication.

The second part of this work describes the results of a study in vivo of IL-7 administration during the acute phase of SIV infection in rhesus macaques, the pathogenic animal model for AIDS. IL-7 treatment prevented the depletion of circulating naïve and memory CD4+ T cells that typically occurs within the very first weeks of infection, primarily by reducing the levels of apoptosis. Moreover, IL-7 treatment also induced sustained increases in all subsets of circulating CD8+ T cells. Of note, treatment with IL-7 did not have any effect on SIV plasma viral load nor on the content of SIV DNA provirus. Finally, IL-7-treated animals developed earlier and stronger CD8+ and CD4+ T-cell responses as compared to untreated animals, although overall no protective effects on disease progression were observed.

Taken together, these data further support the use of IL-7 as an immunomodulatory adjuvant to HAART in HIV-1-infected individuals, and suggest that this cytokine may be a useful tool to preserve or restore the CD4+ T-cell pool during both the acute and chronic phases of the infection.
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PRESENTATIONS AND PUBLICATIONS

Poster Presentations:

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Abstract accepted for poster presentation at the "HIV Immunobiology: From Infection to Immune Control" (Keystone Symposia). March 2009; “Interleukin-7 Treatment Prevents Naïve CD4+ T-Cell Depletion during the Acute Phase of Simian Immunodeficiency Virus Infection in Rhesus Macaques” (Not attended).

Abstract accepted for poster presentation at the meeting "Overcoming the Crisis of TB and AIDS" (Keystone Symposia). September 2009; “Interleukin-7 Treatment Prevents Naïve and Central Memory CD4+ T-Cell Depletion during the Acute Phase of Simian Immunodeficiency Virus Infection in Rhesus Macaques” (Not attended).

Scholarship Awardee for the Keystone Symposia “HIV Biology and Pathogenesis”; January 2010; abstract title: “Interleukin-7 Treatment Prevents the Early Depletion of Naïve and Central Memory CD4+ T Cells during the Acute Phase of Simian Immunodeficiency Virus Infection in Rhesus Macaques” Vassena L, Miao H, Malnati MS, Morre M, Fauci AS, Lusso P.

Oral Presentation:


Publications:


Furci L, Sironi F, Tolazzi M, Vassena L, Lusso P. “Alpha-defensins block the early steps of HIV-1 infection: interference with the binding of gp120 to CD4”. Blood, 2007

CHAPTER ONE

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1.0.1 HIV-1 Pandemics

Since in 1983 a retrovirus (later named the human immunodeficiency virus, HIV) was first isolated from a patient with acquired immunodeficiency syndrome (AIDS), more than 65 million persons have been infected by the HIV type 1 (HIV-1), and more than 25 million have died of AIDS, making it the most destructive disease in the recorded history. According to the “UNAIDS REPORT ON THE GLOBAL AIDS EPIDEMIC | 2010” by the Joint United Nations Programme on HIV/AIDS (UNAIDS), in 2009 over 33 million people were living with AIDS (2.5 million of which were children under 15 years old), 2.6 million people got infected by HIV-1 (370000 children under 15 yo) and 1.8 million died of AIDS (260000 children under 15 yo). The proportion of women living with HIV is slightly less than 52% of the global total.

The pandemic is not homogeneously distributed around the world, some Countries being more affected than others, and it appears to have established in most regions, although prevalence continues to increase in Easter Europe and Central Asia and in other parts of Asia due to a high rate of new HIV-1 infections (Figure 1.1). Sub-Saharan Africa remains the worst region, accounting for 68% of the people living with HIV worldwide (1.3 million below 15 years of age) and for 72% of the world’s AIDS related death in 2009. Globally, 34% of people living with HIV in 2009 resided in the 10 countries in southern Africa; 31% of new HIV infections occurred in these 10 countries, as did 34% of all AIDS-related deaths. About 40% of all adult women with HIV live in southern Africa (Figure 1.1).

The scaling up of treatment is profoundly affecting sub-Saharan Africa. At the end of 2009, 37% of adults and children eligible for antiretroviral therapy were receiving it in the
region overall, compared with only 2% seven years earlier (1). AIDS-related deaths
decreased by 18% in southern Africa (1). Besides this, South Africa is one of the few
countries in the world where child and maternal mortality has risen since the 1990s. AIDS is
the largest cause of maternal mortality in South Africa and also accounts for 35% of deaths
in children younger than five years (2). The second region of prevalence is Asia, where 4.9
million of people were living with HIV in 2009, and where the AIDS picture is dominated
by India epidemic.

AIDS continues to be a major global health priority: the number of people living
with AIDS continues to increase and AIDS-related illnesses remain one of the leading
causes of death globally.

However, there is growing evidence of success in HIV prevention in diverse settings,
particularly thanks to an effective prevention of mother-to-child transmission, through
strategies aimed at the reduction in overall HIV among reproductive-age women and men,
the reduction of unwanted pregnancies among HIV-1-infected women, the provision of
antiretroviral drugs to reduce the chance of infection during pregnancy and delivery and
appropriate treatment and care and support to mothers living with HIV (including infant
feeding). Ideally, the provision of antiretroviral prophylaxis and replacement feeding could
reduce transmission from and estimated 30-35% to around 1-2%. Most countries have not
yet reached all pregnant women with these services, but the data are encouraging. Indeed,
exclusively examining the provision of antiretroviral prophylaxis to HIV-positive pregnant
women, UNAIDS estimates that 200000 cumulative new HIV infections have been avoided
in the past 12 years.
Moreover, there is increased evidence that improved access to treatment is having an impact.

In high-income countries, where antiretroviral drugs have long been widely available, access to treatment has had an extraordinary impact on HIV-related mortality (3), and a 10-fold rise in access to treatment between 2003 and 2008 in low- and middle-income countries is also starting to provide similar reassuring evidences from recent studies in different regions (4-7).

Although current estimates of coverage of antiretroviral treatment for children are close to those of adults (World Health Organization, United Nations Children’s Fund, UNAIDS, 2009), the provision of therapy to children has specific challenges, including the faster progression to AIDS, the difficulty of diagnosing HIV, and the higher costs for developing appropriate antiretroviral drugs for children, that are now starting to be addressed by some controlled studies (8-10). However, even with the impressive medical outcomes achieved through diagnosis and treatment, mortality within the first months of therapy still remains high in HIV-1-infected children in sub-Saharan Africa (11, 12).

One of the main problems that still characterize the fight against AIDS is the failure to match national AIDS strategies to the documented national needs. While high HIV prevalence has been well documented in sex workers for a long time, recent studies conducted have shown elevated levels of infection in other risk populations, including injecting drug users and men who have sex with men, in many countries worldwide. Thus, prevention strategies targeted towards different risk populations according to the national needs seem to be necessary, whereas gaps are still evident in basic prevention approaches. For instance, even though most of new infections in Sub-Saharan African regions occur
among old heterosexual couples, few prevention programmes have been specifically targeted towards them.

1.0.2 HIV History

HIV, the causing agent of AIDS, belongs to the lentivirus family of retroviruses. Of the two types of HIV that infect humans, HIV-1 and HIV-2, HIV-1 is the more virulent and the more easily transmitted and it is the cause of the vast majority of HIV infections globally; by contrast, HIV-2 appears to be less easily transmittable and is associated with significantly slower progression to immune deficiency (13, 14). The two types of HIV have a different geographic localization, with HIV-1 being found globally, and HIV-2 confined primarily to West Africa and India (13, 15). Research conducted using molecular phylogenetics, which compares viral genomic sequences to determine relatedness, revealed that HIV evolved at some point from the closely related Simian immunodeficiency virus (SIV), which was transferred from non-human primates to humans in the recent past (as a type of zoonosis) (16). HIV-2 seems to be closely related to a virus of the sooty mangabey (Cercocebus atys atys), an Old World monkey inhabiting southern Senegal, Guinea-Bissau, Guinea, Sierra Leone, Liberia, and western Ivory Coast (13). Conversely, the known strains of HIV-1 are more closely related to the simian immunodeficiency viruses endemic in West Central African forests and each of the known HIV-1 strains is either closely related to the SIV that infects the chimpanzee subspecies Pan troglodytes troglodytes (SIVcpz), or to the SIV that infects Western lowland gorillas (Gorilla gorilla gorilla), called SIVgor (17). The pandemic HIV-1 group M (main) is most closely related to the SIVcpz collected from the southeastern rain forests of Cameroon (18). Thus, this region is presumably where the virus
was first transmitted from chimpanzees to humans. However, reviews of the epidemiological
evidence of early HIV-1 infection in stored blood samples, and of old cases of AIDS in
Central Africa have led many scientists to believe that HIV-1 group M early human
epicenter was probably not in Cameroon, but rather farther south in the Democratic
Republic of the Congo, more probably in its capital city, Kinshasa (17-20).

Using HIV-1 sequences preserved in human biological samples along with estimates
of viral mutation rates, scientists calculate that the jump from chimpanzee to human
probably happened during the late 19th or early 20th century. Some molecular datation
studies suggest that HIV-1 group M had its most recent common ancestor (MRCA) in the
early 20th century, probably between 1915 and 1941 (21, 22), while another study published
in 2008 suggested a common ancestor between 1873 and 1933 (with central estimates
varying between 1902 and 1921) (23).

According to the 'Natural Transfer' theory (also called 'Hunter Theory' or 'Bushmeat
Theory'), the "simplest and most plausible explanation for the cross-species transmission" of
SIV or HIV (post mutation), the virus was transmitted from an ape or monkey to a human
when a hunter or bushmeat vendor/handler was bitten or cut while hunting or butchering the
animal (24). The resulting exposure to blood or other bodily fluids of the animal can result
in SIV infection (25). How the SIV virus would have transformed into HIV after infection of
the hunter or bushmeat handler from the ape/monkey is still a matter of debate, although
natural selection would favor any virions capable of adjusting so that they could live, infect
and reproduce in the T cells of a human host.

Up to the 1980s, it is not known how many people had developed AIDS. "The
dominant feature of this first period was silence, for the human immunodeficiency virus
(HIV) was unknown and transmission was not accompanied by signs or symptoms salient enough to be noticed. While rare, sporadic case reports of AIDS and sero-archaeological studies have documented human infections with HIV prior to 1970, available data suggest that the current pandemic started in the mid- to late 1970s. By 1980, HIV had spread to at least five continents (North America, South America, Europe, Africa and Australia). During this period of silence, spread was unchecked by awareness or any preventive action and approximately 100,000-300,000 persons may have been infected. Jonathan Mann (26).

The first cases of AIDS appeared in the USA in the early 1980s, when a number of gay men in New York and California started to develop rare opportunistic infections and cancers resistant to treatment. It soon became clear that all these were manifestation of a common syndrome, which was for the first time defined as AIDS (Acquired Immune Deficiency Syndrome) in September 1982 by the Center for Disease Control (CDC) (27). By the end of the year the number of AIDS cases in the USA had risen to 3,064 and of these 1,292 had died (28). In May 1983, doctors in the group of Luc Montagnier at the Institute Pasteur in France reported that they had isolated a new virus, which they suggested might be the cause of AIDS (29). One year later, on May 4, 1984, Robert C. Gallo and his collaborators published a series of four papers in Science demonstrating that a retrovirus they had isolated, called HTLV-III in the belief that the virus was related to the leukemia viruses of Gallo's earlier work, was the cause of AIDS (30-33).

1.0.3 HIV-1 Groups and Subtypes

The strains of HIV-1 can be classified into four groups: the "major" group M, the "outlier" group O and two new groups, N and P, which may represent four separate
introductions of simian immunodeficiency virus into humans. More than 90% of HIV-1 infections belong to HIV-1 group M. Group O appears to be restricted to west-central Africa and group N - a strain discovered in 1998 in Cameroon - is extremely rare. In 2009 a new strain closely related to gorilla simian immunodeficiency virus was discovered in a Cameroonian woman. It was designated HIV-1 group P (34).

Within group M there are at least nine genetically distinct subtypes (or clades) of HIV-1, named A, B, C, D, F, G, H, J and K. Occasionally, two viruses of different subtypes can meet in the cell of an infected person and mix together their genetic material to create a new hybrid virus (35). Many of these new strains do not survive for long, but those that infect more than one person are known as "circulating recombinant forms" or CRFs (36). The classification of HIV strains into subtypes and CRFs is a complex issue and the definitions are subject to change as new discoveries are made. So far 48 CRFs have been identified (37). Within the A and F subtypes, separate sub-clusters have been given the designations A1, A2, A3 and A4, and F1 and F2 (38).

The HIV-1 subtypes and CRFs are typically associated with certain geographical regions, with the most widespread being subtypes A (sub-subtype A1 and the A/G recombinant, CRF02_AG) and C (39) (Figure 1.2). Subtype A and CRF A/G predominate in West and Central Africa, with subtype A possibly also causing much of the Russian epidemic, whereas Subtype C is predominant in Southern and East Africa, India and Nepal. Subtype B, historically the first recognized and most studied variant of HIV-1, continues to predominate in North America, Western and Central Europe, and Australia. This subtype accounts for only a small fraction of the worldwide pandemic (<15%) (39) and is the predominant sub-type found among MSM infected in Europe (40). Subtype D is generally
limited to East and Central Africa with sporadic cases being detected in Southern and Western Africa (41). C/D recombinants are common in Tanzania, and B/C recombinants are prevalent among injecting drug users in China (42). One of the CRFs is called A/E because it is thought to have resulted from hybridization between subtype A and some other "parent" subtype E. However, no one has ever found a pure form of subtype E (37). CRF A/E is prevalent in South-East Asia, but originated in Central Africa. Subtype F has been found in Central Africa, South America and Eastern Europe. Subtype G and CRF A/G have been observed in West and East Africa and Central Europe. Subtype H has only been found in Central Africa; J only in Central America; and K only in the Democratic Republic of Congo and Cameroon. At present, >40% of all new infections in Europe are of the non-B African and Asian subtypes (39, 43) (Figure 1.2). Although this is the nowadays picture, it is continuously evolving, with new HIV genetic subtypes and CRFs being discovered, as virus recombination and mutation continue to occur, and the current subtypes and CRFs continue to spread to new areas, as the global epidemic continues.

1.0.4 Genetic Variability of HIV and Functional Implications

Genetic variability of HIV is high, both within an HIV-1 subtype (15 to 20%,) and between subtypes (25 to 35%) (38) and is primarily due to the high error rates of the viral reverse transcriptase, which results in approximately 10 genomic base changes per replication cycle. The most common errors are substitutions, even if deletions and insertions also occur, although their frequency is more difficult to estimate. The envelope gene (env) seems to be subject to the most extensive genetic variation, although alterations can also occur in other genes (44-46). HIV-1 undergoes continuous genetic variability within
individual patients, who usually harbor a considerable number of highly related but individually distinguishable viral variants, which are referred to as quasispecies, with a heterogeneity usually not exceeding 2-5% in the env gene (47, 48).

A broad range of viral genetic variability (in the range of 20-30% in the env gene) has been documented for isolates from distinct geographical locations, largely attributable to differential geographic distribution of the multiple genetic subtypes of HIV-1. Within a single geographic region, the range of genetic variability in the env gene is estimated to be 6-19%, although differences higher than 30% have been documented (49-51). The extent of genetic variability in a given geographical location increases over time after the introduction of a particular subtype in a population. Initially, the heterogeneity in the env gene can be as low as 3-5%, which is comparable with the range of intra-patient variability, with further diversification at an estimated rate of approximately 1% per year. In addition, recent data have provided evidence that genomic recombination between two different HIV-1 populations frequently occurs in vivo, resulting in biologically viable viruses with mosaic genomes, a phenomenon which may result in additional HIV genetic variability and viral genetic shifts (52-54).

The differential characteristics of viral subtypes and their interactions with the human host may influence HIV transmission and disease progression. HIV-1 viruses can be defined for their capacity to use the chemokine receptors CCR5 (R5 viruses) and/or CXCR4 (X4 viruses) as a co-receptor, and, generally speaking, R5 viruses are more frequently transmitted than X4 viruses that emerge later in infected patients and are associated with more rapid disease progression (55). All HIV-1 subtypes can use both coreceptors, but subtype D may be dual-tropic (i.e., an R5X4 virus) most frequently (56). The percentage of
X4 virus appears to be lower in subtype C than in subtype B, even when the viruses are obtained from patients with advanced AIDS (57). There are suggestions in the published literature that HIV-1 subtype or CRF may affect efficiency of transmission (58-61).

Whether subtype differences result in variable rates of disease progression has been investigated in several prospective, observational studies of the course of HIV-related disease in cohorts infected with various subtypes and the results reported are discordant (62-65). Several studies suggest that subtype D appears to be associated with a more rapid rate of disease progression than other HIV-1 subtypes (66, 67), and the propensity of subtype D to exhibit a greater degree of dual-tropism than other subtypes (56) may help to explain this observation. The notable caveat relevant to all these studies of disease progression is that confounders such as access to medical care, nutritional status, host genetic factors, and mode of viral transmission (e.g., sexual, injection-drug, or vertical) may contribute to the divergent results.

Another crucial point is if HIV subtype diversity may also influence the response to antiretroviral treatment, and this issue is particularly relevant considering that subtype B is the one with more clinical data available, but it is not the most diffused (50% of prevalent HIV infections and 47% of all new HIV-1 infections are with subtype C (38)). Indeed, as documented by several studies, the data on baseline antiretroviral susceptibility derived from studies of subtype B may not be applicable to non-B subtypes (68-70). Moreover, certain HIV-1 subtypes might spread or progress more rapidly than others, making treatment decisions more challenging (66).

Another factor that could be influenced by genetic variability is the effectiveness of treatment. However, even if there are potential problems with comparing responses to
therapy among persons infected with group M, non-B–subtype strains — who frequently
live in settings with limited resources — and those infected with subtype B, the data
available thus far are encouraging. Indeed, it appears that HIV-1 subtypes do not affect
major differences in the response to antiretroviral therapy (71-74).

Of course, the most important implication of HIV-1 subtype diversity regards vaccine
development (75). Hosts infected with HIV-1 have cellular and humoral immune responses
to their infecting strains, but there is evidence of mutational escape by viruses from
responses by CD8+ cytotoxic T cells and neutralizing antibodies over time (76, 77).
Although cross-reactive responses to other viral subtypes have been shown (78, 79), the
strength and breadth of these responses are typically limited (80). Critical to the
development of a successful HIV-1 vaccine will be our ability to decipher the genetic
diversity of the virus, elicit broadly neutralizing antibodies, and generate strong CD4+ and
CD8+ T-cell responses (81, 82). The induction of both neutralizing antibody and strong
cellular responses remains a big challenge that is influenced by the high degree of variability
of the virus. Several approaches are currently under investigation to try to obtain broader
immunological responses that can counteract different isolates simultaneously. This issue is
even of greater importance if we consider that even within a single individual, the virus
continuously evolves with time, to try to escape from the host immune response.

1.0.5 HIV-1 structure and genome

HIV-1 belongs to the lentiviral family of retroviruses, and, like the other members of
the family, this virus has an RNA genome that is transcribed into a DNA molecule by the
enzyme reverse transcriptase and then integrated in the genome of the host cell. Lentiviruses
are typically characterized by prolonged periods of disease progression, have high mutations rates, are transmitted exclusively by the exchange of body fluids and, most importantly, cannot be easily eradicated from infected cells, thus making it very difficult to completely solve the infection. They also differ from other viruses in their ability to infect non-dividing cells (83).

The HIV-1 virion is a roughly spherical particle with a diameter of about 120 nm, quite large for a virus. It’s genome is composed of two copies of positive single-stranded RNA that encodes for the nine genes of the virus, held together by RNA-binding proteins and enclosed by a conical capsid composed of 2,000 copies of the viral protein p24 (83). The viral enzymes (reverse transcriptase, RNase H and integrase), tRNA and the accessory proteins required for viral infectivity, cDNA synthesis and virion assembly during budding from the cell surface are also contained within the capsidic core (83). A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle. The virion is in turns surrounded by the envelope, a double phospholipids layer that originates from the membrane of the cell where the virus buds from, which contains about 70 copies of the HIV-1 envelope glycoprotein, Env. Each Env glycoprotein is a heterodimer composed of the trans-membrane protein gp41 and the surface protein gp120, non-covalently bound (84) and env glycoproteins associate together to form trimeric envelope spikes on the surface of the virus (85).

Like all retroviruses, HIV-1 genome encodes three major genes: gag, pol and env. The gag gene encodes the Gag polyprotein, which is processed during maturation to MA (matrix protein, p17), CA (capsid protein, p24), SP1 (spacer peptide 1, p2), NC (nucleocapsid protein, p7), SP2 (spacer peptide 2, p1) and p6 (83). The pol gene encodes the Pr160\textsuperscript{pol}, a
polyprotein derived by ribosomal frame-shifting, that forms the viral enzymes reverse transcriptase, integrase, and HIV protease and the env gene encodes the glycoprotein gp160, that is then cleaved in gp120 and gp41 by a cellular protease during trafficking towards the cellular surface (83). In addition to gag, pol and env, HIV-1 genome encodes for six other genes (tat, rev, vif, vpr, nef and vpu or vpx in HIV-2), which have regulatory (tat and rev) and accessory functions (vif, vpr, nef and vpu). Tat is a transactivator of the LTR region, whereas rev allows fragments of HIV mRNA that contain a Rev Response Element (RRE) to be exported from the nucleus to the cytoplasm (86). Vif counteracts the antiviral activity of the human enzyme APOBEC by targeting it to cellular degradation (87). Vpr regulates nuclear import of the HIV-1 pre-integration complex, and is required for virus replication in non-dividing cells such as macrophages (88). Nef is expressed early in the viral life cycle and ensures T cell activation and the establishment of a persistent state of infection; moreover, it also promotes the survival of infected cells by downmodulating the expression of several surface molecules important in host immune function, including major histocompatibility complex-I (MHC I) and MHC II, CD4 and CD28 (89). Finally, vpu is involved in viral budding and it seems to counteract the action of the cellular protein BST-2 (CD317, tetherin) (90).

1.0.6 HIV-1 Life Cycle

The HIV-1 life cycle can be overall divided into six phases (Figure 1.3):

1) binding and fusion

2) uncoating and reverse transcription

3) integration
4) transcription
5) assembly
6) budding and maturation.

Phase 1: binding and fusion

HIV-1 selectively infects human CD4⁺ T cells and other cell types that express CD4, including macrophages and dendritic cells. The very first step of HIV binding to the target cell is the interaction of HIV gp120 with the cellular receptor CD4 (91, 92). This interaction triggers a conformational change in HIV gp120 that induces the exposure of the so-called co-receptor binding site, so that the env glycoprotein becomes then capable to interact with the co-receptor, usually CCR5 or CXCR4 (93). This in turns leads to a second conformational change, mainly involving the gp41 moiety of the env glycoprotein, which brings it in close proximity with the cellular membrane (94). Gp41 then experiences a structural rearrangement that leads to the formation of a six-helix bundle structure, a process that gives the protein the free energy state necessary for fusion, pore formation and entry of the viral capsid into the target cell (95).

Co-receptor usage

Several seven transmembrane (7TM) receptors have been identified as potential co-receptors for HIV and SIV by their capacity to support infection of CD4⁺ cell lines in vitro, and they are all members of (or closely related to) the chemokine receptor family. CCR5 and CXCR4 are the major co-receptors and all HIV-1 isolates can use one or both, with CCR5 being used by the majority of isolates worldwide. Basing on their co-receptor usage, HIV-1 isolates are called X4 or R5, depending on the usage of CXCR4 or CCR5 to enter cells.
Isolates capable of using both molecules as co-receptors are called dual tropic (R5X4).

Whereas the interaction between CD4 and gp120 is conserved among all primate lentiviruses and some of the amino acids that form the CD4 binding site on gp120 are already variable on the surface of the protein, the co-receptor binding site is not usually fully exposed until CD4 is bound, being covered, in the CD4-unbound state, by the V1/V2 loop of gp120 (93). The co-receptor binding site is formed by the so-called "bridging sheet" that lies between the V1/V2 loop and the V3 loop, and by some amino acids of the V3 loop itself (93). The determinants of co-receptor usage are likely to be complex and involve parameters of viral replication, host genetics and immune activation, in addition to genetic changes in the HIV-1 envelope gene. The major determinant of co-receptor usage is the V3 region of gp120: positively charged amino acids in this region (particularly at positions 11 and/or 25) have been shown to correlate with CXCR4 use (96-98), whereas R5 isolates have a low number of positively charged residues and R5X4 isolates have a V3 charge pattern that is similar to X4 envelopes, even though their dual tropic properties apparently involve multiple discontinuous regions of Env (99-102).

Initially, primary HIV isolates were classified in two distinct groups, defined as rapid/high or syncytium-inducing (SI), T-cell line tropic, and slow/low or non-syncytium-inducing (NSI) isolates, monocyte-derived macrophage tropic. Subsequently, M-tropic/NSI variants were shown to predominantly use CCR5 for entry into macrophages and were designated R5 viruses and T-tropic/SI variants were shown to use CXCR4 and were designated as X4 viruses (102). However, this distinction is not absolute, as CD4+ T cells can be infected by R5 viruses (103), and macrophages can be infected by some primary X4 isolates (104). Dualtropic R5X4 viruses can infect both macrophages and T cells.
After exposure, R5 M-tropic viruses typically predominate in the early stages of infection, even when the donor is infected by both R5 and X4 viruses (105-107). This may be explained as a consequence of the fact that tissue-resident macrophages, monocytes and dendritic cells may provide the initial cell target during the transmission event. However, it remains unclear if R5 viruses are the only ones transmitted or rather X4 viruses are less fit, even if the mutations responsible for R5/X4 switch have been shown to confer a replication-fitness disadvantage to the resulting viruses (108, 109). Indeed, relatively few mutations seem to be required for the co-receptor switch, and this occurs several years after the first infection (110, 111). Even if the major determinant for co-receptor usage is the V3 loop of gp120, mutations in the V1/V2 region may also play a role (112, 113); interestingly, these mutation seem to compensate for the loss-of-fitness mutations in the V3 region, thus allowing phenotype switch to occur (114). X4 viruses have also been reported as more susceptible to cytotoxic T-lymphocyte (CTL) activity as compared to R5 viruses (115), and this may explain both the prevalence of R5 variants in the early stages of the infection and the appearance of X4 variants at more advanced stages of the disease, when the immune system is more compromised.

X4 variants emerge later, when the virus is trafficked from the site of infection to lymphatic tissues, where replication was shown to occur robustly in T cells; however, this happens only in 50% of cases, demonstrating that X4 viruses are not necessary for progression to AIDS (116-118), although evidences suggest that the switch from R5 (NSI) variants to X4 (SI) variants may increase the rate of disease progression (119, 120). The reasons for the enhanced cytopathic effect of X4 viruses are not fully understood, but this may be due to altered replication kinetics and/or the ability of X4 strains to infect naïve
CD4⁺ T-cells in addition of CD4⁺ memory T-cells (121, 122).

**Phase 2: uncoating and reverse transcription**

Following entry of the virus into the target cell, the capsid (CA) partially disassembles (uncoats) to release the ribonucleoprotein complex (RNC) (123, 124). A correct regulation of core uncoating is essential for completion of the early steps of the HIV-1 replication cycle that include reverse transcription of the HIV-1 RNA, synthesis of the double-stranded viral DNA intermediate and formation of a functional pre-integration complex (PIC). In the PIC, CA appears to be lost, while MA, NC, RT, IN and the accessory protein Vpr remain associated (125, 126). Retroviruses have the ability to convert their RNA genome into double-stranded DNA early post-infection, and this reaction is catalyzed by the RT enzyme (127). In HIV-1, RT is a heterodimer of two subunits, p66 and p51, both derived from the Pr160GagPol precursor. Reverse transcription proceeds in the series of steps described below (128):

- DNA synthesis proceeds to the 5' end of the RNA molecule starting from a tRNA bound to the primer binding site (pbs)
- the RNA strand of the DNA/RNA hybrid is degraded by the RNaseH activity of the RT enzyme
- first strand transfer: the remaining minus-strand strong-stop DNA "jumps" from 5' to 3' end by using short regions of homology ("R" regions)
- the minus-strand fragment is completely synthesized
- the plus-strand is synthesized using RNA fragments remaining from step 2 as primers
- the tRNA is removed from the pbs by RNaseH
- plus-strand is synthesized until the end of the minus-strand.

Considering that reverse transcription involves "jumps" from one template to the other, novel recombinant DNA genomes can arise when the two RNA molecules are not genetically identical (129). This phenomenon, together with the high mutation rate of the RT enzyme \(3 \times 10^6\) per replication cycle (130), results in HIV-1 being very heterogeneous in sequence and rapidly capable of evading the host immune responses.

During the reverse transcription process, HIV-1 genome remains associated with the RTC, and the viral DNA is transported to the nucleus as part of the PIC, even though this process has not been fully elucidated yet and more studies are required to fully understand the role of viral and cellular factors in the translocation of the PIC into the nucleus.

**Phase 3: Integration**

Following nuclear import of the viral PIC, HIV-1 IN catalyzes the insertion of the double stranded DNA genome into the genome of the target cells (83). HIV-1 IN is a protein that functions as a multimer, even if it is unknown how many molecules make the functional holoenzyme (131). The integration process can be divided in several steps:

- **3'-end processing**: IN cuts off some nucleotides from the 3' end of both strands of the viral DNA

- **strand transfer**: IN cleaves the cellular target DNA and joins the 3'-recessed ends from step with the cleaved cellular DNA

- **cellular enzymes** fill in the gap between the integrated viral DNA and the host DNA. In the PIC, the ends of viral DNA are organized into a multi-component complex, the so-called "intasomes", in which both viral and cellular proteins coexist (131, 132).
Phase 4: transcription

The integrated provirus serves as a template for the synthesis of viral RNAs that ultimately are required for the encoding of the full complement of structural, regulatory and accessory proteins used to direct virus replication.

Transcription of HIV-1 proviral DNA is regulated by interactions between cellular transcription factors and the HIV-1 5'-long terminal repeat (LTR) (133). A number of the sequences present in the viral LTR closely resemble the promoter regions present in the human genome, thus HIV-1 is able to utilize the transcription machinery of the host for viral gene expression and replication. The HIV-1 promoter is divided into 3 distinct regions (134, 135). The modulatory enhancer and core promoter regions are located upstream of the transcription start site, whereas the Trans-Activation Response region (TAR) lies downstream of the start point.

The site of initiation of the RNA synthesis is HIV-1 LTR, which is composed of three regions: U3 (unique 3'end), R (repeated) and U5 (unique 5'end). The U3 contains several elements that drive the binding of RNA polymerase II (pol II) to the DNA template, including a TATA box (for transcription factor IID), and three Sp1 and two NF-kB binding sites, plus a "modulatory region" upstream of the NF-kB binding sites (136).

RNA synthesis is greatly increased when the transcriptional transactivator protein Tat, a 101 aminoacid protein that transactivates LTR-driven gene expression through the Trans-Activation Response region (TAR), is present (137-139). In particular, Tat interacts with the cyclin T1 (cycT1) (140), which forms a heterodimer with the cyclin-dependent kinase 9 (CDK9), and together they are part of a large protein complex called positive-transcriptional
elongation factor b (P-TEF-b). Tat recruits the human P-TEF-b compels to TAR, resulting in the phosphorylation of the C-terminal domain of RNA Pol II and in a dramatic stimulation of transcriptional processivity.

Transcription from HIV-1 LTR leads to a large numbers of RNA transcripts of three different types: 1) long unspliced RNAs that encode for Gag and GagPol precursors and constitute the genomic RNA, 2) partially spliced mRNAs, around 5 kb, encoding for Env, Vif, Vpu and Vpr and 3) small multiply spliced mRNAs, translated into Rev, Tat and Nef (141). In this context the role of Rev is crucial to shuttle the unspliced and partially sliced RNAs from the nucleus to the cytoplasm. Rev is a 116-aminoacid protein that interacts with the Rev Responsive element (RRE) located in the Env gene and in all unspliced and partially sliced RNAs with a stoichiometry of eight Rev proteins each RRE forming a complex capable of interacting with the nuclear cellular export machinery (142). As a consequence, RNA molecules are transported into the cytoplasm.

Phase 5: assembly

A major role in the virus assembly process is played by the Gag precursor polyprotein Pr55Gag, which contains domains that target it to the plasma membrane and bind the membrane itself (MA), promote Gag-Gag interactions (CA), encapsidate viral RNA genome (NC), associate with Env glycoprotein and promotes budding from the cell (p6) (126, 143-147).

The Env glycoprotein is synthesized in the rough ER to generate the precursor gp160, where oligomerization also occurs (148). Gp160 is transported to the cell surface via the secretory pathway and in the Golgi it is cleaved by a host protease (furin or furin-like
enzyme) to generate SU glycoprotein, gp120 and TM glycoprotein, gp41. After cleavage, gp41 anchors the complex to the membrane and associates non-covalently with gp120, which results in a high amount of gp120 shedding from Env-expressing cells and virions. Env glycoprotein complexes that reach the cellular membrane are either rapidly internalized or incorporated in new virus particles though a process that still remains almost completely understood (126). Some studies have reported that HIV-1 assembly occurs at the plasma membrane (149-151) whereas others have suggested that virus assembly may take place in an endosomal compartment and that release of the viral particles follows an exosomal pathway in which virus-containing endosomes fuse with the plasma membrane releasing their contents (152-154).

Part 6: budding and maturation

The final step in the process of virus assembly is the budding of the virus particle from the cellular membrane, and this occurs though specific sequences encoded by the virus itself, the so-called “late” or “L” domains. In HIV-1, the L domain is present in p6 and most likely it interacts with host factors. During or shortly after virus release from the plasma membrane, the viral protein PR cleaves the Gag and GagPol polyproteins, setting in motion a series of structural rearrangements that ultimately leads to virus maturation (155). In immature virions, Gag monomers are aligned projecting inward from the membrane associated MA domain at the N-terminus to NC at the C-terminus (156). Following cleavage, CA forms a conical shell around the RNA/protein complex within the core. The failure of the virus to mature properly is associated with a complete loss of infectivity and core condensation is essential for the very early post-entry steps of the replication cycle.
1.1 HIV-1 Immunopathogenesis

1.1.1 Typical course of HIV-1 Infection

The typical course of HIV-1 infection is illustrated in Figure 1.4. It begins with an acute symptomatic illness that lasts a few weeks, associated with high levels of plasma HIV-1 viremia, a sharp decline in the peripheral CD4⁺ T cell count (157-165), establishment of a reservoir of latently infected cells (166), and development of an HIV-1-specific immune response (167-170). After this very first phase, there is a 100- to 1000-fold drop in the levels of plasma viral load, a partial restoration of the peripheral blood CD4⁺ T cell count, and the beginning of an asymptomatic phase that can last up to 10 years, characterized by a slow decline in peripheral blood CD4⁺ T cells and a slow rise in HIV-1 plasma viremia. This sort of "silent" phase continues until peripheral blood CD4⁺ T cells fall below 200 cells/µl and the total CD4⁺ T cells in the body decrease by at least half (171) and at this point opportunistic infections and tumors start to occur, thus stating the beginning of the so-called full-blown AIDS phase.

Acute phase of HIV-1 infection

Exposure to HIV-1 occurs primarily through the mucosal route, either gastrointestinal or reproductive, which is thought to be the initial site for viral replication within the target cells of the mucosal tissue. In this context, CCR5 is usually the main target coreceptor for naturally transmitted viruses (107, 110, 121, 172-174).

Even though little is still known about the primary pathophysiological events for lymphocyte and viral dynamics in acute HIV-1-infection, recently some light has been shed by studies conducted in the animal model for HIV-1 infection, SIV infection in Rhesus macaques, in which the fundamental pattern of infection is highly similar to that observed in
HIV-1-infected individuals (175, 176). After both intravenous and intravaginal inoculation of SIV, the majority of infected cells are CD4+ T cells in the lamina propria of the mucosal tissues (159, 177-183). By contrast, dendritic cells do not seem to be a major target for viral replication, but they can facilitate the infection in trans of CD4+ T cells through binding of intact virions to DC-SIGN (184-186), with the exception of Langerhans cells in the vaginal epithelium that can become productively infected by the virus by 18 hours post-inoculation and migrate afterwards to the local lymph nodes where they propagate the virus to CD4+ T cells (187, 188). After the establishment of the infection, both SIV and HIV-1 infect other secondary lymphoid organs, including lymph node, thymus, spleen and mucosal tracts, and this may result in the formation of a pool of latently infected cells (159, 180, 189-191).

Virus transmission seems to be a local cell-to-cell phenomenon that depends upon the status of the target cell rather than on the total number of target cells in the body. Indeed, the initial target for HIV-1 infection are CD4+ T cell that express the coreceptor CCR5, memory T cells that typically resides in the mucosal surfaces of the intestinal, respiratory and reproductive tracts, and is less common in blood, lymph node and spleen (179, 192-197). Mucosal CCR5 positive cells are usually also activated cells, expressing activation markers such as CD69 and HLA-DR (179, 181, 195), and this further qualifies them as ideal targets for the virus. Moreover, the mucosal environment is rich in inflammatory cytokines that can activate T cells thus promoting the infection and propagation of HIV-1 in resting CD4+ T cells (198, 199). Finally, the gastrointestinal tract is the largest lymphoid compartment in the body, accounting for, at steady state, at least 60% of the total body T cells (200). As a consequence, SIV and HIV-1 infections in the very first phase result in a profound damage of the immune system, with a rapid depletion of CCR5+ CD4+ T cells that is much greater
and earlier in the intestinal mucosa than in peripheral blood, lymph nodes and spleen (178-180). In the macaque model this happens within the first three weeks of infection (177, 178, 180, 182). The loss of CD4+ T cells seems to be biphasic, with a dramatic depletion of preexistent T cell targets during the acute phase of the infection and subsequently a slow progressive decline of the remaining cells that characterizes the chronic phase (Figure 1.4). Interestingly, most of the cells that are lost within the acute phase seem to be Ki67+ proliferating cells, and this may constantly generate new CCR5+ CD4+ memory T cells that can become target for viral replication in the post-acute phase. CD4+ memory T cells presumably die of direct and indirect virus cytopathic mechanisms (201-206) and of CD8+ T cell-mediated destruction (207). Considering that, in this context of T-cell activation, both generalized and mediated by the virus, T-cell homing and trafficking between blood and lymphoid organs are dramatically altered (208, 209), the CD4+ T-cell depletion observed in the peripheral blood may only approximately reflect, and likely underestimate, the total loss of CD4+ T cells.

Besides the immune system seems to have the capacity of reconstituting itself after a profound depletion, this does not happen completely in SIV or HIV-1 infection, at least not in the CD4+ T-cell pool, even following treatment (210-212).

After the brief period of acute infection, the immune system has to face a double problem: the reconstitution of the profoundly depleted CD4+ T-cell pool and the ongoing viral replication that keeps on inducing T-cell activation, impairing thymic output, disrupting lymph node architecture, and inducing CD4+ T-cell apoptosis (213), thus contributing to the propagation of the virus itself and further compromising the homeostatic balance and maintenance capacity of the resting naïve and memory CD4+ T-cell pool.
The chronic phase of HIV-1 infection

Viral and T-cell dynamics during the chronic phase of the infection are markedly different from those observed in the acute phase: plasma viral loads are lower and tend to increase slowly, and CD4+ T cells in the peripheral blood recover partially after the resolution of the acute phase, but then decrease, slowly as well, before the onset of AIDS, over a variable period of on average ten years (214). This overall picture is accompanied by high levels of immune activation, to the point that chronic HIV-1 infection is actually a condition of chronic immune activation (215) and the level of chronic activation can be itself a better predictor of disease progression than plasma viral load (216-219). Chronic immune activation, either virus-specific or non-specific "bystander", in turns determines a state of "high turnover" of both CD4+ and CD8+ T cells (220-224). Besides chronic activation may explain the high death rates of T cells, this per se does not account for the profound CD4+ T-cell depletion observed. Indeed, it has been proposed that CD4+ T-cell death may occur as a result of various mechanisms on the various compartments of the resting T cell pools, including both memory and naïve CD4+ T cells (225). These mechanisms involve direct effects of the virus as well as non-destructive effects and act on both memory CD4+ and CD8+ T cells; however, being CD4+ T cells more vulnerable than CD8+ T cells and CD8+ T cells capable of higher levels of proliferation in response to antigens, this phenomenon is more dramatic for CD4+ T cells, and their reconstitution depends greatly on naïve CD4+ T-cell output from the thymus (211, 226-233).

Besides the direct and indirect effects on the T-cell pool, HIV-1 infection also exerts negative effects on the thymus, bone marrow and lymph nodes. Abnormal thymic morphological changes including thymocyte depletion and advanced involution (234-238)
have been documented in both children and adults (234, 239, 240). Thymic dysfunction has been associated with early progression to the disease in perinatally infected infants (241-243) and SIV infection causes similar changes (244, 245). HIV-1 infection also inhibits the production of hematopoietic lineages other than CD4+ T cells (246) and HIV-1-infected individuals are often pancytopenic. Bone marrow and lymph node architecture is also abnormal (247-249) and stromal auxiliary cells from the bone marrow are infected and dysfunctional (250, 251).

The major features of T-cell dynamics during the chronic phase of the infection are shown in Figure 1.5 and include: a) pathological changes in thymus, bone marrow and lymph nodes, and decrease in thymic output, b) depleted memory CD4+ T-cell pool from the acute phase, c) chronic activation of CD4+ and CD8+ T cells, d) increased t-cell death due to increased immune activation, e) expansion of the activated CD8+ T-cell pool, f) more activated CD8+ T cells than CD4+ T cells re-entering the resting memory pool, g) death of the vast majority of activated T cells, h) activated CD4+ T cells being the major source of virus, i) activated CD4+ T cells destined to die independently of infection and j) infected activated CD4+ T cells failing to re-enter the memory T-cell pool.

The chronic phase of HIV-1 infection is a quasi-stable state in equilibrium between T cell activation, death, and renewal and production and removal of virus. As immune degradation progresses this equilibrium eventually breaks down resulting in clinical immunodeficiency and full-blown AIDS (214). Despite the rapid decrease in viral load observed at the end of the acute phase, it usually takes several months to reach this steady state, and this often occurs after a period of strong fluctuations in the viral load. Several factors may contribute to this initial control of viral replication, including the diminishing
availability of the CD4+ target T cells (252), and the development of an effective immune response. Evidences suggest a temporal correlation between the drop in viremia and the appearance of HIV-1-specific CD8+ T-cell responses, but not with neutralizing antibodies, which seem to have a lower impact on the control of viral replication (253-257). In SIV infection the emergence of SIV-specific CD8+ T-cell responses coincides with viral clearance (189, 258) and changes in viral sequences allowing escape from CD8+ T-cell responses develop rapidly during the acute phase in both monkeys (259-261) and humans (262). Loss of specific immunity may play a role in accelerating viral replication and promoting progression toward the disease (263, 264). The frequency of HIV-1-specific T cells, particularly albeit not exclusively CD8+, is consistently high throughout the infection before the onset of AIDS (265-269), but these cells fail to suppress viral replication, and the reasons for this may be multiple. First, the recruitment of naïve CD4+ T cells together with HIV-1-specific CD8+ T cells into infected lymphoid sites may on one hand increase the rate of viral clearance (270), but on the other hand, it may also provide new targets for viral replication (271), which will in turns increase T cell activation and induce the recruitment of more HIV-1-responding cells, leading to further activation, in a sort of positive feedback loop (272). Second, continuous cycles of activation and infection result in a sort of post-activation state of the remaining HIV-1-specific CD4+ T cells, that may not be capable of proliferating in response to additional stimuli and activation signals (273). Third, HIV-1-specific CD8+ T cells are also defective (high sensitivity to Fas-induced apoptosis (264), low expression of perforin (274), lack of cytokine production (275), TCR with low functional avidity (276), inappropriate signaling (276), skewed maturation profiles (277) and diminished proliferation in response to antigens (278)), as a consequence of chronic antigen
stimulation.

In conclusion, CD4\(^+\) and CD8\(^+\) HIV-1-specific T-cell responses make a partially successful attempt to control the infection; however, this specific response, by driving CD4\(^+\) and CD8\(^+\) T cell activation, providing new targets for viral replication and being itself target for preferential infection, may become part of the problem as well.

Finally, another important factor to take into account is the high mutability and genetic flexibility of HIV-1 and SIV that allow them, in response to immunologic pressure, to adapt to the host and evolve into variants capable of using a broader range of target cells and with increased pathogenicity or decreased susceptibility to the immune response (107, 110, 117, 121, 207, 279, 280).

1.1.2 New Insights: structure and function of the gut

The mucosa-associated lymphoid tissue (MALT), of which the gastrointestinal (GI) immune system (also referred to as gut-associated lymphoid tissue, GALT) is the largest component, plays a critical role in the interaction between primate lentiviruses and the host immune system. Indeed, (i) most of the HIV transmissions occur through the mucosal route, either vaginal or rectal, (ii) a significant amount of viral replication occurs at the level of MALT in all stages of the disease and (iii) a progressive depletion and dysfunction of the mucosal immune system is a key feature of HIV/SIV associated immunodeficiencies (281). Moreover, the typical depletion of CD4\(^+\) T cells that characterizes the progression to AIDS is more rapid and severe at the level of MALT than in the peripheral blood or in secondary lymphoid organs (282).

The GI tract, being by far the largest surface of the body in contact with the external
environment, is the entry route for many human pathogens that enter the body through the GI mucosal surface. On the other hand, the intestinal microbial flora plays an essential role in the digestive function of the GI tract, and its integrity is crucial. Thus, the body has evolved several strategies to maintain the equilibrium between the need to preserve commensal microbes and to protect against enteroinvasive pathogens (283-290): first, a continuous layer of epithelial cells kept together by tight junctions separating the lumen from the intestinal lamina propria that constitutes a physical barrier; second, mucus and glycocalyx lining the apical side of the epithelial cells; third, antimicrobial molecules produced and released in the mucosal environment (291-295). Besides this physical barrier function, the GI mucosa is also actively involved in the innate and adaptive immune response to antigens, by the GALT, which represents the most abundant lymphoid tissue in the body, containing the vast majority (more than 40%) of the lymphocytes of the body (296).

Functionally, the GALT can be divided in two compartments: the inductive and the effective compartments (297) (Figure 1.6). In the inductive compartment, composed of mesenteric lymph nodes, Peyer's patches and isolated lymphoid follicles, overlaid by an epithelial membrane containing M cells, antigens are collected from the lumen and immune response is first induced. These cells transcytose particulate antigens to antigen-presenting macrophages located at the basal surface of the epithelium (298), which constitute the first phagocytic cells to interact with microorganisms that have entered the intestinal mucosa. Intestinal macrophages have avid phagocytic and bactericidal activities that protect the host from pathogens and they regulate the immune response to commensal bacteria (299). Antigen-presenting dendritic cells (DC) sample both commensal and pathogenic microbes
for subsequent transport and presentation to B and T lymphocytes (300). In the effector compartment (epithelial cells and lamina propria), the adaptive immune cells differentiate and exert their immune effector functions, either cellular- or humoral-mediated. The intraepithelial lymphocytes are mainly CD8+ T cells or Tc1 cells, whereas the lamina propria contains CD4+ T cells, as well as CD8+ T cells, natural killer (NK) cells and B cells (301-307).

This complex defense system under normal homeostatic conditions can effectively prevent or restrict the entry and propagation of commensal and pathogenic organisms, including HIV-1 (299). It has been suggested that the anti-inflammatory responses induced by commensal flora protect the intestinal epithelium from pathogenic insults (308). This relationship, however, appears to extremely delicate and anything that perturbs either immune or epithelial homeostasis can lead to inflammation and life-long inflammatory conditions such as Crohn's disease and ulcerative colitis.

1.1.3 HIV-1 infection and the gut

HIV and SIV can be considered as mucosal pathogens, since natural transmission occurs mainly though the mucosal surfaces (309). Even if epithelial cells cannot be productively infected by these lentiviruses, however, they can capture virions and transfer them to dendritic cells, macrophages and CD4+ T cells of the sub epithelial layers (310-312). Dendritic cells then in turns can facilitate infection of CD4+ T cells by HIV and SIV via "infectious synapse", a dendritic cell/T cell conjugate that also promotes the spread of the virus to adjacent cells (313-315). As a consequence, as suggested by several studies conducted in macaques, the GI mucosa is the first and predominant site of SIV infection,
where SIV-infected lymphocytes accumulate and the virus highly replicates (158, 159, 182).

The first description of the early events that occur following SIV infection at the level of the gut was by Veazey and Lackner that in 1998 documented a 70-95% loss of CD4+ T cells in the jejunum, ileum and colon by day 21 post-infection in SIVmac239-infected macaques (182). Subsequent studies confirmed this observation and elucidated the mechanisms underlying SIV-associated mucosal CD4+ T-cell depletion (316-318). The main conclusion from these studies is that the early phase of SIV infection is associated with a rapid dramatic loss of mucosal CD4+ T cells, particularly with a memory phenotype and expressing the HIV-SIV coreceptor CCR5. Indeed, the large population of memory/activated CCR5+ CD4+ T cells residing in the gut represents the ideal target for viral infection and replication (182, 316-322), also considering that the majority of newly transmitted HIV and SIV strains are CCR5-tropic, and activated cells are preferentially infected and killed by lentiviruses (159, 173, 323, 324). Interestingly, in a study conducted in SIVmac251-infected macaques, up to 60% of CD4+ T cells were found to harbor SIV DNA by day 10 post-infection, thus postulating a relevant role of direct virus-mediated T-cell killing during the very early acute phase of the infection (317), even though additional mechanisms have also been proposed, including CD95-mediated T-cell apoptosis (318).

Investigating the early events occurring at mucosal level during the acute primary phase if HIV infection in humans is a challenge for obvious practical and ethical reasons. However, a few studies were performed, by three research groups separately (Dandekar, Markovitz and Douek), on samples obtained from jejunal, colon and terminal ileum biopsies of acutely HIV-1-infected individuals, showing an early, severe and mostly irreversible depletion of CD4+ CCR5+ memory T cells, albeit to a lesser extent as compared to SIV
infections (316-318, 320-322, 325). Thus, the model for HIV pathogenesis involves the selective depletion of memory CD4+ T cells from mucosal tissues during the early acute phase of the infection, inducing a significant impairment of mucosal immunity that may result in a series of pathogenic events mostly apparent during the chronic phase.

Whereas the CD4+ T-cell depletion in the acute phase is mainly due to direct cytopathic effects of an uncontrolled viral replication primarily on memory/activated CD4+CCR5+ T cells, during the chronic phase, in which the immune system is to some extent capable of controlling viral replication, a key role is also played by host-specific factors, such as a chronic, generalized immune activation state (326). Indeed, in addition to the loss of CD4+ T cells, gene expression profiling studies in GI tract biopsies reveal that several genes associated with cell cycle regulation, lipid metabolism, and epithelial cell barrier and digestive functions are dysregulated in HIV-1-infected individuals (327). The enteropathy is characterized by diarrhea, increased inflammation, increased permeability and malabsorption (328) and, histologically, infiltrates of lymphocytes and epithelial damages including villous atrophy and blunting and crypt hyperplasia (329). Recent studies have documented the preferential GI depletion of a subset of CD4+ T cells defined by their secretion of IL-17, the so called Th17 cells, that are critical against bacteria and fungi at mucosal surfaces and contribute to the homeostasis of enterocytes (330).

Taken together, these data indicate that the GI mucosal barrier suffers a serious immunological and structural insult in HIV-1 and pathogenic SIV infections and that this damage may adversely affect the barrier function of the gut. The idea is that the loss of mucosal immune function favors a breakdown of the physical and biological mucosal barrier that results in the translocation of microbial products from the gut to the systemic circulation.
Indeed, chronically HIV-1-infected individuals have significantly increased levels of plasma lipopolysaccharide (LPS), an indicator of microbial translocation, as compared to uninfected individuals (331). Microbial products would in turn cause a broad activation of the immune system through their binding to certain toll-like receptors (TLR-4) and consequent bystander activation of non HIV-specific lymphocytes (331). These findings point to microbial translocation as a cause of immune activation in chronic HIV/SIV infections, thus providing a link between damage to the GI tract and progression to AIDS.

CD4+ T-cell depletion alone does not seem to be sufficient to result in mucosal translocation and inflammation, as indicated by studies in non-pathogenic SIV infections in African green monkeys and sooty mangabeys (332), natural hosts for SIV, which show CD4+ T-cell depletion associated with immune activation and microbial translocation during the acute phase, but do not progress to AIDS, as both phenomena resolve as the animal enters the chronic phase, even in a context of viral replication (Figure 1.7).

Thus, it seems that CD4+ T-cell depletion during the acute phase of the infection, is necessary, but not sufficient nor predictive of progression to AIDS, which seems to be a more complex phenomenon, initially triggered by the virus, but ultimately related to the nature of the host, and resulting from multiple immunologic abnormalities, including but not limited to, CD4+ T-cell depletion and immune activation, that exacerbate proliferation and apoptosis (225, 333-337).

1.1.4 Mechanisms for CD4+ T-cell Depletion

As outlined above, the mechanisms contributing to CD4+ T-cell depletion during the acute and chronic phases of HIV-1 and SIV infection seem to follow different dynamics and
to be mediated by different processes. During the acute phase a key role is played by viral replication and cytolysis. Indeed, HIV-1 can directly induce cell death in the cells it infects by several mechanisms: i) syncytia formation in CXCR4+ cells (338), ii) disruption of the plasma membrane due to continuous virus budding (339) or to viral proteins such as Vpu that can alter membrane permeability (340), iii) cellular toxicity mediated by un-integrated linear viral DNA (341), iv) HIV-1 protease that inactivates the anti-apoptotic protein Bcl-2 and activates procaspase 8, making the cell more susceptible to apoptotic stimuli (342, 343) and v) interference with the apoptotic pathways, through direct action of the virus with cellular pro- and anti-apoptotic proteins.

By contrast, during the chronic phase of HIV/SIV infection, additional mechanisms, including defective T-cell regeneration, limited regenerative capacity, anergy and programmed cell death (PCD) or apoptosis, become increasingly relevant (339).
1.2 Apoptosis

1.2.1 General Overview

Three distinct but overlapping PCD pathways have been described based on differences in the stimuli, cytokine environment and death-inducing signaling cascades. The first two pathways, apoptosis and autophagy, do not affect the integrity of the plasma membrane and thus, do not induce an inflammatory response (344). The third pathway, necrosis, involves mechanical rupture of the membrane, release of cellular organelles and induction of a phagocytic inflammatory response (344). Autophagy and necrosis have been studied in vitro, while apoptosis has been extensively investigated both in vitro and in vivo in HIV-1-infected patients (344), where a positive correlation with lymphocyte activation and disease progression has been documented (345). Apoptosis, also called programmed cell death, is an important mechanism that plays a central role in the development and homeostasis of multicellular organisms, and can be dysregulated in several pathological processes including tumors and viral infections. During this process, a cascade of biochemical events leads to characteristic morphologic changes of the cell and ultimately to death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Unlike necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis produces cell fragments called apoptotic bodies that are quickly engulfed by phagocytic cells and removed from the extracellular environment before the contents can spill out and cause damage, thus preventing inflammation (346).

Apoptosis can follow mainly two distinct pathways: the extrinsic pathway, which is initiated outside the cell by the so-called “death molecules” or by other stress factors, and
the intrinsic pathway, which begins when the stress or damage occurs within the cell itself. In both pathways, signaling results in the activation of a family of Cys (Cysteine) proteases, named caspases that act in a proteolytic cascade to disrupt the cell (347) (Figure 1.8).

The more classical “extrinsic” cascade is triggered by the binding of the “death molecules” to their specific “death receptors” (DRs) on the surface of the cell, and activates caspases within seconds of ligand binding, causing apoptotic damage within hours. Although there are differences in the signaling pathways activated by the different DRs, it is possible to outline a general apoptotic signaling pathway. These receptors belong to the superfamily of TNFR (Tumor Necrosis Factor Receptor) and are characterized by a Cys-rich extracellular domain that binds the ligand and a homologous intracellular domain known as the Death Domain (DD). Binding of the ligand to its receptor can lead to the release of ceramide, which is thought to promote lipid raft fusion, resulting in a large scale clustering of the receptors, thus amplifying the apoptotic signaling. Following ligand binding a conformational change in the intracellular domains of the receptors reveals the DDs that recruit various apoptotic proteins, including FADD (Fas-Associated via Death Domain), TRADD (Tumor Necrosis Factor Receptor-1-Associated Death Domain) or Daxx containing DDs, to the receptor. This protein complex is called the DISC, or Death Inducing Signaling Complex. The final step in this process is the recruitment of caspase-8, to the DISC, and the initiation of apoptosis. The best-characterized DRs are Fas and TNFR1 (Tumor Necrosis Factor Receptor-1), which bind Fas-Ligand (FasL) and TNF-α (Tumor Necrosis Factor-alpha), respectively (348). Other DRs include the TNF-related apoptosis inducing ligand (TRAIL) receptors DR4 and DR5.
Apoptosis can also be activated by various forms of cellular stress, including gamma- and UV radiation, treatment with cytotoxic drugs and removal of cytokines. Stress induced apoptosis involves altering mitochondrial permeability and formation of a channel, the mitochondrial apoptosis-induced channel (MAC), in the outer mitochondrial membrane, with subsequent release of cytochrome C (cytC), with regulatory functions (349). Released cytC binds to Apoptotic protease activating factor - 1 (Apaf-1) and ATP, and subsequently to pro-caspase-9 to create the apoptosome. In this complex, the pro-caspase is cleaved to its active form of caspase-9, which in turn activates the effector caspase-3. The MAC can be regulated by several proteins, including those encoded by the mammalian Bcl-2 family of anti-apoptotic genes, the homologs of the ced-9 gene found in C. elegans (350). Bax and/or Bak form the pore, while Bcl-2, Bcl-xL and Mcl-1 inhibit its formation. Bax, Bid and Bim are initially inactive and require translocation to mitochondria to induce apoptosis. The Bcl-2-associated death promoter (Bad) protein is a pro-apoptotic member of the Bcl-2 family that, after dephosphorylation, is able to form heterodimers with anti-apoptotic proteins (Bcl-2 and Bcl-xL) and prevents them from inhibiting apoptosis (351, 352). When a stress injury, such as oncogenes, direct DNA damage, hypoxia, and survival factor deprivation, occurs within the cell the apoptotic pathway that is triggered is called “intrinsic” pathway. The p53 is a sensor of cellular stress and is a critical activator of the intrinsic pathway. This protein initiates apoptosis by inducing transcriptional activation of pro-apoptotic and repressing anti-apoptotic Bcl2 family members. Additional p53 targets include Bax, Noxa, Puma (p53-Upregulated Modulator of Apoptosis) and Bid. The p53 also transactivates other genes that may contribute to apoptosis including PTEN (Phosphatase and Tensin Homolog Deleted On Chromosome-10), APAF1, Perp, p53AIP1 (p53-regulated Apoptosis-Inducing Protein-1),
and genes that lead to increases in ROS (Reactive Oxygen Species). These ROS lead to a
generalized oxidative damage to all mitochondrial components (353).

Other proteins released from mitochondria, SMAC (Second Mitochondria-Derived
Activator of Caspase)/ Diablo, Arts and Omi/HTRA2 (High Temperature Requirement
Protein-A2), counteract the effect of IAPs (Inhibitor of Apoptosis Proteins), which normally
bind and prevent activation of caspase-3 (354). The interaction between Bcl family
members, IAPs, SMAC and Omi/HTRA2 is central to the intrinsic apoptosis pathway.
Recent studies demonstrated that another nuclease, Endonuclease G, mitochondrionspecific, translocates to the nucleus and cleaves chromatin DNA during apoptosis (355).
Another protein, AIF (Apoptosis Inducing Factor) was also shown to have a role in
apoptosis, becoming active upon translocation from mitochondria to nuclei, where it initiates
chromatin condensation and large-scale DNA fragmentation (356).

1.2.2 Apoptosis in HIV/SIV Infection

Several studies have documented abnormal levels of apoptosis both in vitro (357-
367) and in vivo (368, 369) in CD4+ and CD8+ T cells from HIV-1-infected individuals. Of
note, the vast majority of T cells undergoing apoptosis in HIV-1-infected patients are not
infected by the virus (368). This observation led to the definition of 'bystander' apoptosis,
referring to apoptosis when it is not occurring as a direct cytopathic effect of the virus in an
infected cell. HIV-1 and HIV-2 differ in their natural course of infection: HIV-2 is
characterized by higher CD4 counts, low level of viremia, low transmission rate (370), as
well as by lower immune activation and CD4 T-cell apoptosis (371). The level of CD4+ T-
cell apoptosis in HIV-1-infected individuals has been shown to correlate with the stage of
the disease (372-377) and changes in the levels of T-cell apoptosis following highly active antiretroviral therapy (HAART) were shown to predict the immunological response to therapy (378-380). Taken together, these observations indicate that the increased susceptibility to apoptosis of T lymphocytes from HIV-1-infected individuals can be considered as a marker of disease progression and support the hypothesis that the chronic immune system activation that follows HIV-1 infection could be one of the mechanism responsible for this cell death process (368, 381, 382).

Apoptosis seems to be a relevant mechanism contributing to CD4+ T-cell depletion during both the acute and chronic phases of HIV/SIV infections. Indeed, the level of apoptosis during primary SIV infection was shown to be predictive of the rapid or slow progression towards AIDS (383) and to be greater in primates infected with a pathogenic SIV strain (384). Moreover, studies performed in pathogenic and nonpathogenic primate models of HIV or SIV infection during the chronic asymptomatic phase identified a correlation between the induction of T-cell apoptosis in vitro and the pathogenic nature of the infection in vivo (367, 381, 385-392).

Enhanced levels of apoptosis in CD4+ T cells were observed in HIV-1-infected human individuals, and in rhesus macaques and chimpanzees infected with pathogenic strains of SIV leading to AIDS (386), while enhanced CD8+ T-cell apoptosis was observed in both pathogenic and nonpathogenic primate models. By contrast, no increased propensity of either CD4+ or CD8+ T-cell in vitro apoptosis and normal levels of T-cell apoptosis in the T-cell-dependent areas of the lymph nodes were observed in either naturally or experimentally SIV-infected sooty mangabeys (381).
Taken together, all these reports underscore the relevance of apoptosis in AIDS pathogenesis, and suggest that the capacity to induce apoptosis during primary SIV infection, which seems to be directly correlated with the pathogenicity of the infection itself, is a feature that does not depend solely on the virus, but is also related to specific host–virus interactions that play a key role in determining the potential to induce AIDS.

1.2.3 Mechanisms of Apoptosis in HIV/SIV Infection

Various mechanisms have been proposed to contribute to the spontaneous apoptosis observed during the course of HIV-1 infection. Among them sustained immune activation associated with dysregulated cytokine production (345, 393), loss of extracellular survival signals ("death-by-neglect") (394), inappropriate signaling mediated by HIV-1 envelope binding to CD4 (395-397) or coreceptors (398), direct effect of accessory viral proteins, including Tat and Nef (399-403), defective antigen presentation (404) and activation of death receptors (366, 405-410), seem to play an important role.

*Sustained immune activation, “death by neglect” and dysregulated cytokine production*

Since many of the molecular steps required for apoptosis (such as chromatin condensation or caspase activation) are in common with pathways of cellular activation (411), the two phenomena are closely related and strictly linked in the so called activation-induced cell death (AICD). Priming of a cell can occur in different ways, including repeated stimulation through CD3/TCR (412), via CD4 (395) or activation without co-stimulation (413), and these stimuli upregulate Fas and FasL expression on the surface of the cell (414). Several studies have documented increased immune activation and accelerated T-cell regeneration rates in the chronic phases of HIV/SIV infection (222, 223, 345, 393), and
elevated T-cell turnover is known to lead to the exhaustion of the immune system. Moreover, this excessive immune activation has been suggested to induce apoptosis through Fas/FasL (376, 415, 416). Activated lymphocytes, at the end of an immune response, when antigens and inflammatory cytokines are cleared, can undergo death-by-neglect in the absence of further stimuli. In this case, cell death is preceded by the loss of a positive extrinsic signal rather than the presence of a negative signal, as a consequence of which cells undergo atrophy, characterized by a decrease in cell size and protein content and loss of cellular ATP, and die (417-419).

Another factor that may contribute to apoptosis in HIV-1 infection is cytokine dysregulation, which is likely to occur as a consequence of the death of Th lymphocytes (420). It has been hypothesized that as HIV-1 disease progresses there is a shift in the cytokine response from a predominantly type-1 cellular immune response (IFN-γ, TNF-α, IL-12), to a type-2 humoral response (IL-4, IL-5, IL-10, IL-13), and type-1 cytokine responses decrease as the disease progresses (421). Considering that resistance to apoptosis in vitro seems to be associated with a predominant type-1 response (360, 362), and that IL-2 and Ifn-γ exert anti-apoptotic activities, whereas IL-4 and IL-10 increase susceptibility to apoptosis (364, 422), this type-1/type-2 shift of the immune response could have drastic effects on apoptotic events. Although, gene expression data demonstrated that IFN-α is capable of strongly inducing a number of pro-apoptotic genes in the TNF superfamily (423), suggesting that susceptibility to apoptosis may not strictly fit the type-1/2 paradigm, it is however clear that cytokine dysregulation plays an important role in HIV-induced apoptosis, regardless of the assignation of these cytokines to a type-1 or type-2 phenotype.

The role of other cytokines, such as TNF-α and other TNF-family members, and the
growth factors IL-2 and IL-15, is still a matter of debate. Indeed, although TNF is one of the strongest pro-apoptotic signals and its serum levels were shown to be increased, as well as its modulation being dysregulated, in HIV-1-infected individuals (424), there is only limited evidence for TNF-mediated apoptosis in either infected and bystander lymphocytes and clinical trials of anti-TNF therapies failed to show improvement in either immunological or clinical outcome (425). The role of the T cell growth factors IL-2 and IL-15 in HIV-induced apoptosis is also unclear: treatment with recombinant IL-2 or IL-15 was shown to increase or decrease T-cell apoptosis susceptibility, depending on the activation status of the cell; moreover, IL-2 and IL-15 were shown to increase susceptibility to CD95-mediated apoptosis (426), while protecting against spontaneous apoptosis (427); finally, treatment with IL-2 in vivo was shown to decrease overall the levels of apoptosis in HIV-1-infected individuals (428).

**Direct effect mediated by HIV-1 envelope and other viral proteins**

Inactivated HIV-1 virions (429) and HIV-1 proteins released into the extracellular environment can have dramatic effects on lymphocytes. HIV-1 proteins such as gp120, Tat, Nef and Vpu have been shown to induce cell death in both infected and uninfected cells.

Soluble gp 120 has been shown to promote the cross-linking of CD4 molecules on the surface of the cells (395), and this, in the absence of TCR stimulation, triggers apoptosis (430). Moreover, binding of soluble gp120 to its receptors, CD4 and the coreceptors CCR5 and CXCR4, triggers both Fas-dependent and -independent apoptotic cascades (398, 431, 432). Finally, cell surface presentation of gp120 can induce cell death mediated by close cell-to-cell in both infected and bystander cells (396, 397, 433).

HIV-1 proteins Tat and Nef have also been implicated in directly inducing apoptosis in
both infected and bystander uninfected cells (399-403). HIV-1 Tat protein, secreted from infected cells (434), has been suggested to upregulate caspase 8, Fas-ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (402, 435-437) in bystander cells. However, the role of Tat is not solely pro-apoptotic, as this protein was also shown to protect against TRAIL-mediated apoptosis (438), though upregulation of Bcl-2 (439). HIV-1 Nef protein was shown to induce cell death in neuronal cells as well as in a wide range of blood cells via a Fas-dependent mechanism (440-442).

In HIV-1-infected cells, the virus has evolved strategies for both inducing and preventing apoptosis at the same time, to try to preserve its “source” of new virions. In this context, the role of the same HIV-1 proteins, Tat and Nef, seems to be peculiar in that both proteins were shown to promote apoptosis (see above) or to reduce apoptosis, for instance by decreasing the expression of MHC I molecules that serve for the presentation of non-self viral epitopes on the surface of an infected cell to promote cell-killing by CTLs (443). Similarly to Tat (439), HIV-1 Vpr protein was reported to induce the expression of Bcl-2 and decrease the expression of Bax (444), while at the same time prolonging the G2 cell cycle delay (445) and transactivating the viral promoter at the LTR (446), to boost viral replication. However, despite these strategies, infected cells have a shortened half-life and therefore are more prone to die, thus contributing to the overall decrease in the CD4+ T-cell pool.

**Defective antigen presentation**

Several studies have reported reduced myeloid and/or plasmacytoid dendritic cells (mDC and pDC) numbers in the blood of HIV-1-infected individuals (447, 448). In addition, loss of Langerhans cells has also been reported (449). DCs are susceptible to HIV-1
infection. However, although they are considered to be an early target of HIV in sexual transmission (158), there may not be an absolute requirement for the productive infection of these cells. Indeed, it has been proposed that HIV-1 binds to DC-SIGN on DCs in the genital tract and is internalized into non-lysosomal compartments where it retains infectivity (186), to be then transported by DCs to the draining lymph node, where it is transmitted to permissive T lymphocytes during antigen presentation (185). The virus can also be kept in a silent form in DCs that are thus considered as a potential HIV reservoir. On Langerhans cells this “transfer” process may be mediated by langerin, another C-type lectin expressed on these cells that was shown to bind HIV-1 (313). Several reports suggest a functional impairment in the DC compartment in the course of HIV-1 infection, both in the capacity to induce allogeneic T-cell proliferation (450-453) and in the capability to produce IFN-α following antigen stimulation (454). DC dysfunction could contribute to the observed increase in T-cell apoptosis, as a consequence of the lack of appropriate co-stimulatory signals, that may result in T-cell anergy followed by T-cell deletion (455).

Activation of death receptors

Extensive death-receptors mediated apoptosis is thought to occur in many infectious diseases, including HIV/SIV infection. As outlined above, several HIV-1 proteins may interact directly with the death receptor apoptotic pathway, altering the expression of various death receptors, including Fas and TRAIL-receptors. Indeed, peripheral blood mononuclear cells (PBMC) from HIV-1-infected individuals express higher levels of FasL and Fas (407), and this upregulation further increases with disease progression (365, 405, 456). Augmented levels of soluble Fas were also documented in the plasma of HIV-1-infected individuals, and this can be used as a marker for the prognosis of AIDS (457). An increased susceptibility to
TNF-mediated apoptosis was observed in both CD4\(^+\) and CD8\(^+\) T cells from HIV-1-infected individuals (410), accompanied by an upregulation of TNF-R2 expression (458). High levels of TNF and soluble TNF-R2 were also observed, and were found to be predictive of disease progression (424). TRAIL-dependent AICD was detected in CD4\(^+\) T cells isolated from HIV-1-infected individuals \textit{in vitro}, particularly in infected cells, and the addition of antagonistic TRAIL-specific antibodies reduced apoptosis (459). HIV-1 Tat protein seems to upregulate TRAIL expression, thus triggering apoptosis in bystander uninfected cells (437, 460). Thus, TRAIL seems to be one of the most significant molecules for apoptosis in HIV-1 infection, and was also shown to induce apoptosis in neurons both \textit{in vitro} and \textit{in vivo}, thus potentially explaining neuronal death in HIV-1-associated encephalopathy (461-463).

Finally, in HIV-1 infected individuals, the expression level of other co-stimulatory and death molecules, including CD28, CD40 and CD40-ligand, is also altered (464-466).
1.3 Non-Human Primate (NHP) Models for HIV-1 Infection and AIDS

The challenge in establishing animal models for HIV-1 is that the virus does not replicate in most of the animal species tested (467), with the exceptions of chimpanzee and gibbon apes (468). However, HIV-1 infection of chimpanzees is typically non-pathogenic, with only rare animals developing AIDS-like symptoms after prolonged incubation period (386, 469, 470) and these species are endangered and costly to maintain, limiting their use for research purpose (471).

The family of CD4+ T-lymphotropic primate lentiviruses comprises two human viruses (HIV-1 and HIV-2) and at least 40 simian immunodeficiency viruses (SIV) that can be found naturally in non-human primates (NHPs). The similarities between SIV and HIV with respect to genomic structure and biological features renders infection of various macaque species with SIVs, or with chimeric viruses containing both SIV and HIV sequences (SHIVs), the most reliable animal models to study HIV-1 infection and AIDS. Natural hosts for SIV (sooty mangabeys and African green monkeys) generally do not show any signs of AIDS, despite chronic sustained levels of viral replication. Actually, few reports have demonstrated that SIV infection in natural hosts can eventually lead to the development of immunodeficiency. However, clinical disease seems to occur in the minority of cases, and only when animals have been infected over long periods of time. Indeed: (i) AIDS cases were reported for mandrils infected with SIVmnd-1 and SIVmnd-2 after 17 years of infection (472); (ii) a sooty mangabey (SM) naturally infected with SIVsmm progressed to AIDS after an incubation period of 18 years (473) and (iii) an African green monkeys (AGM) coinfected with SIVagm and simian T-cell leukemia virus (STLV) was also reported to progress to AIDS (474). Transmission of SIV from natural hosts (SMs and
AGMs) to Asian non-natural NHP host species (pigtailed and rhesus macaques) has been shown to lead to AIDS (475). Similar to humans and macaques, naturally or experimentally infected SMs, AGMs and mandrills show viremia levels which are persistently as high or even higher than those known with progression in humans (381, 472, 476-478). Studies of naturally SIV-infected AGMs during the chronic phase of the infection show signs of viral replication in the same tissues as during pathogenic infections, including gut and thymus (479-481) and both T CD4+ lymphocytes and macrophages are infected (479). Among the existing primate lentiviruses, infection of macaques with these SIVs most closely approximates HIV-1 infection in humans. Similarities include CD4+ T-cell and macrophage tropism, CD4+ T-cell depletion, serologic and immunologic responses and pathology, including neuropathology and opportunistic infections. However, the incubation and disease time are usually shorter and there are no lesions similar to Kaposi’s sarcoma, which may occur in HIV-1-infected individuals (482). Considerable efforts have been made to understand the similarities and differences between pathogenic and non-pathogenic SIV infections, with the hope of uncovering new host defenses that will guide conventional AIDS vaccine development. Several mechanisms, such as effective host control of viral replication, effective host control of viral pathogenicity, and failure of the virus to induce immunodeficiency, despite successful persistent infection, have been proposed to contribute to the nonpathogenic nature of SIV infection in natural hosts, and it is likely that more than one concur.

For HIV-1-infected patients and SIV-infected macaques plasma viral load is the best predictor of disease progression (483-486). In captive NHPs naturally infected with SIVs (SIVsmm, SIVagm, SIVmnd-1, and SIVmnd-2), viral loads during the chronic phase of the
infection are higher than in chronically HIV-1-infected asymptomatic patients (472, 476-480, 487, 488), and longitudinal analyses of the dynamics of plasma viremia in naturally SIV-infected animals suggest that the level of viral replication is relatively constant over time (472, 473, 489). Thus, it does not appear that the lack of disease in naturally infected animals is associated with effective host containment of viral replication in these species. Studies of SIV-infected SMs and AGMs showed that, despite a significant CD4+ T-cell depletion, the regenerative capacity of the CD4+ T-cell compartment is fully preserved (332, 381), and this may play a key role in determining the lack of disease progression in naturally SIV-infected monkeys.

Both HIV-1 and SIVmac infections induce immune responses characterized by robust neutralizing and cellular immune responses (490), but a continuous immune escape occurs (491). Conversely, for natural infections of NHPs, the general consensus is that the immune responses are lower compared to those documented in pathogenic models (492) and this has led to the hypothesis that it is the low level of immune activation that protects natural SIV hosts from CD4+ T-cell depletion and AIDS (493). Lower levels of immune activation may in fact favor the preservation of the number and/or function of CD4+ T cells as well as of other immune cell types (including CD8+ T cells, γδ T cells, NK cells, macrophages, etc.), whose functional integrity may contribute to the preservation of the CD4+ T-cell pool. This theory is consistent with the observation that, during pathogenic HIV/SIV infections, generalized immune activation is an important driver and predictor of disease progression (335). An explanation for this lower immune activation could be related to the observation of increased numbers of Tregs, paralleled by an increase in TGF-β and IL-10 levels, in the plasma of SIVagm-infected AGMs, during the acute phase of infection (494). Alternative
mechanisms may involve dendritic cell–T cell interface, proinflammatory cytokine response, regulation of T cell homing to inflamed tissues, and maintenance of the balance of distinct CD4+ T cell subsets (Th1, Th2, Th17) (333).

As described above, a series of studies showed that pathogenic HIV/SIV infections are characterized by an early, severe, and largely irreversible depletion of mucosal CD4+CCR5+ memory T cells. Thereby, a model of AIDS pathogenesis has been formulated in which the selective depletion of memory CD4+ T cells from mucosal tissues during acute HIV infection is a key determinant of disease progression. However, an early, severe, and persistent depletion of mucosal CD4+ T cells was also observed during nonpathogenic SIV infection in natural hosts, with a kinetics that closely resembles that observed in pathogenic infections (495). Of note, the early mucosal CD4+ T cell depletion of natural hosts either does not progress further after reaching a stable plateau (in SMs) or is followed by a significant recovery of these cells (in AGMs) (495), thus implying that the severe loss of MALT CD4+ T cells during the acute phase of the infection may not be sufficient for the development of AIDS and that other factors, maybe related to the nature of the host, are required to induce mucosal and systemic immune dysfunction. Indeed, albeit for the very early acute phase of the infection, the majority of naturally SIV-infected SMs generally maintain normal CD4+ T cell counts in the peripheral blood throughout the infection (496). One possible explanation could be that natural SIV hosts have evolved to be less dependent on CD4+ T cells to maintain the overall function of the mucosal immune system (497). Alternatively, additional factors, such as the lack of local immune activation, would contribute to protect the CD4+ T cell–depleted mucosae of natural SIV hosts from losing their barrier function (495).
Another striking immunologic difference between natural non-natural SIV hosts is the lower level of CCR5 expression on CD4$^+$ T cells in the blood, lymph nodes, and mucosal tissues of natural SIV hosts (498). Although this is not sufficient to confer protection against infection of CCR5-tropic viruses, it is possible that the restricted CCR5 expression only to a limited group of CD4$^+$ T cells, likely those that have already encountered an antigen and are therefore at a more advanced stage of activation, may contribute, in synergy to the overall lower level of immune activation, to preserve the homeostasis of the "resting" CD4$^+$ T-cell pool (333) (Figure 1.9).
1.4 Treatment of HIV-1 Infection

1.4.1 Antiretroviral therapy (ART)

Nowadays, the treatment options for clinicians working with HIV-1-infected individuals include several classes of drugs:

- Entry inhibitors (or fusion inhibitors) interfere with binding, fusion and entry of HIV-1 to the host cell by blocking one of several targets (maraviroc and enfuvirtide)

- CCR5 receptor antagonists target CCR5 receptor on the surface of the T-Cell and block viral attachment to the cell

- Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) inhibit reverse transcription by being incorporated into the newly synthesized viral DNA strand as a faulty nucleotide

- Non-nucleoside reverse transcriptase inhibitors (NNRTIs) inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function

- Protease inhibitors (PIs) target viral assembly by inhibiting the activity of protease, an enzyme used by HIV to cleave nascent proteins for the final assembly of new virions

- Integrase inhibitors inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell (raltegravir, 2007)

- Maturation inhibitors inhibit the last step in gag processing in which the viral capsid polyprotein is cleaved, thereby blocking the conversion of the polyprotein into the mature capsid protein (p24) (Ifn-α).

Historically, the very first antiretroviral drug discovered for the treatment of HIV-1 infection was zidovudine (ZDV), which was approved by the U.S. Food and Drug
Administration (FDA) in 1987. This drug belongs to the class of NRTIs and inhibits the enzyme reverse transcriptase (RT) by inducing chain termination and an interruption of HIV-1 cDNA synthesis (499). The second FDA approved drug was didanosine (ddI), an analog of adenosine, introduced in 1991 in the United States (500). Unfortunately, scientists soon discovered the limitations of monotherapy regimen, mainly related to the appearance of HIV-1-variants that were resistant to the inhibitory effect of this molecule (501-503). It became increasingly clear that a combination of drugs would probably have been more effective and most likely required in order to counteract the emergence of resistant variants and to try to achieve a more profound and hopefully sustained suppression of viral replication. In 1995, David Ho promoted a "hit hard, hit early" approach with aggressive treatment with multiple antiretroviral drugs early in the course of the infection (504), but the associated risks of increasing side effects and developing multidrug resistance were shortly emphasized (505). As the understanding and clarification of the HIV-1 replication cycle progressed, additional viral proteins started to be identified as potential new targets for antiretroviral drugs. This led to the discovery and development of the protease inhibitors (PI), the second class of anti-HIV-1 compounds to be employed for the treatment of HIV-1 infected individuals. This class of compounds acts though directly binding to the active site of HIV-1 protease, thus blocking virus maturation and the generation of new infectious viral particles (506). Shortly after, triple combination therapy consisting of a PI and two nucleoside RT inhibitors (NRTIs), ultimately referred to as highly active anti-retroviral therapy (HAART), was introduced as the standard of care for HIV-1+ patients in the developed world (507-511). Subsequently, a third class of anti-HIV-1 agents, the non-nucleoside RT inhibitors (NNRTIs) became available. NNRTIs are a structurally distinct
group of drugs that bind to the HIV-1 RT at a position other from the active site, causing conformational changes in the active site itself (512). Like PIs, NNRTIs have a synergistic effect when used in combination with two different NRTIs (506). Integrase inhibitors act by blocking the action of viral integrase and, since they target a distinct step in the retroviral life cycle, they may be taken in combination with other types of drugs to minimize adaptation by the virus. They are also useful in salvage therapy for patients whose virus has mutated and acquired resistance to other drugs. The first integrase inhibitor approved by the FDA was raltegravir, approved on October 12, 2007, which was shown to increase the efficacy of optimized background therapy (513).

Other treatment options that were recently introduced for the treatment of HIV-1 infection include two promising entry inhibitors, Enfuvirtide and Maraviroc. Enfuvirtide, or T20, is a fusion inhibitor that has been approved for use in treatment-experienced patients (514). It is a synthetic peptide that mimics amino acids 127-162 of HIV-1 gp41, a key domain involved in viral fusion with the cell membrane. Maraviroc, which was approved by the FDA in 2007, binds to CCR5 preventing interaction with HIV and CCR5-mediated signaling events (515).

The final class of antiretroviral drugs under development for the treatment of HIV-1-infected individuals are maturation inhibitors, whose prototype is bevirimat that targets the internal HIV-1-structural precursor Gag and its function in the final assembly of the mature infectious virus (516). Besides bevirimat, HIV-1 assembly and budding is currently being exploited as a new potential target for antiretroviral therapy. For instance, it is known that HIV-1 Vpu acts by restricting the antiviral activity of tetherin, an integral membrane protein that binds to fully formed virions thus retaining them on the surface of infected cells (517);
therefore, targeting Vpu-tetherin interaction may represent a new therapeutic target. Another interaction that it might be worth to try targeting is that of HIV-1 Vif with APOBEC3G, a DNA editing enzyme that exerts its antiviral activity by introducing C-to-U changes in the newly synthesized viral single stranded cDNA, thus promoting its degradation (518). Small inhibitors aimed at blocking this interaction may provide a novel therapeutic option.

1.4.2 Immune-reconstitution agents

As described above, multiple classes of antiretroviral drugs are now available and HAART regimens are effective in suppressing HIV-1 replication in the majority of patients. However, some patients still have a discordant response to HAART, in that viral load is suppressed, but CD4+ T-cell counts remain low and HAART alone may not be sufficient to completely restore the immune system. In these patients the use of immunomodulatory agents in addition to antiretroviral drugs could help to try to restore the CD4+ T-cell lymphopenia and therefore the immune functions. Current strategies have focused on immunomodulatory cytokines, such as interleukin-2 (IL-2), interleukin-7 (IL-7) and interleukin-15 (IL-15), in virtue of their central role in regulating T-cell proliferation and survival, to try maintain T-cell homeostasis (519). IL-2, IL-15 and IL-7 belong to the γ-chain family and their receptors share a common γ-chain, CD132, which is responsible for the signal transduction via activation of the JAK-STAT and PI3K pathways. Activation of the JAK-STAT pathway is associated with the induction of the anti-apoptotic protein Bcl-2, whereas activation of the PI3K pathway induces cellular proliferation (519). In addition to the common γ-chain, each of these receptors has one or two specific chains responsible for the binding with the cytokine and/or additional signaling pathways. Indeed, IL-2R is a
heterotrimer composed of CD132, a specific α-chain that binds IL-2 (CD125) and the IL-2/IL-15R β chain (CD122); IL-15R is composed of IL-15Rα, CD122 and CD132 and IL-7R is a heterodimer composed of IL-7Rα (CD127) and CD132 (519). Even if these cytokines share signaling pathways, however they have distinct effects, due to 1) unique signaling events, 2) different receptor expression on different T-cell subsets (Figure 1.10) and 3) variable levels of the cytokines in different compartments.

**IL-2**

IL-2 is secreted by activated CD4+ T cells and, although it can support the expansion of antigen-stimulated CD4+ and CD8+ T cells, its primary role is related to the regulation of regulatory T cells (Tregs) and to the maintenance of peripheral tolerance (520).

IL-2 has been used in HIV-1-infected individuals in several types of clinical trials: the earliest based on frequent high doses intravenous administrations and the more recent consisting of infrequent low doses subcutaneous administrations (521-530). Phase I studies of exogenous IL-2 in HIV-1 infection were initiated as early as 1983, before the availability of cART and since then, there have been at least 15 phase II studies employing various formulations of recombinant IL-2 (rIL-2). These trials demonstrated that treatment with IL-2 induces an increase in the absolute number of circulating CD4+ T cells, particularly in patients with pre-treatment CD4+ T-cell counts above 200 cells/μl in the absence of HAART, or below 200 cells/μl but in the presence of HAART, with no increases in CD8+ T-cell counts (521, 522, 525-528, 531-535). IL-2-driven CD4+ T-cell expansion seems to involve mainly naïve CD4+ T cells, and a population of CD4+ CD25+ T cells expressing intermediate levels of CD45RA and CD45RO (526, 527). Of note, this expansion was shown to be polyclonal (521). Moreover, in these studies, treatment with IL-2 was not
associated with any increase in CD8+ T-cell activation (525, 527, 530,). Albeit this positive immune-reconstitution effect, IL-2 administration was also associated with transient increases in the plasma viral loads, both in the absence and presence of HAART (521, 525, 526), as well as with severe side effects. Besides the consistent increase in CD4+ T-cell count observed in these studies, none of them were powerful enough in determining whether the increase in CD4+ T-cell count could be translated into a clinical benefit, although a trend towards a reduction in opportunistic infection resulted from a pooled analysis (536) and in a study in patients with advanced HIV (530). As a consequence of these non-conclusive results, it became clear that clinical endpoint studies were required in order to clarify whether CD4+ T-cell increase could be considered a reliable marker of a successful therapy, and to investigate the antiviral effects. Two phase III clinical studies of recombinant IL-2 administration in HIV-1-infected individuals, named SILCAAT and ESPRIT, were started back in 1999, with enrollment completed at 1695 and 4111 patients in 2002 and 2003, respectively (537). Both studies, besides documenting sustained increases in CD4+ T-cell counts of 19% (SILCAAT) and 26% (ESPRIT) in IL-2 and ART treated individuals, as compared to individuals treated with ART alone, failed to show any kind of clinical benefit. One explanation could be that the CD4+ T cells expanded by IL-2 do not have the same functionality as those depleted during the course of HIV-1 infection or those restored with ART (538). Alternatively, the predominant expansion of naive and central memory cells, without expansion of effector memory cells, could be the explanation for the lack of defense against OD pathogens, or it is possible that this expansion does not specifically fill in the deficits in the T-cell receptor repertoire. Finally, the CD4+ T cells expanded by IL-2, expressing intermediate levels of CD25 and the transcriptional regulator FoxP3, remind of
Tregs cells (527), and therefore they could have suppressor activity, even if additional data are needed to confirm this hypothesis. Despite the uncertainty regarding clinical efficacy of rIL-2, the French Government did allow access to IL-2 for the treatment of patients with CD4+ T-cell counts < 200 cells/μl on combined ART, an access scheme that, however, ceased in 2007. In a very recent study, intermittent IL-2 treatment was also shown not to have any effect on CD4+ T-cell depletion in the gut of HIV-1-infected individuals (539). In light of this “failure” concerning the use of IL-2 to try to improve the clinical outcome of HIV-1-infected individuals, alternative settings in which this cytokine could be useful are currently under investigation. The first is the potential use of IL-2 (with ART) to induce the re-activation and eventually the eradication of HIV from latently infected CD4+ T cells, in order to reduce the pool of viral reservoir (540-542), and studies showing reactivation of latent integrated virus in vitro with IL-2, together with clinical trials demonstrating lower changes from baseline plasma HIV-1 RNA in patients treated with IL-2 plus ART as compared to ART alone, may provide a rationale for this approach (536). However, preliminary studies designed to explore this theory so far failed to give definitive results (542). The second setting in which IL-2 treatment is being investigated is the role that this cytokine may have in delaying the start of continuous combination ART, and this strategy is based on the idea of avoiding ART for as long as the CD4+ T-cell compartment can be preserved. Two examples are the ANRS 119 study and the STALWART study (543, 544). The third potential use of IL-2 is the treatment with combined ART in order to delay the time for re-initiation of ART, an option explored in the TILT and in the ICARUS study (545, 546). Besides the therapeutic described so far, another field in which IL-2, as well as other γ-chain cytokines like IL-15 (see below), could find a place is therapeutic vaccination.
Indeed, these cytokines could be useful to increase T-cell levels and/or their function to boost HIV-1-specific T-cell responses to the vaccine (547). Overall, the main barrier to moving forward with this strategy seems to be, at least for now, the poor performance of these vaccines in controlling the viremia, even when T-cell responses are boosted (548).

**IL-15**

IL-15 exerts its function mainly in a membrane-bound isoform (549) expressed on a variety of cell types, including monocytes/macrophages, dermal fibroblasts, keratinocytes, epithelial cells neuronal cells, dendritic cells and T cells (550-555). It is primarily implicated in the expansion of CD4+ and CD8+ EM T cells, even if it was also shown to promote the survival and growth of NK and NK-T cells (556-558). Moreover, in T cells, IL-15 induces both a proliferative and differentiation signal, so that CM T cells not only proliferate, but also differentiate into EM T cells (559). Finally, IL-15 was shown to induce the homeostatic proliferation of CD4+ and CD8+ EM T cells in the periphery (560). The complexity of the IL-15/IL-15R system is further increased by the fact that IL-15Rα is often shed from the cells, and its soluble form can bind and sequester the active cytokine (558).

The immunologic impact of IL-15 administration *in vivo* during the chronic phase of the infection was tested in SIV-infected rhesus macaques, in which it was shown to induce the expansion of both NK and memory T cells, as well as of CD8+ EM T cells and CD4+ EM T cells with ART, with no effects on viral replication (559). IL-15 administration during the acute phase of SIV infection, however, increased viral set point by three logs, and in 30% of the animals it increased the rate of disease progression (561). Like IL-2, IL-15 is also being tested as an immune adjuvant for vaccination and several studies have been conducted in SIV-infected animals, although the usefulness of IL-15 treatment seems to be strictly
dependent on the virus used for the challenge, thus suggesting that its potential utility for HIV/SIV vaccine or immune-based therapies may be very limited (547).
1.5 IL-7

1.5.1 Phenotype and function of CD4\(^+\) and CD8\(^+\) T cells subsets

T-cell maturation occurs predominantly at the level of the thymus (from here the term "thymocytes" for T cells), where hematopoietic progenitors derived from hematopoietic stem cells of the bone marrow colonize and proliferate to generate a large population of immature thymocytes. The earliest thymocytes in the thymus do not express CD4 nor CD8, and are therefore called "double negative" (CD4\(^-\)CD8\(^-\)), whereas, as they progress through their development, they become "double-positive" (CD4\(^+\)CD8\(^+\)) first, and subsequently "single-positive" (CD4\(^+\)CD8\(^-\) or CD4\(^-\)CD8\(^+\)) before being released into peripheral tissues. The thymus provides an inductive environment that allows for the development and selection of physiologically useful T cells, which occur through the processes of beta-selection, positive selection, and negative selection. These processes shape the population of thymocytes into a peripheral pool of T cells able to respond to foreign pathogens and immunologically tolerant towards self antigens. These cells are called "naïve", and are mature T cells that have not encountered their cognate antigen within the periphery yet.

T-cell maturation was once thought to occur entirely within the thymus, but nowadays consensus exists about the fact that the youngest peripheral T cells, termed recent thymic emigrants (RTEs) comprise a distinct population from their more mature, yet still naïve counterparts (562). Indeed, RTEs undergo a process of post-thymic maturation, involving both phenotype and immune function, that ensures T-cell fitness and self tolerance (562). RTEs constitute a considerable proportion of the T-cell pool in neonates, infants and young adults (563), and they also maintain T cell receptor (TCR) repertoire diversity in
young and middle-aged adults to allow for recovery from lymphopenia (564). Mature naïve T cells, typically expressing L-selectin (CD62L) and lacking the expression of activation markers (CD25, CD44 or CD69) and of CD45RO (565), constantly recirculate through peripheral lymphoid organs, particularly lymph nodes (they also express CCR7 for lymph-node homing) (566), where, eventually, they encounter antigen-presenting cells (dendritic cells and macrophages). In the naïve state, T cells are thought to be quiescent and non-dividing, requiring the common-gamma chain cytokines IL-7 and IL-15 for homeostatic survival mechanisms. Indeed, they also express functional IL-7 receptor, consisting of IL-7 receptor-α, CD127, and common-γ chain, CD132.

In peripheral lymphoid organs, naïve T cells can respond to novel pathogens that the immune system has not yet encountered. In the cortex of lymph nodes, T cells that do not encounter their specific antigen leave the lymph node and return to circulation; by contrast, T cells that encounter their specific antigen on antigen-presenting cells are activated to proliferate and differentiate into effector cells (phenotype: CD25+ , CD44+ , CD62Llow, CD69+ , CD45RO+). Recognition by a naïve T cell clone of its cognate antigen results in the initiation of an immune response. The proliferation and differentiation of activated T cells depends upon the production of cytokines, such as IL-2, and its binding to the high affinity receptor (CD25) expressed on the surface of activated T cells. Armed effector T cells mediate a variety of functions, including killing of infected cells by CD8+ cytotoxic T cells and the activation of macrophages by CD4+ T\textsubscript{H}1 cells and of B cells by CD4+ T\textsubscript{H}1 and T\textsubscript{H}2 cells.

T\textsubscript{H} cells translate antigen stimulation to tissues and instruct tissue cells to increase immune responses and the type of response depends on the properties of the T cells that
interact with the tissue. In addition to T\textsubscript{H1} and T\textsubscript{H2} cells, also T\textsubscript{H17} and regulatory T (Treg) cells are known to play important roles. Each T\textsubscript{H} cell subset secretes a characteristic set of cytokines. Thus, T\textsubscript{H1} and T\textsubscript{H2} typically produce IFN-\textgreek{y} and IL-4, whereas IL-17A is specifically produced by the T\textsubscript{H17} subset and coordinates tissue inflammation through induction of proinflammatory cytokines and chemokines, such as IL-6, TNF-\textalpha, and IL-8 (567). Consistently, T\textsubscript{H17} cells were shown to play a key role in many autoimmune disorders, such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and inflammatory bowel disease (567). Another cytokine that is known to be produced by T\textsubscript{H17} cells is IL-22, whose function has not been fully clarified yet. Recent studies have determined that some T cells express IL-22 independently of IL-17, and these cells were named T\textsubscript{H22} (568). T\textsubscript{H22} are present within the epidermal layer in inflammatory skin diseases and seem to be involved in epidermal immunity and remodeling (569).

Tregs, sometimes referred to as suppressor T cells, are a subpopulation of T cells that contribute to the downregulation of the immune system, the maintenance of the tolerance to self-antigens, and the downregulation of autoimmune diseases. Several types of regulatory cells do exist, some of which are induced in response to infectious challenge (the so-called inducible Treg) and some that are considered natural regulators (natural occurring Treg) (570). Inducible Tregs cells can develop from conventional CD4\textsuperscript{+} T cells that are exposed to specific stimulatory conditions such as the blockade of costimulatory signals, deactivating cytokines or drugs (570). By contrast, natural Treg cells arise during the normal process of maturation in the thymus and survive in the periphery as Treg cells. Natural Tregs express a specific set of markers like CD25, the T cell inhibitory receptor CTLA-4 and the glucocorticoid-inducible tumor necrosis factor receptor (GITR). The unique transcription
factor Foxp3 is required for the generation of natural Treg cells, and this represents their most specific marker identified so far (571). Natural Treg cells can respond to a large variety of self antigens, and they may also respond to antigens expressed by microbes, whereas inducible Treg cells may control various infectious processes (572).

A primary adaptive immune response to an infection serves not only to clear the primary infection from the body, but also to provide protection against a second reencounter with the same pathogen, in a process that is called protective immunity. One of the most important consequences of the adaptive immune response is the immunological memory, generally defined as the ability of the immune system to respond more rapidly and effectively to pathogens that have been previously encountered, and reflects the pre-existence of a clonally expanded population of antigen-specific lymphocytes.

Memory T cells are heterogeneous in terms of both homing capacity and effector functions and they can be broadly classified in central memory (T_{CM}) and effector memory (T_{EM}) (573). According to the model proposed by Lanzavecchia and Sallusto, T_{EM} migrate to inflamed peripheral tissue and display immediate effector function, whereas T_{CM} home to the T-cell areas of secondary lymphoid organs and have little effector function, but immediately proliferate and differentiate in effector cells in response to antigen stimulation (574). T_{CM} are CD45RO+ cells that constitutively express CCR7 and CD62L, two receptors that are also expressed by naïve cells, and are necessary for extravasation and migration to the T-cell areas of secondary lymphoid organs (575, 576). Following TCR triggering they produce IL-2 and proliferate and differentiate into effector cells which in turns produce high amounts of IFN-γ and IL-4. T_{EM} cells are memory cells that have lost the expression of
CCR7 and are heterogeneous for CD62L. As compared to T_{CM}, they are characterized by a more rapid effector function, as they produce high amounts of IFN-\( \gamma \), IL-4 and IL-5 within hours after stimulation. Some CD8\(^+\) T_{EM} express CD45RA (T_{EMRA}) and carry the largest amount of perforin. The distribution of T_{CM} and T_{EM} in the body varies according to the CD4\(^+\) and CD8\(^+\) T-cell compartment and to the tissues: T_{CM} are predominant in CD4\(^+\) T cells and T_{EM} in CD8\(^+\) T cells, and T_{CM} are enriched in lymph nodes and tonsils, whereas T_{EM} are predominant in lung, liver and gut (577). In antigen-primed individuals, antigen-specific T cells are detected in both T_{CM} and T_{EM} compartments, although the relative proportion can be extremely variable according to the condition. For instance, HIV-1-specific T cells are largely T_{EM} (CD45RA\( ' \)CCR7\( ' \)) (578).

In the body several subsets of T_{CM} and T_{EM} cells with distinct functional properties exist, and can be identified according to the expression of surface molecules, being them costimulatory molecules, such as CD27 and CD28, or chemokine receptors, like CCR4 or CCR5, which can be expressed at variable levels in CD4\(^+\) and CD8\(^+\) T cells (Figure 1.11). For instance, the simultaneous expression of CLA and CCR4 identifies skin-homing T cells (579), whereas \( \alpha_4\beta_7 \) and CCR9 characterize gut-homing cells (580). As compared to naïve T cells, memory T cells show low-activation threshold and vigorous proliferation, with the expansion potential progressively decreasing from T_{CM} to T_{EM} and T_{EMRA} (581, 582).

According to the model proposed by Lanzavecchia A. and Sallusto F., it is the strength of the signal delivered by TCR and cytokine stimulation that determines proliferation and differentiation of T cells, driving them through hierarchical thresholds of differentiation, which follows a sequence of proliferation, acquisition of fitness, effector
function and death (Figure 1.12) (583). T cells receive TCR and cytokine stimulation through stochastic interactions with dendritic cells at different levels and, therefore, they reach different levels of differentiation. At low signal levels naïve T cells proliferate but do not acquire effector functions and they retain lymph node-homing capacity. By contrast, at thigh signal strength and in the presence of costimulatory cytokines that polarize differentiation, T cells loose lymph node-homing capacity and acquire effector functions and the capacity to migrate to inflamed peripheral tissues. At even higher signals, T cells undergo activation induced cell death (AICD). At the end of the antigen stimulation phase, activated T cells are selected for their capacity to survive in the presence of homeostatic cytokines: "unfit" cells die by neglect, whereas "fit" cells that home to the appropriate tissue survive as memory T cells. After the proliferative burst most of the effector cells die, but a minor fraction persists as long-term memory T cells that continue to divide slowly in the absence of antigen. Evidences suggest that $T_{CM}$ derive from cells stimulated with sub-threshold amounts of antigen and they are arrested at an intermediate stage of differentiation, retaining lymph node-homing capacity and with low effector functions, that precedes $T_{EM}$; by contrast, $T_{EM}$ seem to arise directly from effector cells (Figure 1.12).

Very recently, a novel human memory T cell subset with stem cell-like properties has been identified (584). These stem cell-like memory T cells ($T_{SCM}$) are long-lived memory T cells that have an enhanced capacity of self-renewal and a multipotent ability to derive central memory, effector memory and effector T cells. They were found within the naïve T cell compartment ($CD45RO^{+}CD45RA^{+}CD62L^{+}CD27^{+}CD28^{+}IL-7R^{+}$), but they also express markers that are typical of memory T cells, like CD95, IL-2Rβ, CCR3 and LFA-1, as well as numerous functional attributes distinctive of memory T cells. The hypothesis
suggested by the authors is that T_{SCM} are a clonally expanded primordial memory subset arising after antigen stimulation with increased proliferative and reconstitutive capacity.

1.5.2 IL-7 in Physiology

IL-7 is the only cytokine of the gamma-chain family to be constitutively produced, instead of being secreted upon stimulation, and low levels of IL-7 are constantly detectable in the human serum (585). IL-7, initially identified as a B-cell growth factor (586), was shortly after recognized as a key factor also for the development and homeostasis of both thymic and peripheral CD4^{+} and CD8^{+} T cells (587), particularly on the naïve and memory compartments. It is produced by stromal tissues, including the bone marrow, as well as by thymic epithelial and intestinal epithelial cells (588). In the absence of perturbation, the number of T cells in the peripheral lymphoid compartment is strictly controlled through several mechanisms, including death, survival and turnover, and IL-7 in this context plays an essential role, also mediated by a feedback control mechanism by which IL-7 stimulation downregulates the expression of IL-7Rα (CD127) (589), to ensure that the cells that have already responded to the cytokine do not compete with those that have not encountered it yet. After the first encounter with an antigen, naïve T cells become activated and undergo several rounds of expansion differentiating into effector cells that produce IL-2, which in turns downregulates IL-7Rα expression (590). This ensures IL-7- independence. At the end of the immune response, T cells progress to a resting state with upregulation of IL-7Rα expression, which promotes the differentiation into memory and their subsequent survival (591-593) (Figure 1.10). IL-7 also contributes to the maintenance of the CD4^{+} and CD8^{+} memory T-cell pools, by supporting their “homeostatic” division in the absence of antigen
stimulation (592). In lymphopenic hosts IL-7 was shown to support the division of both CD4⁺ and CD8⁺ T cells. It seems that the regulation of the survival and proliferative effects of IL-7 is mediated by both IL-7Rα expression and IL-7 concentration. IL-7Rα is expressed at high levels on resting cells, to ensure that the physiological IL-7 concentration (2.7 pg/ml in serum (594)) supports the survival of naïve and memory T cells but not of effector cells, which do not express the receptor, or express it at much lower levels (591). The dose of IL-7 is also crucial for discriminating between proliferation and survival: at low doses receptor-expressing cells respond inducing a pro-survival signal, whereas when the concentration increases, the same cells can undergo proliferation and division, probably through the synergy with TCR stimulation (Figure 1.13) (595). Thus, in lymphopenic conditions CD4⁺ and CD8⁺ memory T cells, as well as naïve T cells albeit to a lesser extent, expand in an L-7-dependent manner, probably as an effect of the increased availability of the cytokine, together with de novo cytokine synthesis (585).

1.5.3 IL-7 in HIV/SIV infection

Several lines of evidence suggest that the IL-7/IL-7R system is impaired in HIV-1-infected individuals, as well as in SIV-infected macaques. Indeed, virus-induces T-cell lymphopenia is accompanied by increased levels of plasma IL-7, which correlate inversely with CD4⁺ T-cell counts and can be normalized after ART treatment has restored the CD4⁺ T-cell subpopulation (585, 596-599); moreover, pre-HAART plasma IL-7 levels were shown to correlate with long-term CD4⁺ T-cell increase after treatment (600). The augmented IL-7 levels seem to be mediated by increased production by dendritic-like cells within the peripheral lymph nodes and by peripheral lymphocyte-depleted lymphoid tissue,
and was proposed as a compensatory mechanism of the body to try to counteract the virus-induced lymphopenia (585). A recent longitudinal study, showing that in patients with low or moderate immunodeficiency CD4+ T-cell counts and IL-7 levels do not evolve in parallel either in the absence or presence of HAART, suggest that factors other than CD4+ T-cell count might contribute to the upregulation of plasma IL-7 in HIV-1-infected individuals (601). Other studies documented reduced IL-7Rα expression on all subsets of CD4+ and CD8+ T cells from HIV-1-infected individuals, correlated with disease progression and restored upon ART treatment, thus suggesting that the increased production of IL-7 to promote homeostatic rebalance may be ineffective, in the absence of therapy (602-608). Indeed, recently, mathematical modeling demonstrated that the normalization of plasma IL-7 levels following ART can be explained by changes in the receptor expression: these data indicate that T-cell restoration after ART is driven predominantly by CD127+ T cells and that the observed decreases in serum IL-7 levels following ART can be simply due to improved CD127-mediated clearance (609). A soluble form of IL-7Rα (sCD127), derived from shedding of CD127 from CD8+ T cells (610), was found to be increased in the plasma of HIV-1-infected individuals as compared to uninfected control subjects, and was recently proposed as an explanation for the reduced membrane-bound expression of CD127 observed in HIV-1-infected individuals, as well as for the lack of effect of IL-7, which would be sequestered by the soluble receptor, thus limiting its bioavailability (611). However, other studies reported lower or equal levels of soluble IL-7Rα in the serum of HIV-1-infected patients (612), thus indicating that this field needs a more extensive investigation. An alternative explanation for lower CD127 expression was recently proposed by Faller et al.,
showing that soluble HIV-1 Tat protein promotes the internalization of CD127 and subsequently targets it for degradation via the proteasome (613).

Functional defects of the IL-7R were also reported for T cells from HIV-1-infected individuals, which may contribute to explain the lack of effect of the IL-7-mediated compensatory response. These include decreased IL-7 binding capability and abnormal activation of the JAK/STAT pathway (614), aging of the cells (615), decreased induction of Bcl-2 and CD25 expression and impaired induction of cellular proliferation (616). Finally, a recent study showed that IL-7 failed to enhance antigen-mediated cellular proliferation on CD8⁺ memory T cells derived from HIV-1-infected individuals, indicating that the effect of IL-7 in the secondary immune responses is impaired in memory CD8⁺ T cells from HIV-1-infected individuals, thus contributing to the loss of CD8⁺ T-cell function observed in HIV-1 infection (617).

1.5.4 Pre-clinical Studies with IL-7

Pre-clinical studies in macaques showed that IL-7 treatment dramatically alters peripheral T-cell homeostasis, of both naïve and non-naïve phenotype, in healthy and T-cell depleted animals (618-620). In these studies naïve as well as memory T-cells increased within days of IL-7 administration, and the number of recent thymic emigrants, measured by the TCR recombination excision circle (TREC) values, decreased, suggesting that peripheral rather than central effects play a major role. In a complementary experiment with cynomolgus macaques, IL-7 was also shown to lead to the expansion of all T-cell subsets, with a subsequent dilution of newly generated T-cells, confirming that homeostatic proliferation, rather than thymic effects, are responsible for the IL-7 effects (618). The same
hypothesis was also suggested in another study of IL-7 administration to baboons after autologous CD34 cell transplantation (621). Nukeyre et al. demonstrated that IL-7 induces increases in the number of CD4+ and CD8+ T-cells in both infected and uninfected animals, persisting over several weeks, and returning to baseline by 11 weeks (620). Importantly, in this and other studies, IL-7 did not increase the viral loads during the chronic phase of SIV infection, even in the absence of ART, and did not seem to promote B-cell expansion nor tumorigenesis (618, 620, 622). In another study with uninfected macaques, IL-7 induced T-cell proliferation in a dose-dependent manner, and led to a marked phenotypic conversion of naïve T-cells to memory phenotype and function (619). Remarkably, these T-cells reverted back to naïve phenotype after IL-7 therapy withdrawal (619), suggesting that transient treatment did not convert these cells to the memory fate. Recent data suggest that the proliferative and pro-survival effects of IL-7 strictly depend upon dosing intervals, which therefore need to be carefully planned during the design of a study according to the desired effects (623). Recently, IL-7 was also shown to stimulate SIV-specific CD8+ T-cell responses in SIV-infected rhesus macaques, thus suggesting another possible useful effect of IL-7 treatment (624).

1.5.5 Clinical Studies with IL-7

The first five studies of rhIL-7 in humans evaluated an E. Coli produced, non-glycosylated rhIL-7 recombinant human IL-7 from Cytheris (France), CYT99007. Two trials involved oncology subjects, one trial involved a subject following allogeneic transplantation for nonlymphoid malignancy and two trials involved HIV+ subjects.
The first two clinical trial involving rhIL-7 were dose escalation studies (3, 10, 30, 60, and 100 μg/kg) of 8 repeated IL-7 administrations, covering respectively two and three weeks, to subjects with refractory malignancies (625, 626). In both studies rhIL-7 was shown to mediate selective increases in circulating CD4+ and CD8+ lymphocytes, and decreases in Treg cells. Moreover, rhIL-7 induced in vivo T cell cycling, Bcl-2 up-regulation and T cell expansion, causing a significant broadening of circulating TCR repertoire diversity with a preferential expansion of naive T cells, including recent thymic emigrants (RTEs) (626).

The study of IL-7 administration following allogeneic transplantation has involved so far one single patient and the results have not been published (627, 628).

The first study on HIV-1-infected individuals was ACTG 5214, a multicenter phase 1, placebo-controlled double-blinded study with a 3:1 randomization (rhIL-7: placebo) for each dose level in 2 strata according to plasma HIV-1 RNA levels at screening (stratum 1: < 50 copies/ml, stratum 2: 50-50000 copies/ml) (629). Participants, with CD4+ T-cell counts higher than 100 cells/μl and plasma HIV-1 RNA lower than 50000 copies/ml on HAART for a minimum of 12 months, received a single subcutaneous dose of rhIL-7 or placebo in a consecutive dose escalation design (3, 10, 30, 60, and 100 μg/kg) on day 0 and were then monitored for toxicity on days 1, 4, 14, and 28 with an additional visit on day 56. Results have documented beneficial effects of IL-7 treatment, which was tolerated for up to 30 μg/kg, with induction of peripheral T-cell cycling in both CD4+ and CD8+ T cells, as well as of antigen specific CD8+ T cells, and transient increases in the absolute numbers of circulating CD4+ and CD8+ T cells, particularly of the memory phenotype. Interestingly, rhIL-7 administration was associated with only limited or no increase in the levels of virus.
replication. Recent data on sequencing of the viruses detected during episodic blips following IL-7 administration indicate that these viruses are similar to those found in the plasma and in PBMC before treatment (630). This suggests that the low level of viremia induced by IL-7 likely reflects transient induction or release of virus from a preexisting pool rather than activation of silent quasispecies. The second study of IL-7 administration in HIV-1 infection was a prospective open-label multicenter phase I/IIa trial, conducted on 14 HIV-1-infected individuals with a CD4+ T cell count of 100–400 cells/μl and plasma HIV-1 RNA levels lower than 50 copies/ml for at least 6 mo while receiving cART for at least 12 months, who received subcutaneous injections of rhIL-7 every other day for a total of 8 doses over 16 days. Visits for safety and immunological efficacy were made at 7, 14, 21, 28, and 35 days and 12 weeks. An extended follow-up period lasted until 48 weeks (631). Six patients received a dose of rhIL-7 of 3 μg/kg and the remaining 8 patients were treated with 10 μg/kg. Results showed that IL-7 administration was clinically and biologically well tolerated, and induced significant increases of CD4+ and CD8+ T cells in a dose-dependent manner, which were sustained for up to 45 weeks after treatment interruption. Phenotypic analysis of CD4+ and CD8+ T-cell subsets revealed that naive and central memory T cells were preferentially increased by IL-7 treatment in both subsets and that increases were not associated with increased immune activation but rather with increased T-cell cycling. Injection of rhIL-7 also expanded CD4+ and CD8+ T cells following polyclonal and antigenic stimulation. In the group of patients treated with the higher dose (10 μg/kg), transient increases were detected in plasma HIV-1 RNA, but not in the content of viral DNA in CD4+ T cells, suggesting that IL-7 treatment did not directly affect the cellular HIV-1 DNA content. This clinical study, the first of repeated IL-7 administration in chronically
lymphopenic HIV-1-infected patients, provided evidence that rhIL-7 therapy is better tolerated as compared to other immunotherapeutic strategies, thus giving good hopes for further investigation.

A second-generation glycosylated rhIL-7 made by Cytheris is CYT107, produced via a recombinant mammalian cell culture system. This new glycosylated rhIL-7 is currently being tested in several Phase II clinical trials for the treatment of HIV-1-infected individuals (INSPIRE, INSPIRE 2 and INSPIRE 3 and EraMune 01), actually ongoing (see Cytheris website: IL-7 HIV Trials). INSPIRE studies involve patients with variable peripheral $CD4^+$ T-cell counts (two ranging from 101 and 400 cells/mm$^3$ and the third above 500 cells/mm$^3$) and suppressed HIV viremia (HIV-1 RNA< 50 copies/ml) following HAART. Interim data released from the INSPIRE study at week 12 of the study were presented at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) held September 12-15, 2009, in San Francisco, CA (Abstract H-1230a) and published in “AIDS Patient Care and STDs” in 2009 ((632) and Drugs.com News Clinical Trials). INSPIRE is a Phase I/IIa study randomized placebo controlled, single-blinded multicenter (Europe, United States, and Canada) dose-escalation study of subcutaneous intermittent injections of glycosylated rhIL-7 (CYT107) in chronically HIV-1-infected patients with $CD4^+$ T lymphocyte counts between 101 to 400 cells/mm$^3$ and plasma HIV-1 RNA<50 copies/ml. These patients were categorized as immunological low or non responders (INR), meaning patients who have not optimally restored their immunity despite at least 12 months of HAART and with complete control of viral replication. Three doses were tested: 10, 20, or 30 $\mu g/kg/week$ (8 patients CYT107 versus 2 placebo per dose). Results show a dose dependent and sustained increase of total $CD4^+$ T cells (>500 cells/mm$^3$), as well as of naïve
CD4+ T cells, and CD8+ T cells and a trend toward higher thymic output at the dose of 20 μg/kg, with a transient increase of plasma HIV-1 RNA in 4 patients. No clinical or laboratory side effects higher than grade 2 were recorded. These data, although preliminary, clearly indicate the potential of this cytokine for the treatments of HIV-1-infected individuals with low CD4+ T-cell counts despite HAART, and justify its further clinical development.

EraMune 01 is a Phase II international, multicenter, randomized, non-comparative controlled study of therapeutic intensification plus immunomodulation in HIV-1-infected patients with long-term viral suppression, designed to test the effect of rhIL-7 together with ART intensification (cART + raltegravir and maraviroc) on HIV-1 viral reservoirs. This trial arises from the observation that in previous studies IL-7 in combination with cART has been demonstrated to induce a certain extent of HIV-1 replication, and is based on the hypothesis that by combining the antiretroviral drugs, with an immunomodulating agent capable of targeting or inducing activation of latently infected cells, the reservoirs of HIV-1 could possibly be decreased and, in the best case scenario, eradication of the virus may be possible (ClinicalTrials.gov).
1.6 Aims of the current study

In the context of innovative therapeutic strategies based on the use of immunomodulatory agents, as a complement to ART, to try to restore the HIV-1-induced CD4+ T-cell lymphopenia, the potential effects of IL-7 treatment are currently being evaluated. As extensively described in paragraph 1.5 of this thesis work, several pre-clinical and clinical studies have documented a positive effect of in vivo IL-7 administration to SIV-infected macaques and HIV-1-infected individuals on the CD4+ and CD8+ T-cell pools. More in details, sustained increases in the absolute numbers of naïve and memory CD4+ and CD8+ T cells in the peripheral blood have been observed during and following treatment. The effects of IL-7 seem to be mediated by both induction of cell cycling and proliferation and reduction of apoptosis. Although protection from apoptosis could be very useful to preserve the CD4+ T-cell pool that is severely depleted during the course of HIV/SIV infection during both the acute and chronic phases, induction of cellular proliferation, particularly on CD4+ T cells, is looked at skeptically, as this could on one hand increase the pool of cells target for viral infection, and on the other hand it could induce the reactivation of latent proviruses. Therefore, we could postulate that the “safety” and efficacy of IL-7 treatment strictly depends upon a fine modulation of this “dual” effect.

The overall aim of the current study was to investigate the effects of IL-7 on spontaneous apoptosis in HIV/SIV infection ex vivo, on T cells freshly isolated from HIV-1-infected individuals (CHAPTER TWO), and in vivo, during the acute phase of SIV infection in rhesus macaques, the pathogenic animal model for AIDS (CHAPTER THREE).

 Increased spontaneous apoptosis ex vivo have been documented on T cells from HIV-1-infected individuals as compared to uninfected control subjects, and the levels of apoptosis
were shown to correlate inversely with the CD4+ T-cell counts of the patient *in vivo*, emphasizing the relevance of apoptosis in disease progression. Thus, we designed a study aimed at evaluating the effects of IL-7 *ex vivo* on the survival of T cells derived from HIV-1-infected individuals and cultured *in vitro* for several days in the absence of additional pro-survival stimuli. Specific aims of this part of the study were also to correlate the pro-survival effect of the cytokine with immunological and virological parameters and to discriminate between anti-apoptotic and proliferative effects, to eventually find an ideal concentration of the cytokine at which T cells could be preserved without inducing high levels of cell cycling and proliferation.

The results of the *ex vivo* studies are described in CHAPTER TWO and provided a strong rationale for testing the protective effects of IL-7 *in vivo*, during the acute phase of SIV infection in rhesus macaques (described in CHAPTER THREE). Indeed, several studies had previously evaluated the consequences of IL-7 administration to both SIV-infected macaques and HIV-1-infected individuals during the chronic phase of the infection, documenting sustained increases in both CD4+ and CD8+ circulating T cells mediated by the induction of cellular proliferation and the reduction of apoptosis (outlined in paragraph 1.5 in this thesis work). However, no previous studies have investigated the effects of IL-7 administration during the acute phase of SIV infection. Specific aims for this second study were to evaluate the effect of IL-7 on various CD4+ and CD8+ T-cell subpopulations, with a particular focus on naïve and memory CD4+ T cells that are typically depleted within the very first weeks after infection, on viral replication, on humoral and cellular responses to the virus and, eventually, on disease progression.
To summarize, the main objective of this study was to evaluate the immunomodulatory effects of IL-7 in HIV/SIV infection, with a particular focus on the pro-survival properties of this cytokine. An extensive investigation of IL-7-mediated effects during both the acute and chronic phases of the infection could lead on one hand to a deeper understanding of the pathogenesis of the disease and on the other hand to designing new regimens of immune-based therapeutic strategies.
1.7 Figures and Figure Legends

Figure 1.1 *Global prevalence of HIV, 2009*. Map showing the distribution and frequency of HIV-1/AIDS in different regions of the globe relative to the total number of HIV-1 infections worldwide for 2009. Source: “UNAIDS REPORT ON THE GLOBAL AIDS EPIDEMIC | 2010”.

Figure 1.2 *Current Global Distribution of HIV-1 Subtypes and Recombinant Forms*. Map showing the distribution of HIV-1 subtypes and Circulating Recombinant Forms (CRFs) at the global level. (From Taylor et al. N Engl J Med 2008; 358:1590).
Figure 1.3 Overview of the HIV-1 life cycle. The infectious life cycle begins when the HIV-1 gp120 glycoprotein binds to the CD4 receptor on the surface of helper T cells, macrophages or dendritic cells. This interaction induces conformational changes in the core region of gp120, leading to unmasking of the chemokine co-receptor (CCR5 or CXCR4) binding site. Further interaction with CCR5 (or CXCR4) leads to further conformational changes, activation of gp41 and virus fusion with the cell membrane (1.). The viral core is then inserted into the cytoplasm where it is reverse-transcribed into cDNA (2.) before being transported to the nucleus and integrated into the human genome, a process that is catalyzed by HIV-1 integrase (3.). Transcription of the integrated proviral DNA leads to the production of multiply, singly and unspliced mRNAs that are then transported back to the cytoplasm where they are translated and processed into HIV-1 structural and accessory proteins (4.). Env and immature precursors of the capsid (Gag) and viral polymerase (Pol) are transported to the cell membrane and assembled into HIV-1 virions (5.). Virus infectivity is acquired after Gag and Gag-Pol cleavage, particle maturation and budding from the cell surface (6.). (Adapted from Monini et al. Nat Rev Cancer 2004;4:861).
Figure 1.4 Typical course of HIV-1 infection. Schematic representation of the typical course of HIV-1 infection showing changes in CD4+ and CD8+ T-cell counts in peripheral blood and plasma viral load. (From Munier et al. Immunol and Cell Biol, 2007; 85: 6–15).

Figure 1.5 Diagrammatic representation of T-cell dynamics in an individual with chronic HIV-1 infection. Resting cells along the bone marrow axis to naïve T cells are shown in yellow, activated T cells in green, resting memory T cells in blue, dying cells in black, HIV-1-infected cells in red. The red arrows depict negative effects of HIV-1 on cell production and survival, including destruction of the lymphoid organs architecture. The green arrows depict HIV-specific and bystander HIV-1-induced T-cell activation. (From Douek et al., Annu Rev Immunol, 2003; 21:265–304).
Figure 1.6 **Schematic representation of gut-associated lymphoid tissue (GALT).** Peyer's patches and isolated lymphoid follicles (ILFs) are composed of a specialized follicle-associated epithelium (FAE) containing M cells, a subepithelial dome (SED) rich in dendritic cells (DCs), and B-cell follicle(s) that contain germinal centres (GCs), where follicular B cells efficiently undergo class-switch recombination (CSR) and somatic hypermutation (SHM). The diffuse tissues of the lamina propria contain a large number of IgA⁺ plasma cells, T and B cells, macrophages, dendritic cells (DCs) and stromal cells (SCs). IgA⁺ B cells and plasma cells are shown in red, IgG⁺ cells in blue and IgM⁺ cells in pink. (From Fagarasan S. and Honjo T., Nat Rev Immunol 2003 Jan;3(1):63-72).

Figure 1.7 **Mechanistic link between mucosal functionality and systemic levels of immune activation in HIV/SIV infection.** Right: during pathogenic HIV/SIV infections, CD4⁺ T-cell depletion and chronic levels of immune activation (IA) result in a loss of mucosal immune function and breakdown of the mucosal barrier, thus resulting in microbial translocation. Microbial products in turns may cause a toll-like receptor-mediated broad immune system activation, with consequent activation-induced cell death of bystander lymphocytes. Left: despite similar levels of CD4⁺ T-cell depletion, in the context of normal IA, natural SIV host preserve mucosal immune functions and show no microbial translocation. (From Paiardini M. et al. AIDS Rev. 2008;10:36-46).
Figure 1.8 Overview of extrinsic and intrinsic apoptotic pathways. Arrows indicate positive effects, whereas block arrows indicate inhibition. Extrinsic apoptotic pathway with death receptors on the surface of the cell and intracellular mediators of the cascade which activates the mediator pro-caspase 8 is shown. The intrinsic pathway initiated by intracellular stress and leading to the release form mitochondria of CytC, which in turns activates the mediator pro-caspase 9 is also shown. Both pathways converge on the activation of the effector caspases 3, 6 and 7. (From Cell Signaling Technology website).
Figure 1.9 Potential synergy between low immune activation and reduced CCR5 expression in preserving CD4+ T cell homeostasis in SIV-infected SMs despite high virus replication. This figure shows how, in SIV-infected SMs (natural hosts), low immune activation, expressed as the fraction of CD4+ T cell clones that undergo activation at any given time, may act in concert with reduced and/or delayed expression of CCR5 in promoting a steady state where immune system homeostasis is preserved despite high virus replication. The top row shows how in HIV-infected humans and SIV-infected RMs, presence of a high fraction of activated CD4+ T cell clones results in the rapid accumulation of CD4+ T cells expressing CCR5 that are infected and killed by the virus, resulting in the disruption of the homeostasis of these activated clones. In SIV-infected SMs (bottom row), a smaller fraction of CD4+ T cells clones are activated, but a delayed and reduced expression of CCR5 on these cells may allow for their accumulation, resulting in an equally high level of virus replication when CCR5 is finally expressed. In this case, however, the homeostasis of fewer CD4+ T cells clones is disrupted at any given time. (From Silvestri G. et al. J Clin Invest. 2007 Nov;117(11):3148-54).
Figure 1.10 Differential role of γ-chain cytokines on T cells. Receptor expression and cytokine availability dictate the effects of common γ-chain cytokines on T cells. Naïve CD4 and CD8 T cells express high levels of the IL-7R and low levels of the receptors for IL-2, -4 and -15. On T-cell activation through antigen stimulation of the TCR, IL-7R expression reduces, whereas receptor expression for IL-2, -4 and -15 increases. On clearance of antigen and the formation of a stable memory population, cytokine receptor expression returns to that seen on the naïve population. (From Bradley, L. M. et al. Trends Immunol. 2005 Mar;26(3):172-6).

Figure 1.11 Phenotypic heterogeneity of human memory T cells. Expression of various cell surface markers on T\textsubscript{CM} and T\textsubscript{EM} CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and on T\textsubscript{EMRA} CD8\textsuperscript{+} T cells. (From Lanzavecchia, A. and Sallusto F., Annu Rev Immunol. 2004 22:745-763).
Figure 1.12 Signal strength model for T cell differentiation and memory T cell generation. The duration and intensity of antigenic stimulation is indicated by the length and thickness of the solid arrows. Antigen-independent events leading to T-cell proliferation and differentiation are indicated by the dotted lines. AICD, activation-induced cell death. (From Lanzavecchia, A. and Sallusto F., Annu Rev Immunol. 2004 22:745-763).

Figure 1.13 Role of IL-7 and TCR signaling in regulating T-cell responses. At low doses of IL-7, CD4+ T cells are induced to survive but not divide, whereas at higher doses, division is also induced. Evidence suggests that signals to the TCR synergize in the induction of division. T-cell division in lymphopenic hosts is likely to be due to higher IL-7 levels, probably as a result of the deletion of IL-7R-expressing lymphocytes. (From Bradley, L. M. et al. Trends Immunol. 2005 Mar;26(3):172-6).
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CHAPTER TWO

Primary cells in vitro

Effect of IL-7 on Spontaneous Apoptosis of CD4\(^+\) and CD8\(^+\) T cells

2.0 Introduction

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3.0 Introduction

Infection with human immunodeficiency virus type 1 (HIV-1) causes a progressive depletion of CD4+ T cells that ultimately leads to AIDS (1). Several mechanisms have been implicated in the loss of CD4+ T cells, including direct virus-induced cytolysis, defective T-cell regeneration, anergy, and apoptosis (2). Both CD4+ and CD8+ T cells derived from HIV-infected individuals show an increased propensity to undergo spontaneous apoptosis both in vivo (3-5) and after short-term ex vivo culture (6). Remarkably, the vast majority of the cells that undergo apoptosis are uninfected (5). Similar observations were made in macaques infected with simian immunodeficiency viruses (SIV) (7, 8). The level of apoptosis in HIV-1 infected individuals was shown to correlate with the levels of circulating CD4+ T lymphocytes and stage of disease (9, 10), reinforcing the concept that apoptosis may be one of the primary mechanisms of CD4 depletion in HIV infection. Various mechanisms have been invoked to account for the spontaneous apoptosis observed during the course of HIV-1 infection, including sustained immune activation associated with dysregulated cytokine production (10, 11), inappropriate signaling mediated by HIV-1 envelope binding to CD4 (12) or coreceptors (13), defective antigen presentation (14), activation of death receptors (15, 16), and loss of extracellular survival signals (17).

The increase in apoptosis levels seen in HIV-infected individuals implies that therapeutic interventions aimed at reducing apoptosis may also decrease the rate of T-cell depletion. For example, interleukin-2 (IL-2), a cytokine that induces a marked increase in the number of total and naïve CD4+ T cells in HIV-1-infected patients (18), was shown to reduce the levels of T-cell apoptosis both in vivo (19) and ex vivo (20); the long-term benefits of this therapeutic regimen are being determined in clinical trials. Another cytokine that is under
evaluation as a potential agent of immune reconstitution in HIV-1 infection is interleukin-7 (IL-7), a key factor in the generation, activation and homeostasis of the T-cell compartment of the immune system (21). The prosurvival effects of IL-7 are primarily mediated by upregulation of Bcl-2 synthesis and inactivation of pro-apoptotic proteins like Bad and Bax (22). Conflicting evidence has been reported on the anti-apoptotic effects of IL-7 on T cells from HIV-1-infected individuals studied after short-term ex vivo culture. Zaunders et al. found no effects of IL-7 on peripheral blood mononuclear cells (PBMC) from patients with acute primary HIV-1 infection (23), whereas others have reported a limited anti-apoptotic effect in patients with chronic HIV-1 infection, which was less pronounced than in uninfected individuals (24, 25). Likewise, a limited, but not statistically significant, effect of IL-7 was reported on CD4+ and CD8+ T cells from macaques chronically infected with SIV (8). Another controversial issue is the putative ability of IL-7 to induce HIV-1 replication. At doses that promote T-cell proliferation, IL-7 has been reported to induce the transcription of latent HIV-1 in resting peripheral blood CD4+ T cells derived from infected patients on anti-retroviral therapy cultured for several weeks ex vivo (26). However, in vivo administration of IL-7 to SIV-infected macaques did not cause a detectable increase in viral load both in lymph nodes and in peripheral blood, in spite of a widespread induction of T-cell proliferation (27, 28).

In the present study, we investigated the protective effects of IL-7 against spontaneous T-cell apoptosis in a cohort of HIV-1-infected subjects at different stages of disease. An extensive time-course analysis over 7 days of ex vivo culture permitted us to document a potent anti-apoptotic effect of IL-7 on both CD4+ and CD8+ T cells from HIV-infected
individuals. This effect was not associated with T-cell proliferation or reactivation of latent provirus.
3.1 Materials and Methods

2.1.1 Study subjects

Twenty-nine HIV-1-infected individuals and 14 HIV-1-seronegative age-matched controls were included in this study. The HIV-1-infected individuals were selected among those attending the National Institute of Allergy and Infectious Diseases (NIAID) Outpatient Clinic to represent different clinical stages. All patients provided informed consent, in accordance with the NIAID Institutional Review Board, and were subjected to leukapheresis according to approved protocols to obtain PBMC.

2.1.2 Isolation of PBMC and purification of CD4+ and CD8+ T cells

PBMC were isolated by gradient centrifugation from leukapheresis packs using Lymphocyte Separation Medium (MP Biomedicals). CD4+ and CD8+ T cells were purified by negative selection using Dynabeads goat anti-mouse IgG (Dynal) and a cocktail of purified monoclonal antibodies (mAb) against human CD14 and CD19 (Biodesign International), CD16 and CD56 (BD Pharmingen) and either CD8 (BD Pharmingen) or CD4 (Biodesign International). The purity of the CD4+ and CD8+ populations was consistently greater than 95%, as determined by cytofluorimetry.

2.1.3 Cell cultures

Isolated PBMC or purified CD4+ and CD8+ T-cell populations were cultured for up to 21 days at 37°C in 25 cm² flasks at a density of 10⁶ cells/ml in complete culture medium [RPMI 1640 with L-glutamine (Gibco) supplemented with Penicillin-Streptomycin at 10000 U/ml each (Gibco) and 10% fetal bovine serum (FBS) (HyClone)]. Recombinant human IL-
7 (Peprotech) at different concentrations (0.04-50 ng/ml) or IL-2 (Roche) at 100 U/ml were added at the beginning of the culture.

2.1.4 Measurement of apoptosis

Analysis of apoptosis was performed every day over the first 7 days of culture by the detection of Annexin V binding (Figure 2.1) and caspase 3 activation. Annexin V binding was measured using the Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen). Briefly, at each time point, 5 x 10^5 cells were stained with mAbs anti-CD3-APC, anti-CD4-FITC and anti-CD8-PerCP (BD Pharmingen), and in selected experiments anti-CD45RA-FITC or anti-CD45RO-FITC (BD Pharmingen), and then washed and incubated with 2μl of Annexin V-PE or 5μl of propidium iodide (PI). Cells were then immediately analyzed using a FACScalibur Flow Cytometer (Beckton Dickinson). Both live and apoptotic cells were included in the analysis, while cellular debris was excluded. Apoptosis was demonstrated by transition from a single-positive (Annexin-V+ PI') towards a double-positive phenotype. Reduction of apoptosis by IL-7 or IL-2 was calculated by subtracting the percent Annexin V+ cells in cytokine-treated cultures from the percent Annexin V+ cells in untreated controls. In selected experiments, apoptosis was confirmed by evaluating the activation of caspase 3. After surface staining as described above, the cells were fixed using BD FACS Lysing Solution, permeabilized using BD FACS Permeabilizing Solution 2 and incubated with a PE-conjugated rabbit mAb against the active form of caspase 3 (BD Pharmingen). The cells were then analyzed by flow cytometry. All the flow data were analyzed using the Flowjo software (Tree Star).
2.1.5 Analysis of cellular proliferation

Cellular proliferation was evaluated using intranuclear expression of Ki67, dilution of the vital dye CFSE, and absolute cell counting. Ki67 was assessed using a PE-conjugated anti-Ki67 mAb (BD Pharmingen) after fixation and permeabilization of the cells. Labeling with CFSE was performed using the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen), following the provided protocol. Labeled cells were then cultured in complete medium for up to 7 days with or without rhIL-7 or rhIL-2, as described above and analyzed by flow cytometry. Absolute cell counting was obtained by flow cytometry analysis of a fixed volume of cell culture at high flow pressure for 30 seconds. To calculate the absolute number of cells in each sample, the number of events acquired was multiplied by the flow rate.

2.1.6 Measurement of HIV-1 replication

HIV-1 replication was evaluated by measuring the levels of HIV-1 p24 antigen released into the culture supernatants using an HIV-1 p24 Antigen capture EIA (Beckman Coulter). To maximize sensitivity, the samples were analyzed undiluted. The plates were read according to a kinetic protocol using a microplate spectrophotometer (Bio-Rad Instruments).

2.1.7 Statistical analysis

Statistical analysis was conducted using the softwares SAS (version 9.1 for Windows), S-Plus (version 6.2 for Windows), and StatView (version 5.0.1). Paired t-tests were used to compare differences in apoptosis between IL-7-treated and untreated cells on each day and
averaged over all 7 days. Regression analysis was used to compare the effect of IL-7, averaged across the first 6 days of culture, to other covariates. Non-parametric Mann Whitney tests were used for comparisons between different groups of patients. Mixed model methodology was used in analyses comparing slopes relating the IL-7 effect and days. Pitman's test was used to compare pairs of days with respect to the variability of IL-7 effects.
3.2 Results

2.2.1 Anti-apoptotic effect of IL-7 on spontaneous apoptosis in PBMC from HIV-1-infected subjects

The effects of IL-7 were initially evaluated in unfractionated PBMC from a cohort of HIV-1-infected subjects (n = 24) at different stages of disease as reflected by levels of peripheral CD4+ T-cell counts and HIV-1 plasma viremia (nos. 1-24, Table 2.1). The cells were cultured \textit{ex vivo} in the presence or absence of recombinant human IL-7; IL-2 was tested in parallel as a positive control for the reduction of apoptosis. Apoptosis was evaluated on freshly isolated cells (baseline), and then every day for 7 days by using two unrelated techniques: Annexin V binding (Figure 2.1) and caspase 3 activation. To determine most accurately the effect of IL-7 on apoptosis over the 7-day period, we calculated the mean of all daily apoptosis measurements for each patient and then from those data we determined the mean value for the 24 patients.

The proportion of Annexin V-binding cells at baseline was similar in HIV-1-infected patients and controls (p = 0.61); however, it significantly increased upon \textit{ex vivo} culture in HIV-1-infected subjects (p < 0.0001), while the increase was only marginal in seronegative controls (p = 0.52) (Figure 2.2 A). The addition of exogenous IL-7 (5 ng/ml) induced a dramatic reduction in the levels of spontaneous apoptosis in cells from all the HIV-1-infected patients tested. The mean level of Annexin V binding over the 7 days of culture was significantly lower in cells treated with IL-7 than in untreated homologous cells (p < 0.0001) (Figure 2.2 A). As expected, treatment with IL-2 (100 U/ml) also significantly reduced the levels of Annexin V binding (p = 0.002) in PBMC from HIV-1-infected individuals (Figure 2.2 A). At each time point between day 1 and 6, Annexin V binding was significantly
reduced in IL-7-treated cells compared to controls; the difference was no longer significant at day 7 (Figure 2.2 B). In PBMC from selected patients, IL-7 was titrated over a wide dose range (0.04-50 ng/ml). Reduction of apoptosis was consistently seen at doses higher than 0.5 ng/ml (Figure 2.10 A).

The anti-apoptotic effect of IL-7 was also investigated by measuring the activation of caspase 3. Overall, the mean levels of caspase 3 activation during the 7 days of culture were significantly lower in IL-7-treated than in untreated cells (n = 7; p = 0.01). The levels of caspase 3 activation paralleled those of Annexin V binding in the presence and absence of IL-7 (Figure 2.2 C). In line with previous observations (29), the reduction of apoptosis mediated by IL-7 was associated with an increase in intracellular levels of Bcl-2 (Figure 2.3).

Despite the consistency of the anti-apoptotic effect of IL-7, its kinetics and magnitude showed a marked variability among patients. The peak reduction in the proportion of apoptotic cells in different patients ranged from 5.1% to 28.4% (mean, 14.1±5.7%), and occurred at different time points during ex vivo culture (range, 1-6 days; mean, 3.9±1.5). In some patients the effect of IL-7 increased over time (positive slope), while in others it remained constant or even decreased (negative slope). Z-score values comparing each patient's slope to the overall slope confirmed that such differences were real (p < 0.0001). The fact that patients had relatively similar baseline values but different trajectories over time explains the increased variability in the last days of culture (Figure 2.2 B; also confirmed by Pitman's test for paired variances).

As stated above, exposure of PBMC from HIV-1-infected individuals to IL-2 (100 U/ml) effectively reduced the levels of spontaneous apoptosis (Figure 2.2 A) with peak
reductions ranging from −0.9% to 31.0% (mean, 16.6±7.1%); the peak effect of IL-2 tended to occur slightly earlier than that of IL-7 (range, 1-4 days; mean, 2.3±1.1).

2.2.2 Limited anti-apoptotic effects of IL-7 on PBMC from HIV-1-seronegative individuals

Treatment with IL-7 had limited effects in PBMC from a group of age-matched HIV-uninfected subjects (n = 14). The mean levels of spontaneous apoptosis during the 7-day culture period were not significantly different in IL-7-treated and untreated cultures (Figure 2.2 A), and comparisons at each time point showed a significant apoptosis reduction only at day 2 (p = 0.002) (data not shown). Thus, the mean effect of IL-7 over 7 days of culture was significantly different in cells from HIV-1-infected and uninfected subjects (mean: 8.2±5.4% vs. 0.6±3.3%; p = <0.0001) (Figure 2.2 A); a similar difference was seen considering the peak levels of apoptosis reduction (mean: 14.1±5.7% vs. 5.3±4.1; p <0.0001). Analogous results were obtained in IL-2-treated cells with regard to both the mean (7.6±8.9% vs. -2.0±5.2%; p = 0.002) (Figure 2.2 A) and peak (16.6±7.1% vs. 6.5±8.9%; p = 0.01) levels of reduction of apoptosis.

2.2.3 Correlation between the sensitivity to the anti-apoptotic effect of IL-7 ex vivo and the CD4+ T-cell count in vivo

The propensity to undergo spontaneous apoptosis in HIV-1-infected subjects was previously shown to correlate directly with the plasma viral load and inversely with the levels of circulating CD4+ T cells (30, 31). Thus, we evaluated the potential correlations between the sensitivity to the anti-apoptotic effects of IL-7 and several demographic, clinical
and immunologic parameters at the time of sampling (see Tables 2.1 and 2.2). No significant correlations were observed between the mean levels of apoptosis reduction by IL-7 over the first 6 days in culture and the plasma HIV-1 load, CD8 count and age (Figure 2.4 A, left panel), as well as with the expression of CD8\(^+\) T-cell naïve/memory markers, CD8\(^+\) T-cell activation markers (HLA-DR\(^+\), CD38\(^+\), CD38\(^-\)HLA-DR\(^+\), CD25\(^+\)), selected CD4\(^+\) T-cell activation markers (HLA-DR\(^+\), CD38\(^+\)HLA-DR\(^+\), CD25\(^+\))(Figure 2.5 A), and treatment status (Figure 2.5 B). In contrast, a significant inverse correlation was observed with the circulating CD4\(^+\) T-cell count \((R^2 = 0.266, p = 0.0099)\) (Figure 2.4, left panel). For selected parameters, these findings were confirmed by comparing the levels of reduction of apoptosis between different groups of patients defined by specific cut-off values (Figure 2.4, right panel). Moreover, the mean levels of reduction of apoptosis by IL-7 over the first 6 days in culture were inversely correlated with the absolute number of memory CD4\(^+\) T cells \((R^2 = 0.229, p = 0.018)\), as well as with the proportion of CD4\(^+\) T cells expressing the activation marker CD38 \((R^2 = 0.221, p = 0.020)\) (Figure 2.5 A).

Interestingly, within patients with low CD4 counts (< 350 cells/\(\mu\)l), no significant differences were observed in the mean levels of apoptosis reduction between patients that were naïve to treatment and patients that were on therapy at the time of testing or had been treated in the past, as well as between patients with different plasma viremia (two cut-off values were used: 50 and 30000 copies/ml), irrespective to their treatment status (Figure 2.5 C).

The CD4\(^+\) T-cell counts were also predictive of the slopes of IL-7-mediated reduction of apoptosis over time, with a greater increase in IL-7 effectiveness over time in patients with lower CD4 counts (Figure 2.6).
2.2.4 Anti-apoptotic effect of IL-7 on both CD4\(^+\) and CD8\(^+\) T cells from HIV-1-infected subjects

As a preliminary approach to elucidate whether IL-7 can protect from apoptosis both CD4\(^+\) and CD8\(^+\) T cells derived from HIV-1-infected individuals, we analyzed separately the two subpopulations by multiple color flow cytometry within unfractionated PBMC cultures. The results indicated that IL-7 treatment reduced the level of Annexin V binding in both CD3\(^+\)CD4\(^+\) and CD3\(^+\)CD8\(^+\) gated cells (Figure 2.7 A). To address this issue more formally, we enriched CD4\(^+\) and CD8\(^+\) T-cell subpopulations by negative selection from PBMC of 5 HIV-1-infected individuals, and then cultured the cells for 6 days in the presence or absence of IL-7 (5 ng/ml). IL-7 had strong anti-apoptotic effects on both CD4\(^+\) and CD8\(^+\) purified T cells from all the HIV-1-infected individuals analyzed (Figure 2.7 B): the peak levels of reduction of apoptosis measured by Annexin V binding ranged from 10.2% to 40.5% for CD4\(^+\) T cells (mean, 20.8±13.1%; p=0.02 for the comparison between IL-7-treated and untreated cells) and from 15.5% to 52.8% for CD8\(^+\) T cells (mean, 27.2±16.2%; p=0.02); likewise, there was a marked reduction in the mean levels of apoptosis over the first 6 days of culture in both CD4\(^+\) T cells (11.7±6.6%; p=0.02) and CD8\(^+\) T cells (14.6±9.1%; p=0.02). Similar data were obtained when caspase 3 activation was used as an indicator of apoptosis: representative time courses of Annexin V binding and caspase 3 activation in purified CD4\(^+\) and CD8\(^+\) T cells in the presence or absence of IL-7 are presented in Figure 2.8.

When naive and memory CD4\(^+\) and CD8\(^+\) T cells were separately analyzed either on unfractionated PBMC cultures by multiple color flow cytometry or on purified CD4\(^+\) and
CD8⁺ T cells, IL-7 was shown to reduce apoptosis in both the naïve and memory subsets (Figure 2.9). As expected, treatment with IL-2, used as a positive control, reduced the levels of spontaneous apoptosis in both naïve and memory CD4⁺ and CD8⁺ T cells from HIV-1-infected individuals (Figure 2.9).

2.2.5 Uncoupling of the anti-apoptotic effects of IL-7 from the induction of cellular proliferation

Since IL-7 exerts concentration-dependent proliferative effects on CD4⁺ and CD8⁺ T cells (32), we measured the fraction of cells undergoing cycle progression and proliferation in IL-7-treated cells from 10 selected patients using three different methods: expression of the nuclear antigen Ki67, dilution of the vital dye CFSE and absolute cell counting (Figure 2.10). Although IL-7 at 5 ng/ml induced cellular proliferation in PBMC from all the patients analyzed, we consistently observed a temporal dissociation with the anti-apoptotic effect. As illustrated in Figure 2.10 A, IL-7 induced an anti-apoptotic effect from the earliest days of culture; in contrast, Ki67 expression appeared later (day 5) and was seen in a high proportion of cells only at 5 ng/ml of IL-7 (Figure 2.10 B). At lower IL-7 concentrations, Ki67 was expressed in less than 10% of the cells, with negligible levels in cells treated with 0.6 ng/ml. Analogous results were obtained by analysis of absolute cell counting and CFSE dilution over time (Figure 2.10 C and D). Unlike IL-7, IL-2 at a concentration of 100 U/ml induced an early proliferative effect that was first detectable at day 3 of culture and peaked at day 6 (Figure 2.10 C). However, even with IL-2 we observed a temporal dissociation between reduction in apoptosis and Ki67 expression, since its anti-apoptotic effect was already evident at day 1 of culture and peaked at day 2 (Figure 2.10 A).
2.2.6 Lack of effect of IL-7 on the induction of HIV-1 replication in purified CD4\(^+\) T cells from HIV-1-infected individuals

*Ex vivo* treatment of purified resting CD4\(^+\) T cells from a fraction of HIV-1-infected patients with high doses of IL-7 has been reported to induce the activation of latent provirus after several weeks in culture (26, 33). Thus, we measured the levels of endogenous HIV-1 replication over time in purified CD4\(^+\) T cells derived from 7 HIV-1-infected individuals, cultured in the presence or absence of IL-7 at 5 ng/ml or IL-2 at 100 U/ml. HIV-1 replication was evaluated by measuring secretion of HIV1 p24 in the tissue culture media. No quantification of cell- or media-associated HIV-1 RNA/DNA was performed.

Table 2.3 shows that supernatants from 5 out of 7 CD4\(^+\) T-cell cultures treated with IL-7 remained negative for HIV-1 p24 throughout the culture period, while the remaining two showed only minimal levels of p24 release (0.01 ng/ml). Of note, similar levels of p24 were also seen in IL-7-untreated cultures approximately at the same time points, suggesting that HIV-1 replication was unlikely a result of IL-7 treatment and actually represented spontaneous release of virus. Likewise, treatment with IL-2 at 100 U/ml resulted in the appearance of low levels of p24 (0.01-0.142 ng/ml) in CD4\(^+\) T-cell cultures from 4 of the 7 patients, including one (no. 29) in which IL-7 did not induce any detectable viral replication; IL-2 treatment yielded the highest levels of p24 release. Analogous data (not shown) were obtained using unfractionated PBMC followed for up to 21 days after establishment in culture.
2.3 Discussion

Although the introduction of effective antiretroviral therapy (ART) has resulted in a
dramatic decline in the morbidity and mortality among HIV-1-infected individuals,
phenotypic and functional immunologic abnormalities may persist even in patients with
sustained suppression of viremia for several years (34). Thence, innovative approaches
aimed at improving immune reconstitution have been proposed as a complement to ART. In
particular, cytokines that play an essential role in T-cell homeostasis and proliferation, such
as IL-2 and IL-7, are currently under clinical investigation (18). In this study, we
demonstrated that IL-7 exerts protective effects against spontaneous apoptosis on both CD4+
and CD8+ T cells derived from HIV-1-infected individuals. In agreement with previous
reports (6, 9, 35), we found that the level of spontaneous apoptosis was significantly higher
in HIV-1-infected subjects than in seronegative controls and that, among the former, it
correlated with the degree of CD4+ T-cell depletion in vivo, reinforcing the concept that
apoptosis is a key mechanism of T-cell destruction during HIV-1 infection. Thus, the anti-
apoptotic activity of IL-7 provides an additional rationale for consideration of this cytokine
as a potential immunotherapeutic agent in the treatment of HIV-1-infected individuals.
An unexpected finding emerging from our study is that the reduction of apoptosis mediated
by IL-7 in HIV-1-infected individuals was inversely correlated with the level of circulating
CD4+ T cells. Thus, in spite of a more severe immune dysfunction, patients with lower CD4+
T-cell counts showed a higher sensitivity to IL-7, while those with higher CD4+ T-cell
counts, whose levels of spontaneous apoptosis are generally lower, exhibited a lesser
sensitivity. In line with this trend, the effect of IL-7 on spontaneous apoptosis was even
lower, failing to reach statistical significance, on cells derived from HIV-1-seronegative
individuals. These findings suggest that under physiological conditions, there is a basal level of spontaneous apoptosis that is largely insensitive to IL-7. With the progression of HIV-1 disease, the proportion of cells that are prone to spontaneous apoptosis increases, and a large fraction of these cells is sensitive to the anti-apoptotic effects of IL-7. The *ex vivo* effects of IL-7 in chronically HIV-1-infected individuals have been previously investigated in only two studies, which reported a limited, if any, reduction of spontaneous apoptosis, while a more pronounced effect was seen with cells derived from HIV-seronegative individuals (24, 25). However, these studies evaluated apoptosis only at a single time point after several days (6 or 7) of *ex vivo* culture. By contrast, the present study was designed to provide a detailed longitudinal representation of apoptosis levels, with daily measurements for 7 days of *ex vivo* culture in the presence or absence of IL-7. This extensive time-course analysis permitted to document variable kinetics of IL-7-mediated reduction of apoptosis in different patients, with some patients showing an increasing effect over time, and others manifesting an effect that remained constant or even decreased over time. Thus, there was an increasing variability of the IL-7 effect at the late time points of culture, as attested by a reduced statistical significance at days 5 and 6, and a loss of significance at day 7. These observations suggest that a single determination is unlikely to accurately represent the effects of IL-7 on spontaneous apoptosis. The role played by IL-7 *in vivo* in the course of HIV-1 infection is still poorly understood. Increased plasma levels of IL-7 were documented in HIV-1-infected individuals and inversely correlated with CD4 counts (30, 31, 36). In parallel, the expression of the α-chain of the IL-7 receptor (CD127), was shown to be downmodulated (24, 36, 37). Since the levels of IL-7 decreased when the CD4⁺ T cell counts were restored during therapy, the increases in IL-7 levels have been interpreted as a
homeostatic response to lymphopenia (31). However, the fact that in the absence of ART the number of circulating CD4+ T cells remains low suggests that IL-7 per se is unable to reconstitute the loss of CD4+ T cells resulting from the effects of HIV viremia. Different mechanisms may account for this inability. The rate of CD4+ T-cell destruction in patients with advanced HIV-1 disease may exceed the regenerative capacity of IL-7 or the pool of progenitor T cells may be irreversibly damaged or depleted. Alternatively, the IL-7/IL-7 receptor axis may be impaired (37). The marked sensitivity to the effects of IL-7 that we documented in patients with low CD4+ T cell counts indicates that circulating T cells from these patients did express a functional IL-7 receptor. Interestingly, however, kinetic analysis demonstrated that the ex vivo effect of IL-7 in these patients was delayed, suggesting that their T cells might be in a state of IL-7 refractoriness in vivo. Responsiveness to IL-7 would then be gradually restored upon ex vivo culturing, presumably due to the removal of putative inhibitory factor(s).

Studies of IL-7 administration to uninfected and SIV-infected macaques have demonstrated a proliferative effect on CD4+ and CD8+ T cells expressing both memory and naïve phenotypes (27, 28, 38). Analogous results were more recently obtained in human cancer patients (39), as well as in HIV-1-infected individuals (40). In the present study, we observed that IL-7 given ex vivo at concentrations higher than 2.5 ng/ml consistently induced cellular proliferation. However, this effect became detectable only after several days in culture and was temporally dissociated from the effect of IL-7 on apoptosis. Moreover, at concentrations below 1 ng/ml, IL-7 was still effective in reducing apoptosis, but failed to induce cellular proliferation. The evidence that the different activities of IL-7 can be dissociated is encouraging with regard to the potential therapeutic use of this cytokine in
HIV-1-infected subjects, since it mitigates concerns related to the putative risk of enhancing the level of immune activation and viral replication. Previous studies have shown that *ex vivo* treatment with IL-7 increases the levels of HIV-1 replication in naturally infected CD8-depleted mononuclear cells (33), and may lead to reactivation of latent provirus in purified resting CD4 T lymphocytes isolated from HIV-1-infected subjects receiving ART with undetectable plasma viremia (26). At variance with these observations, however, we detected only minimal levels, if any, of HIV-1 replication in purified CD4 T cells treated *ex vivo* with IL-7. Our results are in agreement with the findings in SIV-infected macaques treated *in vivo* with IL-7, which showed no increase in viral load both in the presence (27) and in the absence (28) of concomitant ART.

In conclusion, results of the present study provide a further rationale for consideration of IL-7 as a potential adjuvant therapy in HIV-infected individuals in association with ART. Studies are under way to elucidate the precise molecular mechanisms underlying the increased propensity to spontaneous apoptosis in HIV-1-infected individuals, as well as the anti-apoptotic action of IL-7. Further experimental studies in nonhuman primates and clinical studies in HIV-1-infected individuals should allow elucidation as to whether IL-7 administration effectively leads to a reduction in the levels of spontaneous apoptosis *in vivo*, and whether this effect is associated with a stable immune reconstitution in patients with sustained suppression of viremia.
### 2.4 Tables

**Table 2.1** Demographic, immunologic and clinical characteristics of the HIV-1-infected patients included in the study.

<table>
<thead>
<tr>
<th>Patient</th>
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<th>CD8 count (cells/ml)</th>
<th>Anti-retroviral therapy</th>
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n.a. = not available
Table 2.2 Immunologic characteristics of the HIV-1-infected patients included in the study.

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Table 2.3 HIV-1 replication in purified CD4$^+$ T cells derived from selected HIV-1-infected subjects, cultured *ex vivo* for 6 days in the absence of additional stimuli (TCM = tissue culture medium) and in the presence of IL-7 (5 ng/ml) or IL-2 (100 U/ml). Reduction of apoptosis at the same time point is shown in parenthesis.

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2.5 Figure Legends

Figure 2.1 Schematic representation of the Annexin V assay. (A) Annexin V is a molecule that binds in a calcium-dependent manner to phosphatidylserine (PS), a component of the cellular membrane. On a viable cell PS is mainly in the cytoplasmic face of the membrane, and therefore Annexin V binding does not occur in the absence of permeabilization. By contrast, when a cell undergoes apoptosis, structural changes occur at the membrane level, leading to the externalization of PS on the extracellular surface, thus allowing for Annexin V binding. If Annexin V is conjugated with a fluorochrome this binding can be measure by FACS analysis. (B) Discrimination between apoptotic and necrotic cells by double staining with propidium iodide (PI) and Annexin V: double negative cells are viable cells; double positive cells are dead cells (by necrosis or apoptosis); cells that are positive for Annexin V and negative for PI are apoptotic cells.

Figure 2.2 Protective effects of IL-7 against spontaneous apoptosis in ex vivo-cultured PBMC from HIV-1-infected patients and uninfected controls. (A) Mean levels of Annexin V binding at baseline (day 0) and over 7 days of ex vivo culture in the presence or absence of IL-7 at 5 ng/ml or IL-2 (100 U/ml). C = untreated controls. (B) Time-course analysis of IL-7-mediated reduction of Annexin V binding over 7 days of ex vivo culture in PBMC from HIV-1-infected patients. Reduction of apoptosis by IL-7 was calculated by subtraction of the percent Annexin V+ cells in IL-7-treated cultures from the percent Annexin V+ cells in untreated controls. * = p<0.0001 for the comparison between IL-7-treated and -untreated cultures; † = p<0.005; ‡ = not significant. The comparisons
were performed by paired Student's t-test. (C) Time-course analysis of Annexin V binding and caspase 3 activation in PBMC from a HIV-1-infected patient, representative of 7 HIV-1-infected patients (n=7), cultured in the presence or absence of IL-7 at 5 ng/ml.

Figure 2.3 Upregulation of Bcl-2 expression by IL-7. (A) Mean fluorescence intensity (MFI) of Bcl-2 expression by FACS analysis in unfractionated PBMC from a HIV-1-infected individual, #14 representative of 5 HIV-1-infected individuals (n = 5) on freshly isolated cells (Day 0) and after 5 days of culture in the absence or presence of various concentration of IL-7 (5.0-2.5-1.2-0.6 ng/ml) or IL-2 (100 U/ml). numbers in red indicate the MFI for Bcl-2 expression in each condition. (B) Histogram overlay of Bcl-2 expression at Day 5 in PBMC cultured in the absence or presence of various concentration of IL-7 (5.0-2.5-1.2-0.6 ng/ml) or IL-2 (100 U/ml).

Figure 2.4 Correlation between the mean levels of IL-7-mediated reduction of apoptosis in PBMC from 24 HIV-1-infected patients over the first 6 days in culture and various demographic, clinical and immunologic parameters. The left panel shows scatterplots with regression lines relating differences in apoptosis to covariates. Also shown are R-squared values (squares of the Pearson's correlation coefficients) and p values. The right panel shows the comparisons between different groups of patients defined by specific cut-off values for each parameter. Apoptosis was measured by Annexin V binding.
Figure 2.5 Correlation between the mean levels of IL-7-mediated reduction of apoptosis in PBMC from 24 HIV-1-infected patients over the first 6 days in culture and various demographic, clinical and immunologic parameters. (A) Scatterplots with regression lines relating differences in apoptosis to covariates. Also shown are R-squared values (squares of the Pearson's correlation coefficients) and p values. Apoptosis was measured by Annexin V binding. (B) Comparison of the mean levels of Annexin V binding in patients with different treatment status (on-therapy, off-therapy, naïve to treatment). (C) Statistical analysis restricted to patients with CD4 counts < 350 cells/μl (n = 11): comparison of the mean levels of Annexin V binding between patients naïve to treatment and patients on/off therapy at the time of testing, and between patients divided according to their level of viremia (two different cut-off values were used, 50 and 30000 copies/ml). Non-parametric Mann Whitney test was used for statistical comparisons between different groups of patients.

Figure 2.6 Correlation between the slope of IL-7-mediated reduction of apoptosis and the CD4+ T-cell counts. The figure shows the scatterplots with the regression line relating the slope of IL-7-mediated reduction in Annexin V-binding over 7 days of culture in PBMC from 24 HIV-1-infected patients and the number of circulating CD4+ T cells. The upper panel shows the analysis performed on cells from all 24 HIV-1-infected patients; the lower panel shows the same analysis after removal of a single outlier (patient no. 13). Also shown are the R-squared values and p values calculated by Pearson's correlation test.
Figure 2.7 Protective effects of IL-7 against spontaneous apoptosis in CD4⁺ and CD8⁺ T cells from HIV-1-infected patients. (A) Mean levels of Annexin V binding over 6 days of culture in CD4⁺ or CD8⁺ T cells identified by multicolor flow cytometry in unfractionated PBMC cultured in the presence or absence of IL-7 (5 ng/ml). (B) Mean levels of Annexin V binding over 6 days of culture in CD4⁺ or CD8⁺ T-cell populations cultured separately as purified subpopulations in the absence or presence of IL-7 (5 ng/ml).

Figure 2.8 Protective effects of IL-7 against spontaneous apoptosis in ex vivo-cultured purified populations of CD4⁺ and CD8⁺ T cells from one representative HIV-1-Infected patient. The figure shows time-course analyses of the levels of Annexin V binding and caspase 3 activation over 6 days of culture in the presence or absence of IL-7 (5 ng/ml) in purified CD4⁺ (A) or CD8⁺ (B) T-cell populations from a HIV-1-infected patient, # 25 representative of 5 HIV-1-infected individuals (n = 5).

Figure 2.9 Protective effects of IL-7 against spontaneous apoptosis in ex vivo-cultured naïve and memory CD4⁺ and CD8⁺ T cells from 7 HIV-1-Infected patients. Mean levels of Annexin V binding over 6 days of culture in the presence or absence of IL-7 (5 ng/ml) or IL-2 (100 U/ml) are shown. Naïve and memory T cells were identified by multiple color staining with antibodies to CD45RA and CD45RO, respectively, and Annexin V. In 5 patients, the cultures were established with unfractionated PBMC, and the CD4⁺ and CD8⁺ T-cell subsets were separately analyzed by gating. In 2 patients (# 5 and #
27, indicated by an asterisk), the CD4+ and CD8+ T-cell populations were purified by negative selection before culturing.

Figure 2.10 Temporal dissociation between the anti-apoptotic and proliferative effects of IL-7 and IL-2 from a representative HIV-1-infected individual (# 14 of 4 patients). Time-course analysis of Annexin V-binding reduction (A) and Ki67 expression (B) in PBMC from a representative HIV-1-infected patient (# 14) cultured for 6 days in the presence or absence of IL-7 (0.6, 1.2, 2.5 and 5 ng/ml) or IL-2 (100 U/ml). (C) and (D) Proliferative effects of IL-7 and IL-2 in PBMC from a representative HIV-1-infected patient (# 14), analyzed by absolute cell counting by flow cytometry (C) and CFSE dye dilution (D), in PBMC cultured in the absence or presence of IL-7 (0.6, 1.2, 2.5 and 5 ng/ml) or IL-2 (100 U/ml).
2.6 Figures

Figure 2.1

A

Schematic representation of the Annexin V assay

B

Dead cells

Apoptotic cells
Figure 2.2

A

HIV+ HIV-

% annexin V+ cells

Day 0 C IL-7 IL-2 Day 0 C IL-7 IL-2

Days 1-7 Days 1-7

B

Reduction of annexin V+ cells (% of total cell population)

1 2 3 4 5 6 7

Days in culture

Annexin V control

Caspase 3 control

C

% apoptotic cells

0 1 2 3 4 5 6 7

Days in culture

Annexin V control

Annexin V + IL-7

Caspase 3 control

Caspase 3 + IL-7
R2 = 0.119
p = NS

HIV-VL
(RNA copies/mL)

R2 = 0.003
p = NS

CD8 count
(cells/µl)

R2 = 0.266
p = 0.0099

CD8 count

R2 = 0.004
p = NS

CD4 count
(cells/µl)

R2 = 0.148
p = 0.076

Disease duration
(years)

Age
(years)

Disease duration
(years)

Age
(years)

HIV-VL

< 30000
> 30000

p = NS

< 800
> 800

p = NS

< 350
> 350

p = 0.0004

< 42 yrs
> 42 yrs

p = NS

< 3 yrs
> 3 yrs

p = NS
Figure 2.5

A

CD4+ CD8+

% CD4+ HLA-DR+

% CD8+ HLA-DR+

% CD4+ CD38+

% CD8+ CD38+

% CD4+ CD25+

% CD8+ CD25+

% CD4+ naive

% CD8+ naive

% CD4+ memory

% CD8+ memory

B

Mean apoptosis reduction

Treatment Status

C

Mean apoptosis reduction

on/off naive

< 50 > 50

< 30000 > 30000
Figure 2.7

A

CD4+ T cells

% Annexin V+ Cells

Patients

B

CD4+ T cells

% Annexin V+ Cells

Patients

CD8+ T cells

% Annexin V+ Cells

Patients

CD8+ T cells

% Annexin V+ Cells

Patients
Figure 2.8

A

CD4+ T cells

% Apoptotic cells

0 1 2 3 4 5 6

Days in culture

B

CD8+ T cells

% Apoptotic cells

0 1 2 3 4 5 6

Days in culture
Figure 2.9

A  CD4⁺ T cells

B  CD8⁺ T cells
Figure 2.10

A

Reduction of Annexin V Cells

B

% ki67+ Cells

C

Cells no.

Days in culture

D

Day 3

Day 6

Untreated

IL-2 100 U/ml

IL-7 5 ng/ml

IL-7 2.5 ng/ml

IL-7 1.2 ng/ml

IL-7 0.6 ng/ml

CFSE

Untreated

IL-2 100 U/ml

IL-7 5 ng/ml

IL-7 2.5 ng/ml

IL-7 1.2 ng/ml

IL-7 0.6 ng/ml
2.7 References

CHAPTER THREE

In vivo

Effects of Treatment with IL-7 during the Acute Phase of SIV Infection in Rhesus Macaques

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3.0 Introduction

Although HIV-1 establishes a chronic active infection that evolves toward clinical immunodeficiency over a span of several years, accumulating evidence indicates that critical pathogenic events take place during the acute phase of infection, leading to a massive and seemingly irreversible depletion of memory CD4\(^+\) T cells (1,2). The extent of such depletion is not accurately mirrored by circulating CD4\(^+\) T-cell counts, as peripheral blood contains only a minor fraction of the total T cells in the body (3). Gut-associated lymphoid tissue (GALT), a compartment that harbors the largest fraction of the T-cell pool (3), has been identified as a primary anatomical site for CD4\(^+\) T-cell depletion in both HIV-1-infected patients (4-6) and SIV-infected nonhuman primates (1,2,7,8). Yet, the loss of memory CD4\(^+\) T cells within the early phase of infection appears to be a systemic phenomenon that involves all secondary lymphoid organs (1,9). Taken together, these observations suggest that interventions aimed at preventing or reducing the long-term immunologic damage caused by HIV-1 would be most effective if implemented during the earliest stages of infection, before the pool of memory CD4\(^+\) T cells becomes irreversibly depleted.

In spite of extensive research over the past three decades, the mechanism of CD4\(^+\) T-cell depletion during the course of HIV-1 infection is still debated. Studies in SIV-infected macaques have highlighted a major role of cytopathic effects due to direct virus infection during the course of acute primary infection (1,9). However, indirect mechanisms, including bystander apoptosis, may also be important, as suggested by the detection of increased levels of apoptosis in the blood and lymphoid organs of macaques acutely infected with pathogenic SIV strains (2,10-14), as well as in ex vivo-cultured T
cells from individuals with acute HIV-1 infection (15-17). Thus, the use of anti-apoptotic agents during primary HIV-1 infection may have beneficial effects for preserving the integrity of the CD4- T-cell pool. We previously demonstrated that interleukin-7 (IL-7), a nonredundant cytokine that plays a critical role in the development and homeostasis of the T-lymphoid compartment of the immune system (18-20), effectively reduces the levels of spontaneous apoptosis in both CD4+ and CD8+ T cells from HIV-1-infected individuals (21). In hosts who are lymphopenic from a variety of causes, the levels of endogenous IL-7 increase, causing transient proliferation of naïve and central memory (CM) CD4+ and CD8+ T cells, leading to the reconstitution of the physiological T-lymphocyte pool (18-20). Owing to these unique biological properties, IL-7 is currently under clinical investigation as an immune reconstitution agent in various forms of natural and iatrogenic immunodeficiencies, including those associated with AIDS and cancer (22,23).

Pre-clinical studies in macaques chronically infected with SIV (24-27), as well as clinical studies in patients chronically infected with HIV-1 or treated with immunosuppressive anti-neoplastic drugs (28-31), have documented beneficial effects of short-term courses of IL-7 treatment, resulting in proliferation and expansion of naïve and CM T cells in peripheral blood and secondary lymphoid organs. However, the effects of IL-7 treatment in acute HIV-1 (or SIV) infection have never hitherto been investigated, and the anti-apoptotic action of IL-7 was never evaluated in vivo. In the present study, we administered fully glycosylated recombinant macaque IL-7 to rhesus macaques during the acute phase of infection with a pathogenic SIV strain (mac251). We deliberately avoided the concomitant use of ART in order to exclude the confounding effects of ART-induced...
suppression of virus replication on T-lymphocyte pathogenesis as well as to verify the
effects of IL-7 on SIV replication. We demonstrated that treatment with IL-7 is safe and
prevents the early depletion of naïve and CM CD4⁺ T cells without increasing the levels of
SIV replication. These results provide a scientific basis for consideration of IL-7 as an
adjuvant therapy during the acute phase of HIV-1 infection.
3.3 Materials and Methods

3.1.1 Animals and study design

The study protocol enrolled 12 colony-bred juvenile Rhesus macaques, which were housed at Bioqual Inc. (Rockville, MD) and handled in accordance to the standards of the American Association for the Accreditation of Laboratory Animal Care. The animals were quarantined and tested to exclude infections with SIV, STLV and herpes B virus. The study included two groups of animals: untreated controls (group 1) and IL-7 treatment (group 2). Blood samples were obtained three times during the first week of IL-7 treatment, then twice weekly for the entire treatment period and then once per week; terminal ileum biopsies were obtained on day 14-16 post-infection; lymph node biopsies were obtained on day 25-27 post-SIV infection. Since one animal in the control group (H744) was lost on day 14 post-infection during the procedure to obtain intestinal biopsies, all the data for time points subsequent to day 14 refer to 11 animals (5 untreated and 6 IL-7-treated).

3.1.2 IL-7 treatment and SIV infection

Recombinant glycosylated simian IL-7 (Cytheris, France) was employed in this study since it has a considerably longer half-life and greater plasmatic stability (M. Morre et al., unpublished results) than the non-glycosylated form that had been employed in several previous studies (32, 33). A schematic diagram of the design of the study is provided in Figure 3.1. IL-7 was administered subcutaneously to the 6 monkeys included in group 2 at a concentration of 50 μg/kg of body weight once per week for a total of 7 consecutive administrations. To allow for the achievement of steady-state plasmatic levels of the cytokine prior to SIV infection, treatment was initiated 1 week before SIV inoculation (day -
All 12 monkeys were inoculated intravenously with 100 macaque infectious doses of the pathogenic strain SIV<sub>mac251</sub>, kindly provided by Dr. R. Desrosier. Multiple clinical, immunological and virological parameters were monitored throughout the acute phase of the infection, as well as for a follow-up period of 6 months post-infection.

3.1.3 Measurement of plasma IL-7 concentrations

The concentration of IL-7 in serial plasma samples was measured using a high-sensitivity commercial ELISA (Quantikine HS, R&D Systems, Minneapolis, MN).

3.1.4 Peripheral blood cell counts and cytofluorimetric analysis

Peripheral blood was collected under sterile conditions in vacutainer tubes with EDTA as anticoagulant. A complete blood cell count (CBC) with differential was performed by a commercial laboratory (Antech Diagnostics, Rockville, MD). Plasma was separated by spinning whole blood at 500xg for 20 min at 4°C without brake and stored at -80°C. PBMC were isolated by gradient centrifugation using Lymphocyte Separation Medium (LSM; MP Biomedicals). Blood was diluted in Phosphate Buffered Saline (PBS), stratified over LSM and centrifuged at 500xg for 25 min at 4°C without brake. The mononuclear cell ring was collected and the PBMC were washed twice with PBS, counted and used for cytofluorimetric analyses. The following monoclonal antibodies (mAbs) were used for surface staining: CD28-FITC (clone CD28.2), CD4-PerCP-Cy5.5 (clone L200), CD95-APC or -PE (clone DX2), CD8-PE-Cy7 (clone SK1), CD3-APC-Cy7 (clone SP34-2), all from BD Biosciences. The naïve (CD28<sup>+</sup>CD95<sup>-</sup>), memory (CD28<sup>-</sup>CD95<sup>+</sup>) and effector (CD28<sup>-</sup>CD95<sup>-</sup>) T-cell subsets were identified as previously described (34). At selected time points, a more
detailed characterization of the memory T-cell compartment was performed by using a combination of the following mAbs: CD45RA-FITC (clone 5H9), CD28-PECy7, CCR7-APC (clone 3D12), CD95-Biotin (followed by Streptavidin-PerCpCy5.5), CD3-V450, CD4-APC7 (all from BD Biosciences), and CD8-eFLOUR 605NC (clone RPA-T8; eBioScience). Additional mAbs were used to measure expression levels of other cell-surface markers, including IL-7 receptor-α/CD127-PE (clone hIL-7R-M21), CCR5-PE (clone 3A9), CXCR4-PE (clone 12G5) and the activation markers HLA-DR-PE (clone L243/G46-6) and CD25-PE (clone M-A251) (all from BD Biosciences). Apoptosis was assessed by measuring the levels of Annexin V binding, using Annexin V-APC conjugated (BD Biosciences), as follows: after surface staining, the cells were washed with Annexin V-binding buffer, incubated for 15 min at room temperature with Annexin V-APC, and analyzed immediately. Bcl-2 expression levels and cellular proliferation were assessed by intracellular staining using mAbs anti-Bcl-2-PE (clone Bcl-2/100) and anti-Ki67-PE (clone B56) (BD Biosciences). Briefly, after surface staining, the cells were fixed and permeabilized using BD Cytofix/Cytoperm solution (BD Biosciences) and incubated with the appropriate antibody for 20 min at 4°C. Stained cells were analyzed by flow cytometry using a BD FACS Canto collecting a minimum of 100,000 events per sample. Flow data were analyzed using the Flowjo software (Tree Star) or FCS Express (DeNovo Software).

3.1.5 Measurement of SIV plasma viremia, proviral DNA and antigenemia

Plasma levels of SIV RNA were measured using a quantitative real-time RT-PCR assay. Viral RNA was purified from 280 ml of cell-free plasma using the QIAamp Viral RNA kit (Qiagen, USA), and stored at -80°C. The number of SIV RNA genome equivalents
(ge) was determined using a real-time RT-RCR assay based on the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, USA). Briefly, reverse transcription and polymerase chain reaction phases were carried out in a single tube, under the following amplification conditions: 10 min at 45°C (RT), 10 min at 95°C and 40 cycles of 15 sec at 95°C and 45 sec at 60°C (PCR). Primers and probe used for reverse transcription and amplification were specifically designed within the SIV gag gene, in order to amplify a fragment of 91 bp. Forward primer: 5’-GCAGAGGAGGAAAATTACCCAGT-3’; reverse primer: 5’-ATTCTACCCAGGCATTTAATGTTC-3’ (used for the RT phase); TaqMan MGB probe, FAM-labelled: 5’-ACAATAAGGTGGTAACGTATAG-3’. Primers and probe were used at a concentration of 300 nm and 200 nm respectively. The copy number was determined by interpolation on a standard curve of a DNA plasmid carrying a fragment of the SIV gag gene containing the RT-PCR amplicon (serial 10-fold dilutions from 10⁰ copies/reaction to 10⁶ copies/reaction). Forward cloning primer: 5’-GCAGAGACACCTAGTGATGGAAAC-3’; reverse cloning primer: 5’-TCTCCCCACACAATTTAATCTG-3’.

The SIV proviral DNA load was measured by real-time PCR using the same primers and probe as in the plasma SIV viremia assay. Normalization for cell number was performed by quantification of a non-polymorphic single-copy gene, CCR5, and the copy number per million of CD4⁺ T cells was obtained by normalizing for the percentage of CD4⁺ T cells in the corresponding sample, as determined by flow cytometry. Additional details about the method have been reported (35).

SIV p27Gag antigenemia was measured by using the SIV Core Antigen Assay (Coulter Corporation, Miami, USA) according to the manufacturer’s instructions. All samples were initially tested undiluted and retested at 1:10 dilution if the p27Gag content was
higher than the highest point of the standard curve. Plates were read using an end-point protocol with a microplate spectrophotometer (Bio-Rad Instruments).

3.1.6 Terminal ileum and lymph node biopsies

Ileum biopsies were performed by retrograde ileoscopy on day 14-16 post-infection (Figure 3.1). A pediatric colonoscope was passed through the large intestine and the ileocecal valve. At least 6-8 punch biopsies were obtained with cold forceps from the terminal ileum of each animal, immediately placed in cold RPMI medium and then processed within 3 hours of excision. During the first procedure, one animal (H744) suffered an intestinal perforation and was lost. Ileum biopsies were digested in Iscove’s media supplemented with 2 mg/ml Type II collagenase (Sigma-Aldrich) and 1 U/ml DNase I (Sigma-Aldrich) for 30 min at 37°C. After digestion, the samples were passed through a 70μm strainer, and the suspended cells were washed twice with RPMI media supplemented with 10% heat-inactivated FBS.

Lymph node excisional biopsies were performed on day 25-27 post-infection (Figure 3.1) on axillary lymph nodes from all animals using sterile instruments and aseptic technique. Lymph nodes were cut longitudinally in two halves, one of which was stored at -80°C for subsequent analysis. The remaining half was finely minced using sterile scalpels and mechanically smashed to release lymphoid cells into the media. Cells were then washed, passed through a 70μm strainer and stained for surface and intracellular markers as described above.
3.1.7 Analysis of SIV-specific T-cell responses

SIV-specific CD4⁺ and CD8⁺ T-cell responses were analyzed by measuring intracellular cytokine production after antigen stimulation as follows. Frozen samples of unfractionated PBMC collected during the acute phase of SIV infection and during the follow-up period were thawed in complete RPMI medium supplemented with 10% FBS and rested at 37°C for at least 5 hrs. Cells were then plated at a concentration of 5×10⁵ cells/well in a 96-well round-bottom plate in RPMI 10% FBS in the presence of purified anti-CD28 and anti-CD49d mAbs both at a final concentration of 1 ng/ml. Two pools of SIV-gag peptides and one pool of SIV-tat peptides (from NIBSC, EVA Centre for AIDS reagents) at a final concentration of 1 µg/ml (each peptide) and Staphylococcus aureus Enterotoxin B (SEB, Sigma) as positive control at a final concentration of 2 µg/ml were added to the samples and the volume was adjusted to 200µl with RPMI 10% FBS. A negative control with no stimulation was also included. After 1 hr incubation at 37°C, 5% CO₂, 1 µl of Brefeldin A (BD GolgiPlug, BD Biosciences) was added to each well, and the plates were then incubated for an additional 11 hrs. At the end of the incubation period the cells were transferred to 96-well V-bottom plates and washed twice with PBS before surface intracellular staining. Cells were stained first with LIVE/DEAD® Fixable Dead Cell Stain reagent (Invitrogen by Life Technologies) at a concentration of 1 µl/10⁶ cells for 20 min at 4°C and then with the mAbs to lineage antigens (CD3-V450 and CD4-APCH7, BD Biosciences), before fixation and permeabilization with BD Cytofix/Cytoperm Buffer (BD Biosciences). After two washing steps with BD PermWash Buffer (BD Biosciences), the cells were then incubated for 20 min at 4°C in the presence of anti-MIP-1β-PE (clone D21-1351), anti-IFN-γ-PECy7 (clone P2G10) and anti-IL-2-APC (clone MQ1-17H12; all from...
BD Biosciences), washed twice with PBS and analyzed on a BD FACSCanto instrument. T-cell responses were then quantified and analyzed qualitatively using the Flowjo (Tree Star) and Spice softwares.

3.1.8 Statistical analysis

Statistical analysis was conducted using the softwares SAS (version 9.1 for Windows), S-Plus (version 6.2 for Windows), StatView (version 5.0.1 for Macintosh) and GraphPad Prism (version 4.0b for Macintosh). Paired Student’s t-tests were used for the comparison between different time points within the same animal group (untreated or IL-7-treated). Non-parametric Wilcoxon rank sum tests were used to analyze differences between IL-7-treated and untreated animals.

To compare untreated and IL-7-treated animals with respect to changes from baseline to multiple time points simultaneously the O’Brien test was used: it is a natural extension of the Wilcoxon rank sum test to accommodate multiple time points per subject. The analysis was used to compare simultaneously the changes from baseline to each time point during the treatment period (day 7, 14, 21, 28, and 35). First, changes from baseline to each of the time points were ranked separately, and then the ranks for each animal were averaged across the time points. To assess statistical significance, a permutation test was used. Under the null hypothesis of no effect of IL-7 treatment, one should not be able to distinguish treated monkeys from controls; the control monkeys could have been any group of 5 selected from the 11 total (excluding monkey #744 for which several time points were missing). Considering that there are 462 ways to select 5 subjects from 11, for each of these relabelings, the difference between the average for animals labeled as “IL-7-treated” and the
average for animals labeled as "untreated" was computed. Afterwards, we computed the proportion of relabeled datasets that produced a mean difference at least as extreme as the actual trial data.
3.2 Results

3.2.1 MHC screening and study groups

A critical issue in the study design was the assignment of the 12 animals to the two study groups (i.e., treatment and control) in order to avoid potential biases in SIV-disease susceptibility. Since previous work had identified MHC class-I alleles that confer disease-protective (Mamu-A01, Mamu-A08) or disease-enhancing (Mamu-B01) effects (36-39), we pre-screened the animals for defined Mamu haplotypes and attributed a positive score (+1) to the presence of Mamu-A01 or Mamu-A08 and a negative score (-1) to the presence of Mamu-B01 (Table 3.1). The final study groups (no treatment and IL-7 treatment) were balanced considering both the MHC scores and the baseline circulating CD3+ and CD4+ T-lymphocyte counts (Table 3.1). Indeed, not significant p values for the comparison of CD3+ and CD4+ T-lymphocyte counts between the two groups of animals were obtained even after the exclusion of the single outlier animal (#749) in the untreated group.

3.2.2 Safety and pharmacokinetics of glycosylated macaque IL-7

None of the animals treated with IL-7 exhibited adverse clinical side effects such as fever, weight loss or neurological signs, or specific hematological and biochemical alterations. After the first IL-7 injection (day -7 relative to SIV infection), plasma IL-7 levels peaked on day -5 to return to baseline after 7 days (Figure 3.2 A). The two subsequent injections (day 0 and 7) induced higher peak levels and a greater area under the curve (AUC), resulting in markedly elevated trough levels before each of the following injections. This pattern likely reflects the initial distribution of the cytokine to a high-affinity compartment that became saturated at subsequent injections. A significant increase in
plasma IL-7 levels was also observed in untreated animals, albeit to a considerably lesser extent than in IL-7-treated animals, starting on day 28 in parallel with the most pronounced reductions in circulating lymphocyte counts (Figure 3.2 A). No significant correlations were found between plasma IL-7 levels and several immunological or virological parameters (data not shown). In agreement with previous studies (24-31), IL-7 treatment initially caused a significant downmodulation of the IL-7 receptor (CD127) in both naïve and memory T cells (Figure 3.2 B).

3.2.3 IL-7 treatment during the acute phase of SIV infection does not increase the levels of SIV replication and proviral SIV DNA load

The effects of IL-7 administration on SIV replication were evaluated by measuring the levels of SIV viremia and antigenemia on sequential plasma samples. Treatment with IL-7 did not increase the mean levels of SIV plasma viremia (Figure 3.3 A) at any time during the acute phase of infection, including the peak, viral set point and AUC, with the only exception of the earliest point analyzed (day 4 post-infection). Likewise, there were no differences between treated and untreated animals in the levels of SIV antigenemia (Figure 3.3 B) and proviral SIV DNA load measured in blood mononuclear cells on days 14 and 77, as well as in the GALT (ileum) on days 14-16 and in axillary lymph nodes on days 25-27 post-infection (Figure 3.3 C and D). All animals developed SIV-specific IgG antibodies, which became detectable between days 11 and 21 after infection (data not shown).

3.2.4 IL-7 treatment during the acute phase of SIV infection prevents the depletion of naïve and memory CD4\(^+\) T cells
While all the animals in the untreated group experienced a marked and sustained depletion of circulating CD4+ T lymphocytes starting at the time of peak SIV replication (day 14 post-infection), IL-7-treated animals showed no depletion of CD4+ T cells over the entire treatment period, with even a significant increase in CD4+ T-cell counts, relative to baseline, on day 41 (Figure 3.4 A). When the two groups of animals were compared, IL-7-treated macaques showed higher absolute numbers of peripheral CD4+ and CD8+ T cells at multiple time points, including day 14 post-infection.

To better characterize the effects of IL-7 on the different CD4+ T-cell populations, the naïve, memory and effector subpopulations were separately analyzed. In the absence of IL-7 treatment, SIV infection resulted in significant decreases in the absolute numbers of naïve and memory CD4+ T cells, compared to pre-infection levels, throughout the acute phase of the infection (Figure 3.4 A). In contrast, IL-7 caused an initial increase in memory CD4+ T cells (day 7) and subsequently prevented the depletion of naïve and memory CD4+ T cells throughout the treatment period; effector CD4+ T cells were increased at several time points (Figure 3.4 A). When the absolute numbers of circulating naïve, memory and effector CD4+ T cells in the two groups of animals were compared, significant differences were detected at several time points (Figure 3.4 A). A detailed subset analysis of the memory CD4+ T-cell population was performed at selected time points: while in untreated animals all memory CD4+ T-cell subsets (CM, transitional memory [TM] and effector memory [EM]) were depleted during the acute phase of the infection, no depletion was observed in any subset in animals receiving IL-7 (Figure 3.4 B). However, the protective effects of IL-7 on CD4+ T cells were not sustained after treatment interruption. In fact, both naïve CD4+ T cells and all subsets of memory CD4+ T
cells became significantly depleted, compared to baseline values, on day 62 post-infection, 4 weeks after the last injection of IL-7 (Figure 3.4 A and B).

3.2.5 IL-7 treatment expands all subsets of CD8$^+$ T cells

IL-7-treated animals experienced sustained increases in all subsets of CD8$^+$ T cells throughout the acute phase of infection (Figure 3.4 A), associated with increased expression of T-cell activation markers (Figure 3.5). In contrast, untreated animals experienced a transient depletion of naïve and memory CD8$^+$ T cells (Figure 3.4 A). When the two groups of animals were compared, IL-7-treated monkeys showed higher absolute numbers of peripheral CD8$^+$ T cells at multiple time points (Figure 3.4 A). Subset analysis of memory CD8$^+$ T cells revealed no major changes in untreated animals, whereas IL-7 induced significant increases at several time points in all memory CD8$^+$ T-cell subsets (Figure 3.4 B), with significant differences in comparison to untreated animals (Figure 3.4 B). However, on the last determination (day 62 post-infection), CM CD8$^+$ T cells returned to baseline values, while TM and EM CD8$^+$ T cells became significantly depleted in both IL-7-treated and untreated animals (Figure 3.4 A and B).

3.2.6 Changes in T-cell subpopulations mediated by IL-7 in acutely SIV-infected macaques

Further analysis was conducted using the O'Brien permutation test to simultaneously compare the changes in naïve, memory and effector CD3$^+$, CD4$^+$ and CD8$^+$ T cells from baseline to all the time points during the treatment period (day 7-35 post-infection) in IL-7-treated versus untreated animals. As shown in Figure 3.6 A, this analysis demonstrated that
the changes were significantly different in IL-7-treated and untreated macaques. Likewise, significant differences between IL-7-treated and untreated animals were also observed for naïve, memory and effector CD4+ and CD8+ T cells, as well as for naïve and effector CD3+ T cells, but not for memory CD3+ T cells (Figure 3.6 B). When the analysis of changes was restricted to day 14 post-infection, the differences between IL-7-treated and untreated animals were significant for all T-cell subpopulations (not shown).

3.2.7 Repeated administrations of IL-7 induce only transient T-cell proliferation but persistent reduction of apoptosis

To investigate to what extent the effects of IL-7 on CD4+ and CD8+ T cells were due to cellular proliferation or rather to prolonged survival of the pre-existing cellular pools, the levels of in vivo proliferation and apoptosis were longitudinally monitored in freshly isolated peripheral blood T cells. As shown in Figure 3.7, IL-7 treatment induced only a transient increase in the proportion of Ki67-expressing CD4+ T cells during the first week of treatment (day -5 and -3 prior to SIV infection), which returned to baseline at the time of SIV inoculation (day 0) (Figure 3.7 A); remarkably, there were no further changes in Ki67 expression in circulating CD4+ T cells after each of the subsequent IL-7 injections (Figure 3.7 B). In contrast, CD8+ T cells showed elevated Ki67 expression both before SIV inoculation and at two time points (day 4 and 11) after infection, although the proportion of cycling cells returned to baseline thereafter (Figure 3.7 A and B). Analysis of apoptosis in peripheral blood did not show significant elevations of Annexin-V-binding cells in both untreated and IL-7-treated animals throughout the acute phase of SIV infection (Figure 3.7 C); however, IL-7-
treated animals exhibited significant and sustained increases in the intracellular levels of the anti-apoptotic protein Bcl-2 in both CD4+ and CD8+ T cells during the first three weeks of treatment (Figure 3.7 D). These results suggest that the lack of depletion of CD4+ T cells in IL-7-treated animals was primarily due to a decrease in apoptosis rather than to the induction of cellular proliferation, whereas both phenomena likely contributed to the sustained increases in circulating CD8+ T cells.

Terminal ileum biopsies were obtained on days 14-16 post-infection. In both groups of animals, the yield of CD3+ T cells from intestinal biopsies was highly variable (range = 1.25-15% of the total cell population), underscoring the inherent difficulties in obtaining specimens with comparable representation of the GALT via retrograde ileoscopy. Regardless of these limitations, no significant differences were detected in the relative proportions of total, naïve, memory and effector CD4+ T cells in IL-7-treated vs. untreated macaques (Figure 3.8 A, upper panels); likewise, the proportion of Annexin-V-positive CD4+ T cells was similar in the two groups (Figure 3.8 A, lower panels). In contrast, the proportion of Annexin-V-positive CD8+ T cells was lower in IL-7-treated animals (p = 0.041), primarily due to reduced apoptosis among memory CD8+ T cells (p = 0.048), associated with a lower proportion of apoptosis-prone naïve CD8+ T cells (p = 0.024) (Figure 3.8 A). Both the proportion of effector CD8+ T cells (Figure 3.8 A) and the CD8/CD4 ratio (not shown) tended to be higher in the intestine of IL-7-treated animals, but the differences did not reach statistical significance.

Lymph node biopsies were collected on days 25-27 post-infection. The relative proportions of total, naïve, memory and effector CD4+ and CD8+ T cells were not significantly different between IL-7-treated and untreated macaques (Figure 3.8 B,
upper panels), and the level of T-cell proliferation, as measured by Ki67 expression, was very low in both groups of animals (data not shown). Analysis of T-cell apoptosis by Annexin-V binding (Figure 3.8 B, lower panels) and intracellular Bcl-2 expression did not show significant differences between the two groups of animals despite a trend toward reduced apoptosis in memory CD4⁺ T cells, memory CD8⁺ T cells and effector CD8⁺ T cells in IL-7-treated macaques.

3.2.8 Rapid disease progression in 4 SIV-infected macaques: re-analysis of virological and immunological data after exclusion of rapid progressors

During follow-up, two monkeys in each group (H745, H749 untreated; H751, H752 IL-7-treated) developed early signs of SIV-disease progression and were euthanized for humanitarian reasons after a mean of 143.5 ± 20.4 days (range, 115-163). Albeit infrequently, SIV-infected macaques have been shown to undergo a rapid disease course characterized by high-level virus replication in cells of the mononuclear phagocytic lineage rather than in CD4⁺ T cells (40). In agreement with these previous observations, our rapid progressors (RP) showed persistently higher levels of SIV plasma viremia (Figure 3.9 A) and antigenemia (Figure 3.9 B), compared to conventional progressors (CP), as well as overall higher numbers of circulating CD4⁺ T cells, even though the difference reached statistical significance only on day 62 post-infection (Figure 3.9 C).

Although it is impossible, without extending the study to a larger group of animals, to establish if the RP course was affected by IL-7 treatment, the fact that two animals in each group progressed rapidly suggests that IL-7 did not influence this unique form of SIV-disease evolution.
Since the rapid disease course has been associated with an unusual pathogenesis (40), which is likely influenced by genetic factors and insensitive to IL-7 treatment, we reasoned that the inclusion of RP animals could be a confounding factor in our study, and therefore all the immunological and virological data were re-analyzed after exclusion of these 4 animals. This re-analysis showed no major changes in the statistical comparisons between treated and untreated animals regarding SIV viremia and antigenemia (Figure 3.10 A), CD4+, CD8+, naïve, memory and effector T cells in peripheral blood (Figure 3.10 B), ileum and lymph nodes. However, the exclusion of RP animals had important effects on the analysis of T-cell apoptosis in lymph nodes. Unlike in the GALT (Figure 3.11, upper panels), re-analysis of Annexin-V binding in lymph nodes demonstrated lower levels of apoptosis in memory CD4+ T cells, memory CD8+ T cells and effector CD8+ T cells in spite of the smaller sample size (Figure 3.11, lower panels). Likewise, when the analysis was restricted to CP animals, CD4+ T cells in lymph nodes exhibited higher levels of intracellular Bcl-2 (not shown).

3.2.9 IL-7 treatment elicits earlier and stronger SIV-specific CD4+ and CD8+ T-cell responses

SIV-specific T-cell responses were evaluated at multiple time points during and after the IL-7 treatment period by measuring the intracellular production of IFN-γ, IL-2 and MIP-1β in CD4+ and CD8+ T cells stimulated with peptide pools derived from SIV Gag and Tat proteins. Vigorous SIV-specific responses were detected at multiple time points starting from the first time point analyzed (day 21 post-infection). In contrast, we were unable to document the induction of SIV-neutralizing antibodies at any time during
acute primary infection in both untreated and IL-7-treated macaques. The total number of Tat-specific CD8\(^+\) T cells on day 21 was significantly higher in IL-7-treated than in untreated animals (p = 0.017), whereas the difference in Tat-specific CD4\(^+\) T-cell responses did not reach statistical significance (p = 0.051) (Figure 3.12). IL-7 treated animals also displayed overall higher numbers of Gag-responding T cells, particularly CD4\(^+\) T cells on day 35 post-infection; however, the difference did not reach statistical significance (Figure 3.12).

Qualitative analysis of SIV-specific T-cell responses revealed that initially most Tat-specific (Figure 3.13 A) and Gag-specific (Figure 3.14 A) CD4\(^+\) and CD8\(^+\) T cells were monofunctional in both groups of animals, producing a single cytokine (single-producing, SP). When SP cells were analyzed separately from double- and triple-producing cells (DP and TP), the difference between IL-7-treated and untreated animals was significant for both CD4\(^+\) and CD8\(^+\) Tat-specific T cells (p = 0.030 and 0.017, respectively; Figure 3.13 B). The quality of the T-cell responses evolved over time, with both Tat-specific (Figure 3.13 B) and Gag-specific (Figure 3.14 B) cells acquiring some degree of polyfunctionality over time. This phenomenon was more prominent in IL-7-treated animals, as shown by a significant difference in the proportion of the various functional subpopulations of Tat-specific CD8\(^+\) T cells in the two groups of animals on day 62 post-infection (Figure 3.13 C). These results indicate that IL-7 treatment led to the early elicitation of vigorous Tat-specific CD4\(^+\) and CD8\(^+\) T-cell responses following the peak of viral replication, which was not observed in the absence of treatment.
3.3 Discussion

While the progressive refinement of multi-drug ART protocols has led to extraordinary advances in the treatment of chronic HIV-1 infection, the clinical management of acute primary HIV-1 infection remains a challenge. A critical hurdle is the inherent difficulty in identifying and treating patients at the earliest possible stage in order to reduce the peak of HIV-1 replication and its deleterious effects on the immune system. This issue is particularly relevant considering that a profound depletion of the CD4+ T-cell pool occurs within the first few weeks of infection (1,2), marking a critical and seemingly irreversible event in the pathogenesis of HIV-1 disease. Although a beneficial effect of early ART treatment on the induction and maintenance of HIV-specific cellular immune responses has been reported (41-43), additional studies in patients (44) and macaques (45) have shown limited effects of ART alone on T-cell preservation in the intestinal lamina propria, underscoring the importance of devising effective adjunctive therapies aimed at preventing the immunologic damage that occurs during the acute disease. A potential candidate in this respect is the homeostatic cytokine IL-7, which is currently under investigation as an immune reconstitution agent in chronic HIV-1 infection and other immunodeficiencies (28-31), but whose effects during acute primary HIV-1 (or SIV) infection have not been evaluated. We found that treatment of macaques with IL-7 during the acute phase of SIV infection prevented the depletion of naïve and CM CD4+ T cells that occurred, as expected, in untreated animals, without significantly increasing the levels of virus replication. In striking contrast, treatment of acutely SIV-infected macaques with another common-γ-chain cytokine, IL-15, was recently shown to induce a 3-log increase in the viral set-point associated with increased
CD4+ T-cell activation, leading to accelerated disease progression (46). We have to point out, however, that the analysis of the SIV DNA load in CD4+ T cells performed in the blood as well as in peripheral lymphoid tissues (GALT and lymph nodes) of infected monkeys revealed no significant differences between IL-7-treated and untreated animals, thus suggesting that the CD4+ T cells preserved by IL-7 might actually harbor the virus, or, alternatively, that IL-7 does not preferentially protect uninfected cells.

To elucidate the mechanisms responsible for the CD4-protective effects of IL-7 in our macaques, we examined the kinetics of CD4+ T-cell proliferation and apoptosis, as well as the induction of SIV-specific cellular immune responses during IL-7 treatment. We documented only low and transient levels of CD4+ T-cell proliferation, suggesting that repeated injections of glycosylated IL-7 may in fact induce tachyphylaxis, at least concerning the proliferative effects of the cytokine. Conversely, in agreement with our ex vivo findings (21), several observations pointed to reduction of apoptosis as a major mechanism for the preservation of CD4+ T cells in IL-7-treated macaques. First, IL-7 induced a sustained increase in the expression of the anti-apoptotic protein Bcl-2 in both CD4+ and CD8+ T cells. Second, analysis of lymphoid tissues demonstrated reduced levels of apoptosis in various CD4+ and CD8+ T-cell populations in IL-7-treated macaques, even though for CD4+ T cells in lymph nodes statistical significance was only achieved when the analysis was restricted to animals with a conventional disease course. In contrast, analysis of the GALT at the time of peak SIV replication demonstrated that IL-7 reduced apoptosis in CD8+ T cells, but not in CD4+ T cells. An important caveat that must be considered in this setting is the inconsistent quality of ileum specimens obtained by retrograde ileoscopy, which have a high
probability of sampling error due to a highly variable yield of lymphoid cells. Nevertheless, the fact that we did not observe reduced apoptosis among GALT CD4+ T cells most likely reflects the fact that IL-7 was inactive against the direct cytopathic effects of the virus (1,9). IL-7 does not per se exert antiviral effects, and indeed similar levels of SIV proviral DNA were measured in the GALT of untreated and IL-7-treated animals. However, we demonstrated that IL-7 treatment elicited early and vigorous Tat-specific CD4+ and CD8+ T-cell responses associated with the expansion, activation and proliferation of all CD8+ T-cell subpopulations, which may help explain the lack of increase in SIV replication in the blood and lymphoid tissues of IL-7-treated animals, despite the preservation of SIV-harboring CD4+ T cells. Since Tat is a regulatory protein expressed early in the viral life cycle, Tat-specific T-cell responses presumably were able to halt the infection before the completion of a full replicative cycle. Moreover, CD8+ Tat-responding cells in IL-7-treated animals were shown to be more polyfunctional as compared to untreated animals, a profile that has been proposed as a correlate of protective antiviral immunity in HIV-1- and other chronic viral infections (47). Of note, a particularly pronounced numerical increase was detected in EM CD8+ T cells, a functionally competent subset that was recently associated with effective vaccine-elicited protection in macaques (48). An interesting correlate of our findings was illustrated in a recent study in mice acutely infected with LCMV, in which early treatment with IL-7 augmented the number and functionality of specific effector T cells, thereby reducing organ pathology and promoting viral clearance (49). Thus, altogether, our data suggest that IL-7 treatment prevented the depletion of naïve and CM CD4+ T cells during the acute phase of SIV infection by inducing an early expansion of the CD4+
T-cell pool due to initial proliferation combined with preservation of CD4+ T cells via sustained reduction of bystander apoptosis and induction of vigorous virus-specific cellular immune responses.

The results of our study may have implications for devising new treatment strategies for the acute phase of HIV-1 infection. Even if ART is promptly initiated during primary infection, its effects may not be sufficient for fully preventing the immunologic damage caused by HIV-1 due to dishomogeneous drug biodistribution or inactivation by P-glycoproteins present within the intestinal mucosa; moreover, complete suppression of viral replication could take several weeks, and indirect mechanisms of cell destruction, such as bystander apoptosis, may remain active for some time after the virus has ceased to replicate. Our results provide a scientific rationale for the initiation of clinical trials involving the use of IL-7 as an adjunct therapy, in combination with ART, in acute primary HIV-1 infection.
### Table 3.1: HLA haplotype and baseline peripheral blood CD3⁺ and CD4⁺ T-lymphocyte counts in untreated and IL-7-treated macaques.

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<tr>
<th>MHC haplotype</th>
<th>MHC score*</th>
<th>CD3⁺ (cells/μl)</th>
<th>CD4⁺ (cells/μl)</th>
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<tr>
<td><strong>Untreated:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H743</td>
<td>A01-A08</td>
<td>+2</td>
<td>1831</td>
</tr>
<tr>
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<td>B01</td>
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</tr>
<tr>
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<td>A08</td>
<td>+1</td>
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<td>A08-B01</td>
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<td></td>
<td></td>
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</tr>
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<td>B01</td>
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</tr>
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<td>A02</td>
<td>0</td>
<td>2233</td>
</tr>
<tr>
<td><strong>Mean (±SD):</strong></td>
<td></td>
<td>+1</td>
<td>2994±603</td>
</tr>
</tbody>
</table>

* The MHC score was calculated by attributing a positive score (+1) to alleles associated with protection from SIV disease and a negative score (-1) to alleles associated with accelerated disease progression; no score was attributed to alleles with no known effects on SIV disease progression.

** Animals for which none of the MHC alleles tested (A01, A02, A08, A11, B01, B03, B04, B17) were detected.
3.5 Figure Legends

Figure 3.1 Schematic diagram of the study design. IL-7-treatment period of six weeks is shown in grey with black arrows indicating the 7 weekly injections. SIV inoculation, blood draws, intestinal and lymph node biopsies are also shown.

Figure 3.2 IL-7 pharmacokinetics and CD127 expression in circulating T cells in IL-7-treated and untreated animals. (A) Plasma IL-7 concentrations in IL-7-treated and untreated macaques. IL-7-treated animals received 7 weekly injections of 50 µg/kg of body weight of a recombinant, fully glycosylated form of simian IL-7 (the grey shaded area indicates the IL-7-treatment period). The asterisks denote significant differences with baseline values (p < 0.05 by paired Student’s t test). (B) CD127 expression on total, naïve, memory and effector CD3+ T cells from untreated (blue) and IL-7-treated (red) animals. Average values of mean fluorescence intensity (MFI) ± standard error of the mean (SEM) from each group of macaques are shown. Blue and red asterisks denote significant differences with baseline values (day -7) in untreated and treated animals, respectively (* p < 0.05, ** p < 0.01, by paired Student’s t test).

Figure 3.3 Effect of IL-7 treatment on SIV replication. Mean levels (± SEM) of SIV plasma viremia (A) and p27Gag antigenemia (B) in untreated (blue) and IL-7-treated (red) animals. No significant differences were observed between the two groups of animals, with the only exception of SIV plasma viremia on day 4 post-infection, when IL-7-treated animals showed higher levels compared to untreated controls (indicated by the asterisk; p < 0.05, by Wilcoxon rank sum test). The grey-shaded area indicates the
IL-7-treatment period. (C) Mean number of genome equivalent copies (± SEM) of SIV proviral DNA in mononuclear cells from peripheral blood (days 14 and 77 post-infection) and lymphoid tissues (GALT, days 14-16; axillary lymph nodes, days 25-27 post-infection). SIV DNA genome equivalents normalized for million of CD4+ T cells are shown.

Figure 3.4 Effect of IL-7-treatment on peripheral blood T-lymphocyte kinetics. (A) Mean absolute numbers (± SEM) of circulating total, naïve, memory and effector CD4+ and CD8+ T cells in untreated (blue) and IL-7-treated (red) animals. Naïve (CD28'95'), memory (CD28'95') and effector (CD28'95') T-cell subsets were identified using a combination of mAbs against CD28 and CD95. (B) Subset analysis of memory CD4+ and CD8+ T cells in untreated (blue) and IL-7-treated (red) animals. The various memory T-cell subsets (central memory, CM; transitional memory, TM; and effector memory, EM) were identified using a combination of CD28, CD95, CD62L and CD197/CCR7. Absolute counts for each T-lymphocyte subpopulation were calculated by multiplying the percent values obtained by flow cytometry by the total lymphocyte counts obtained from the complete blood counts (CBC). The grey shaded area indicates the IL-7-treatment period; blue and red asterisks denote significant differences versus baseline values in untreated and IL-7-treated animals, respectively (* p < 0.05, ** p < 0.01, by paired Student's t test).

Figure 3.5 Effect of IL-7-treatment on peripheral blood CD8+ T-lymphocyte activation. Relative proportion of activated (HLA-DR/CD25 double expressing) cells
among circulating CD8\textsuperscript{+} T-cells in untreated (blue bars) and IL-7-treated (red bars) monkeys at multiple time points throughout the IL-7-treatment period. Relative significant p values obtained by unpaired t tests are shown.

**Figure 3.6 Comparison of changes in peripheral blood T-cell subpopulations between IL-7-treated and untreated macaques during the acute phase of SIV infection.** Simultaneous comparisons of changes from baseline to all the time points during acute SIV infection as analyzed by the O'Brien permutation tests. (A) Analyses for total CD3\textsuperscript{+}, CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. (B) Analyses for the naïve, memory and effector T-cell subsets. The histograms represent the distribution of all possible values of the difference between IL-7-treated and untreated animals considering all the possible relabelings of the animals in the two groups (by permutation test); dots indicate the values of the differences between IL-7-treated and untreated animals for the actual trial assignments with the relative p values. Significant differences were observed in all T-cell subsets, except in memory CD3\textsuperscript{+} T cells.

**Figure 3.7 Effect of IL-7-treatment on cellular proliferation, apoptosis and Bcl-2 expression in peripheral blood T cells.** (A) Mean levels of cellular proliferation, as measured by expression of the cell-cycling marker Ki67, in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells freshly isolated from untreated (blue) and IL-7-treated (red) animals during the first two weeks of IL-7-treatment. IL-7 injections and SIV inoculation are indicated by arrows. (B) Mean levels (± SEM) of Ki67 expression in CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells freshly isolated from untreated (blue) and IL-7-treated (red) animals throughout the entire study period. (C) Mean levels (± SEM) of spontaneous apoptosis, as measured by Annexin-V binding,
in circulating CD4⁺ and CD8⁺ T cells from untreated (blue) and IL-7-treated (red) animals. (D) Average MFI levels (± SEM) of Bcl-2 expression in circulating CD4⁺ and CD8⁺ T cells from untreated (blue) and IL-7-treated (red) animals. The grey shaded area indicates the IL-7-treatment period. The asterisks denote significant differences between untreated and IL-7-treated animals (* p < 0.05, ** p < 0.01, by Wilcoxon rank sum test).

Figure 3.8 Frequency of CD4⁺ and CD8⁺ T-cell subsets and analysis of spontaneous apoptosis in terminal ileum and lymph node biopsies from untreated and IL-7-treated macaques. (A) Intestinal biopsies were obtained from all animals on days 14-16 post-infection. Upper panels: Relative proportion of total, naïve, memory and effector CD4⁺ and CD8⁺ T cells in terminal ileum biopsies from untreated (blue) and IL-7-treated (red) animals. Lower panels: Mean levels of spontaneous apoptosis, as measured by Annexin-V binding, in total, naïve, memory and effector CD4⁺ and CD8⁺ T cells isolated from terminal ileum biopsies from untreated (blue) and IL-7-treated (red) animals. The differences between untreated and IL-7-treated animals were analyzed by unpaired t-test (similar results were obtained by Wilcoxon rank sum tests). (B) Lymph node biopsies were obtained from all animals on day 25-27 post-infection. Upper panels: Relative proportion of total, naïve, memory and effector CD4⁺ and CD8⁺ T cells in lymph node biopsies from untreated (blue) and IL-7-treated (red) animals. Lower panels: Mean levels of spontaneous apoptosis, as measured by Annexin-V binding, on total, naïve, memory and effector CD4⁺ and CD8⁺ T cells freshly isolated from lymph node biopsies from untreated (blue) and IL-7-treated (red) animals. The differences between untreated and IL-7-treated animals were analyzed by unpaired t-test.
Figure 3.9 Levels of SIV replication and CD4+ T cells in conventional progressor (CP) versus rapid progressor (RP) SIV-infected macaques. Mean levels (± SEM) of SIV plasma viremia (A) and p27Gag antigenemia (B) in CP (orange) and RP (green). RP animals showed higher levels of both SIV viremia and antigenemia compared to CP at several time points, starting on day 18 post-infection, as indicated by the asterisks (*p < 0.05, ** p < 0.01, by Wilcoxon rank sum test). (C) Mean absolute numbers (± SEM) of circulating CD4+ T cells in CP (orange) and RP (green). A significant difference was observed on day 62 post-infection, when RP had higher mean levels of circulating CD4+ T cells compared to CP, as indicated by the asterisk (p < 0.042, by Wilcoxon rank sum test).

Figure 3.10 Reanalysis of virological and immunological parameters in IL-7-treated and untreated macaques after exclusion of macaques with rapidly progressive (RP) disease course. (A) Mean levels (± SEM) of SIV plasma viremia and p27Gag antigenemia in untreated (blue) and IL-7-treated (red) animals. (B) Mean absolute numbers (± SEM) of circulating total, naïve, memory and effector CD4+ and CD8+ T cells in untreated (blue) and IL-7-treated (red) animals. The grey shaded area indicates the IL-7-treatment period; blue and red asterisks denote significant differences with baseline values in untreated and IL-7-treated animals, respectively (*p < 0.05, ** p < 0.01, by paired Student’s t test).

Figure 3.11 Reanalysis of apoptosis in intestinal and lymph node biopsies from IL-7-treated and untreated macaques after exclusion of animals with rapidly
progressive (RP) disease course. Mean levels of spontaneous apoptosis, as measured by Annexin-V binding, in total, naïve, memory and effector CD4\(^+\) and CD8\(^+\) T cells freshly isolated from terminal ileum (A) and lymph node biopsies (B) from untreated (blue) and IL-7-treated (red) animals. The differences between untreated and IL-7-treated animals were analyzed by unpaired t-test.

Figure 3.12 SIV-specific T-cell responses in untreated and IL-7-treated macaques. Mean absolute numbers (± SEM) of total CD4\(^+\) and CD8\(^+\) T cells responding to overlapping peptides derived from the Tat (A) and Gag (B) proteins of SIV in untreated (blue) and IL-7-treated (red) animals. The asterisk indicates a significant difference between total responses in IL-7-treated vs. untreated macaques. The numbers above each line point indicate the fraction of monkeys that gave a measurable response over background in each group at the corresponding time point.

Figure 3.13 Qualitative analysis of SIV-specific T-cell responses in untreated and IL-7-treated macaques. (A) Mean absolute numbers (± SEM) of CD4\(^+\) and CD8\(^+\) T cells producing one cytokine (single-producing, SP), two cytokines (double-producing, DP) or all three cytokines (triple-producing, TP) in response to SIV Tat peptide stimulation in untreated (shades of blue) and IL-7-treated (shades of red) animals. The asterisks indicate significant differences between SP T cells in untreated vs. IL-7-treated animals, as analyzed by Wilcoxon rank sum test. (B). Mean absolute numbers of SIV Tat-responding CD4\(^+\) and CD8\(^+\) T cells in untreated (U) and IL-7-treated (IL-7) animals. The bars indicate the mean numbers of total responding cells; the
colors indicate the mean numbers of IFN-γ, IL-2, or MIP-1β SP cells (shades of blue), IFN-γ/IL-2, IFN-γ/MIP-1β or IL-2/MIP-1β DP cells (shades of green), and IFN-γ/IL-2/MIP-1β TP cells (purple). The numbers above each bar indicate the fraction of monkeys that gave a measurable response over background to SIV Tat peptides at the corresponding time point. The grey shaded areas indicate the IL-7-treatment period. (C) Qualitative analysis of SIV Tat-specific CD8+ T-cell responses in IL-7-treated and untreated macaques on day 62 post-infection. The pie charts indicate the average contribution of the various functional subpopulations of Tat-responding CD8+ T cells (single-, double- and triple-producing, SP, DP, TP, respectively) to the total number of Tat-responding cells in untreated and IL-7-treated animals at day 62 post-infection. The p value was calculated by permutation analysis using the Spice software.

Figure 3.14 SIV Gag-specific T-cell responses in IL-7-treated and untreated macaques. (A) Mean absolute numbers (± SEM) of CD4+ and CD8+ T cells producing one cytokine (single-producing, SP), two cytokines (double-producing, DP) or all three cytokines (triple-producing, TP) in response to SIV Gag peptide stimulation in untreated (shades of blue) and IL-7-treated (shades of red) animals. (B) Mean absolute numbers of SIV Gag-responding CD4+ and CD8+ T cells in untreated controls (c) and IL-7-treated animals (IL-7). The bars indicate the mean numbers of total responding cells; the colors indicate the mean numbers of IFN-γ, IL-2, or MIP-1β single-producing cells (shades of blue), IFN-γ/IL-2, IFN-γ/MIP-1β or IL-2/MIP-1β double-producing cells (shades of green), and IFN-γ/IL-2/MIP-1β triple-producing cells (purple). The numbers above each bar indicate the fraction of monkeys that gave a measurable response over background to SIV
Gag peptides at the corresponding time point. The grey-shaded areas indicate the IL-7-treatment period.
3.6 Figures

Figure 3.1

Intestinal biopsy

 Peripheral blood

SIV biopsy

IL-7

Weeks of SIV inoculation
Figure 3.2

A

Untreated

IL-7-treated

Plasma IL-7 (pg/mL)

Days of SIV infection

B

CD3 total

CD3 Naive

CD3 Memory

CD3 Effector

Days of SIV infection
Figure 3.3

A

Plasma SIV viremia (genome equivalent/ml)

Days of SIV infection

0 10 20 30 40 50 60 70 80

10^1 10^2 10^3 10^4 10^5 10^6 10^7

untreated IL-7-treated

B

Plasma SIV antigenemia (pg/ml)

Days of SIV infection

0 10 20 30 40 50 60 70 80

17.5 15.0 12.5 10.0 7.5 5.0 2.5 0.0

untreated IL-7-treated

C

SIV DNA load (genome equivalent/s/g CD4+ cells)

PB day 14 PB day 77 GALT LN

untreated IL-7-treated
Figure 3.4

A

- CD4 total
- CD8 total
- CD4 Naive
- CD8 Naive
- CD4 Memory
- CD8 Memory
- CD4 Effector
- CD8 Effector

B

- CD4 CM
- CD4 TM
- CD4 EM
- CD8 CM
- CD8 TM
- CD8 EM

Days of SIV infection

CD4 total

CD8 total

CD4 Naive

CD8 Naive

CD4 Memory

CD8 Memory

CD4 Effector

CD8 Effector

Untreated

IL-7-treated
Figure 3.5

![Bar chart showing the relative proportion of activated cells (%) over different days of SIV inoculation. The chart compares Untreated and IL-7 treated groups.](image)

- **Untreated**
- **IL-7**

Days of SIV inoculation:
- 0
- 14
- 21
- 28

Relative proportion of activated cells (%):
- Untreated
- IL-7

Significance levels:
- P = 0.008
- P = 0.017
Figure 3.6

A

CD3 total

CD4 total

CD8 total

Permutation distribution of O'Brien statistic

B

CD3 Naive

CD4 Naive

CD8 Naive

CD3 Memory

CD4 Memory

CD8 Memory

CD3 Effector

CD4 Effector

CD8 Effector

Permutation distribution of O'Brien statistic
Figure 3.7

A. KI67 expression (%)

IL-7

IL-7

IL-7

Day of SIV inoculation

B. CD4

CD4

CD4 IL-7-treated

CD8 Untreated

CD8 IL-7-treated

Days of SIV infection

C. CD8

Annexin-V cells (%)

Untreated

IL-7-treated

Days of SIV infection

D. Bcl-2 (MFI)

Untreated

IL-7-treated

Days of SIV infection
Figure 3.8

A Total Naïve Memory Effector

CD4

% of CD4+ Cells

Untreated IL-7 Untreated IL-7 Untreated IL-7 Untreated IL-7

CD8

% of CD8+ Cells

Untreated IL-7 Untreated IL-7 Untreated IL-7 Untreated IL-7

B Total Naïve Memory Effector

CD4

% of CD4+ Cells

Untreated IL-7 Untreated IL-7 Untreated IL-7 Untreated IL-7

CD8

% of CD8+ Cells

Untreated IL-7 Untreated IL-7 Untreated IL-7 Untreated IL-7

CD4

Annexin V+ cells (%)

Untreated IL-7 Untreated IL-7 Untreated IL-7 Untreated IL-7

CD8

Annexin V+ cells (%)

Untreated IL-7 Untreated IL-7 Untreated IL-7 Untreated IL-7

P = 0.024

P = 0.048
Figure 3.9

A

[Graph showing plasma SIV viremia over days of SIV infection for CP and RP groups]

B

[Graph showing plasma SIV antigenemia over days of SIV infection for CP and RP groups]

C

[Graph showing CD4+ cell count over days of SIV infection for CP and RP groups]

Figure 3.10

A

[Graphs showing plasma SIV viremia and antigenemia over days of SIV infection for untreated and IL-7-treated groups]

B

[Graphs showing CD3, CD4, and CD8 cell counts over days of SIV infection for untreated and IL-7-treated groups]
Figure 3.11

A

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B

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</tbody>
</table>
Figure 3.12

A

- CD4 Tat
- CD8 Tat

B

- CD4 Gag
- CD8 Gag
Figure 3.13

A

CD4 Tat

CD8 Tat

B

Mean number of responding cells/mm²

Day 21 Day 28 Day 35 Day 54 Day 62

Day 21 Day 28 Day 35 Day 54 Day 62

Day 21 Day 28 Day 35 Day 54 Day 62

Day 21 Day 28 Day 35 Day 54 Day 62

C

p = 0.040

Untreated IL-7-treated

Day 62
References


CHAPTER FOUR
Concluding Remarks

4.0 Main Findings

CHAPTER TWO: Primary cells \textit{in vitro}

Effect of IL-7 on Spontaneous Apoptosis of CD4\(^+\) and CD8\(^+\) T cells
- IL-7 exerts strong anti-apoptotic effects on PBMC from HIV-1-infected individuals
- IL-7 exerts anti-apoptotic effects on both CD4\(^+\) and CD8\(^+\) T-cell subsets
- The anti-apoptotic effect of IL-7 \textit{ex vivo} inversely correlates with the CD4\(^+\) T-cell count \textit{in vivo}
- The anti-apoptotic effect of IL-7 can be uncoupled from the induction of cellular proliferation
- IL-7 does not induce HIV-1 replication \textit{ex vivo} in purified CD4\(^+\) T cells from HIV-1-infected individuals

CHAPTER THREE: \textit{In vivo}

Effects of Treatment with IL-7 during the Acute Phase of SIV Infection in Rhesus Macaques
- IL-7 treatment in rhesus macaques is safe and well-tolerated
- IL-7 treatment during the acute phase of SIV infection does not increase the levels of SIV replication and proviral SIV DNA load
- IL-7 treatment prevents the depletion of naïve and memory CD4\(^+\) T cells
- IL-7 treatment expands all subsets of CD8\(^+\) T cells
- Repeated IL-7 administrations induce only transient T-cell proliferation but persistent reduction of apoptosis
- IL-7 treatment does not impact rapid disease progression in SIV-infected macaques
IL-7 treatment elicits earlier and stronger SIV-specific CD4+ and CD8+ T-cell responses

4.1 Discussion

Although the progressive refinement of drug-combination therapeutic regimens for HIV-1 infection, since their first introduction in the late 90's, has determined major improvements in the quality of life and disease outcome of HIV-1-infected individuals, the achievement of complete immunological restoration remains a challenge. Indeed, the majority of the patients treated with ART experience a sustained increase in peripheral CD4+ T-cell counts associated with a sustained control of viral replication; yet, CD4+ T-cell counts often fail to return to normal levels, suggesting a persistent immunologic damage that may reduce the individual's ability to mount effective immune responses against infectious agents (1). This constitutes an important drawback considering that HIV-1 cannot be eradicated from the host, and therefore ART must be administered for the entire lifetime (2). As outlined in the Introduction, adjuvant therapeutic approaches based on the use of cytokines of the γ-chain family, whose immunomodulatory activities are well established, are currently under investigation in an attempt to restore the full immunologic function in ART-treated HIV-1-infected individuals (3). Indeed, considering that key features of SIV/HIV infection are the rapid depletion of mucosal memory T cells, gradual depletion of peripheral blood T cells and high levels of immune activation, it has been hypothesized that these immunomodulatory cytokines could have several beneficial effects, including enhancement of immunological memory, de novo naïve T-cell
production and increased survival and proliferation of the SIV/HIV depleted T-cell subsets.

The first cytokine of this family used in phase I/phase II clinical trials was IL-2. Its beneficial effects, mainly an increase in the absolute number of circulating CD4+ T cells, particularly with a naïve phenotype, have been well documented and confirmed by several studies in HIV-1-infected individuals concomitantly receiving ART, including two recent large-scale phase II studies (SILCAAT and ESPRIT) (4, 5). Nevertheless, the observed immunological improvements failed to result in a significant clinical improvement, to the point that the idea of using IL-2 as an immunomodulatory adjuvant for the treatment of chronic HIV-1-infected individuals was brought into question. Moreover, IL-2 treatment was shown to have adverse side effects, in some case relatively severe, including a flu-like syndrome, mild capillary leak syndromes, gastrointestinal disturbance, and dermatological problems including injection site reactions, rashes and dry skin. Another relatively common adverse effect was endocrine disturbance in the form of chemical hypothyroidism (5). These adverse effects were shown to be usually short-lived after the end of a dosing cycle, but nevertheless they represented a further challenge to the clinical use of this cytokine. After the failure of the SILCAAT and ESPRIT clinical trials (4), the use of IL-2 as a support “immonorestorative” therapy to ART is slowly being abandoned, and alternative settings for its use in HIV-1-infected individuals are currently under investigation. Potential benefits of treatment with IL-2 that are being explored in ongoing clinical trials include its potential role in delaying the start of continued combination ART during the natural course of the infection and in prolonging the intervals in between
consecutive ART cycles. Moreover, IL-2 is under evaluation also as an adjuvant to therapeutic vaccination.

Another cytokine that has been tested in pre-clinical trials is IL-15, which was shown to have positive effects on several cellular compartments of the immune system, like NK cells, CD8+ and CD4+ T cells, in SIV-infected macaques receiving ART, as well as in SIV challenged animals in vaccine-based trials (6-9). To date, IL-15 has never been tested in HIV-1-infected humans. However, one of the major concerns related to the use of γ-chain cytokines like IL-2 and IL-15, which have strong proliferative effects, is the risk of augmenting viral replication both directly, by inducing re-activation of latent provirus, and indirectly, by increasing the pool of CD4+ T cells that would provide new targets for viral infection. Indeed, in several trials IL-2 administration was associated with transient increases in plasma viral load, and this occurred even with the concomitant use of ART (10-12). Albeit initially considered as an unwanted side effect, this IL-2-mediated increase of viral replication was afterwards looked at as a potential strategy to induce re-activation of silent provirus from latently-infected cells in an attempt to break latency and eradicate the virus, resurfaced from its hiding sanctuaries, using aggressive treatment strategies. Viral eradication may be the only possible strategy to definitively cure HIV-1 infection and studies to test the effect of IL-2 administration in this regard are currently under way.

In contrast with the results obtained with IL-2, administration of IL-15 to rhesus macaques during the chronic phase of SIV infection did not result in any change in plasma SIV viremia (6, 7). However, IL-15 administration during the acute phase of SIV infection was shown to increase the viral set point by several logs, and in some animals to accelerate the disease progression (13). This result suggests that the mechanisms of pathogenesis and
T-cell killing may be very different during the acute and chronic phases of HIV/SIV infection, as different may be the level of integrity and functionality of the immune system. Indeed, during the very early phase of infection, T cells may still be functional and able to respond properly to physiological stimuli, including cytokines like IL-15, entering the cell cycle and proliferating, thus becoming more susceptible to productive HIV/SIV infection. Therefore, treatment with IL-15 during the acute phase of the infection may be even more risky than treatment with IL-2. Of course, the same data provide a rationale for the use of IL-15, as discussed above for IL-2, in experiments aimed at re-activating the replication of latent provirus.

For what concerns the effects of IL-7 on viral replication, conflicting results can be found in the literature. On one hand, IL-7 was documented to be, \textit{ex vivo}, “a potent and proviral strain-specific inducer of latent HIV-1 cellular reservoirs of infected individuals on virally suppressive HAART” by the group of Pomerantz (14), and to induce transient “blips” of viral replication in individuals chronically infected with HIV-1 treated with IL-7 (15). However, recent sequencing data of the viruses emerged during these “blips” indicate that they are very close to those found in plasma and PBMC before treatment (16), thus inferring that the low level of viremia induced by IL-7 likely reflects transient induction or release of virus from the replicating pool rather than reactivation of a latent archival quasispecies. Moreover, studies of IL-7 administration to SIV-infected macaques failed to reveal any significant inductive effect of the cytokine on viral replication, even in the absence of ART (17-19).

The results presented in the present thesis work, showing that IL-7 treatment does not boost viral replication from CD4$^+$ T cells derived from HIV-1-infected individuals \textit{ex}
vivo (CHAPTER TWO) and that IL-7 administration in vivo to SIV-infected macaques
does not induce significant increases in plasma SIV viral load (CHAPTER THREE), are in
line with the latter observations. Taken together, these findings suggest that treatment with
IL-7 may be “safer” in a strictly “therapeutic” design as compared to the other γ-chain
cytokines (IL-2 and IL-15) explored so far. The difference may be related to the
homeostatic properties of the cytokine and/or to the intrinsic features of the IL-7/IL-7R
system, which is tightly and finely tuned both at the level of plasmatic concentration of the
cytokine and expression of the specific chain of the receptor (IL-7Rα, CD127). In fact, as
described in the Introduction section, the pro-survival and proliferative effects of IL-7 are
strictly dependent upon the IL-7 concentration: at homeostatic, physiological
concentrations, IL-7 is mainly causing a pro-survival response; at supra-homeostatic
concentrations, those typical of lymphopenic conditions, IL-7 induces vigorous
proliferation aimed at reconstituting the original T-cell pool. In CHAPTERS TWO and
THREE of the present thesis, we show that CD4+ and CD8+ T cells derived from both
HIV-1-infected individuals (ex vivo) and SIV-infected macaques (in vivo) respond to IL-7
stimulation with very limited, and in any case dose-dependent (ex vivo), levels of cell
cycling and proliferation but with significant reduction of apoptosis, as documented by the
low induction of ki67 expression and higher induction of Bcl-2 expression, indicating that
the two effects of IL-7 can be “uncoupled” by finely regulating its concentration. The
effects of IL-7 seem to be mainly anti-apoptotic even at later stages of the disease, at least
ex vivo, and even stronger than at earlier stages, as indicated by the negative correlation
between the extent of IL-7-mediated apoptosis reduction ex vivo and the CD4+ T-cell
counts of the patient in vivo. These findings led to suggest that IL-7-treatment in vivo may
be useful even at a more advanced disease stage (CHAPTER THREE). This is apparently in contrast with the observation that plasma IL-7 levels increase as the disease progresses both in HIV-1-infected individuals and in SIV-infected macaques (20-25), but such increase is not paralleled by the restoration of the CD4⁺ T-cell pool, implying a low induction of cellular proliferation or an insufficient compensation for the accelerated loss of CD4⁺ T cells. At supra-homeostatic concentrations IL-7 should in fact be primarily proliferative, whereas our data ex vivo suggest mainly an anti-apoptotic effect. A possible explanation could be that, at an advanced disease stage, the T-cell pool may be severely and irreversibly compromised in both number and function, and therefore these cells would be unable to respond in a "physiological" way. In line with this concept, several reports have documented impairments in the IL-7/IL-7R system in HIV/SIV infection (26-32) that could explain the reduced or in any case the non-physiological signaling response to IL-7. Studies aimed at investigating the intracellular signaling pathways involved in spontaneous apoptosis in HIV/SIV infection and the mechanisms of IL-7-mediated apoptosis reduction are currently under way, although the results obtained so far are very preliminary and were not included in the present work.

CHAPTER THREE of this work describes the results of a study of IL-7 administration in vivo to SIV-infected macaques, and it shows that IL-7 administration during the acute phase of the infection, when high levels of viral replication and a severe CD4⁺ T-cell depletion typically occur, protects the naïve and memory CD4⁺ T cell pools without inducing any increase in viral replication, in the absence of ART. To our knowledge, this is the first study documenting positive effects of IL-7 (or other γ-chain cytokines) during the acute phase of the infection, and is in sharp contrast with the study
conducted by the group of Mueller et al. in which IL-15 administration during the acute phase of SIV infection was shown to dramatically increase the viral load and to accelerate disease progression (13).

Although there is still debate regarding the exact mechanisms of CD4\(^+\) T-cell depletion during acute HIV-1 infection, apoptosis has been proposed to play a role, and our observation that the lack of depletion of naïve and central memory CD4\(^+\) T cells in IL-7-treated macaques was associated with decreased levels of apoptosis seems to add support to the concept that apoptosis plays a key role in the depletion of CD4\(^+\) T cells during the early phase of HIV-1 infection. Indeed, if the prevalent view that the dice in HIV/SIV pathogenesis are cast within the very first weeks of the infection is correct, the results presented herein provide a strong rationale for further pursuing the possibility of an early IL-7-based immunomodulatory therapy, maybe in association with ART to try to preserve the CD4\(^+\) T-cell pool and therefore the immune system. Indeed, the early acute phase of infection is when the most severe CD4\(^+\) T-cell depletion is believed to occur, together with high levels of viral replication, leading to a dramatic and seemingly irreversible damage to the immune system, to the point that its recovery is very difficult to achieve.

Of course, a critical factor related to this strategy would the inherent difficulty in identifying and treating patients at an early stage during the acute phase of the infection, in order to reduce the peak phase of HIV-1 replication. Some studies have documented the potential benefit of early ART on the induction of HIV-specific cellular immune responses (33, 34). Our data showing that IL-7 treatment in the absence of ART during the acute phase of SIV infection increases SIV-specific CD4\(^+\) and CD8\(^+\) T-cell responses, including
the effector-memory subset that is fully competent for effector functions and has recently been associated with effective vaccine-elicited protection in macaques (35), point towards the same direction and suggest that ART and IL-7 might even have a synergistic effect on T-cell responses. However, other reports in patients (36) and macaques (37) have demonstrated a limited effect of ART alone on the preservation of lamina propria T lymphocytes and other immunologic functions, underscoring the importance of designing salvage immune-based therapies aimed at preventing or at least reducing the immunologic damage associated with the acute disease. In this work, IL-7 treatment was shown to preserve both the naïve and memory CD4+ T-cell pools in peripheral blood, and to reduce the level of apoptosis in CD8+ T cells in the gut and in CD4+ and CD8+ T cells in the lymph nodes of SIV-infected animals. Even if these data are not conclusive and a more extensive analysis aimed specifically at investigating the effect of IL-7 on gut-associated T cells, particularly CD4+ T cells, and on tissue-associated viral load is needed, they nevertheless point to IL-7 as a good candidate for immune-salvage therapies. Of course, additional studies exploring the combined effects of ART and IL-7 during the acute phase of SIV infection are required before moving into clinical trials, which are anyway challenging due to the difficulties in recruiting subjects immediately after acquisition of HIV-1 infection.

An interesting correlate of the results presented in CHAPTER THREE is the observation that in natural SIV infection in sooty mangabeys, which do not progress to AIDS, there is an early increase in IL-7 levels within the very first weeks after infection (1 through 5), that is not observed in disease-prone macaques, in which the levels of IL-7 increase only during the late stages of the disease (38). In the present study, however, no
differences were observed in terms of disease progression between rhesus macaques infected with a pathogenic strain of SIV treated with IL-7 for 7 weeks during the acute phase and untreated infected animals, suggesting that the increase in IL-7 observed in non-pathogenic infections may not be sufficient to confer protection against AIDS progression, and confirming the hypothesis that additional factors, including host-related factors may be crucial, as suggested elsewhere (39, 40).

We also found that IL-7 treatment did not have any effect on rapid progression (RP), a disease that shows distinct features compared to classical AIDS, which was documented in a certain percentage of SIV-infected macaques but does not seem to occur in HIV-1-infected individuals (41). Indeed, four animals developed signs of RP within the first six months after infection, irrespective of IL-7 treatment (two animals in each groups).

Of course, we have to point out that IL-7 was only administered for a relatively short time (7 weeks) and therefore we do not know what would have happened if IL-7 treatment was continued after the resolution of the acute phase of the infection. We could speculate that the effects of the cytokine on the peripheral blood lymphocyte kinetics would have been sustained and that maybe this would have contributed to confer a bigger advantage to treated animals and towards the progression of the infection, but this is just pure speculation.

It must be emphasized that the CD4+ T cell-protective effects of IL-7 in our animals were transient and rapidly reversed after treatment interruption, at a time when the number of CD8+ T cells in the circulation was still significantly augmented. However, considering its favorable safety profile, IL-7 treatment could be continued for a longer
period than in the present study, and the concomitant use of ART could greatly reduce the ability of HIV-1 to cause severe immunologic damage after the acute phase.

In summary, the results of this work provide new insights for a deeper understanding of the mechanisms of HIV-1-induced pathogenesis during both the acute and chronic phases of the infection, with a particular focus on the role played by spontaneous and induced apoptosis. Moreover, they provide further rationale for designing new immune-based treatment strategies aimed at preserving or replenishing the damaged CD4+ T-cell pool at different stages of the disease.
4.2 References


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